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Identification of aspirin resistance using a PDW-miR92a-score: Validation in an intermittent claudication cohort

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ABSTRACT

Objective: Aspirin is a widely used platelet inhibitor to prevent thrombotic events. However, in 25% of patients the antiplatelet effect is insufficient. The current study aimed to validate a newly developed PDW-miR92a-score as a biomarker of the individual response to aspirin enabling targeted antithrombotic therapy.

Methods: Blood samples were collected from 209 patients with intermittent claudication on daily aspirin therapy. Based on results from the arachidonic acid stimulated aggregation test, patients were defined as aspirin resistant (n = 92) or responders (n = 117). Using the cut-off values for platelet distribution width (PDW) and plasma levels of microRNA-92a (miR-92a) defined in our pilot study, we investigated the performance of the combined PDW-miR92a-score in the validation study. Furthermore, receiver operating characteristic curve analysis was performed in the validation cohort in order to optimize the cut-off values of the two score parameters.

Results: PDW and miR-92a levels were significantly higher in aspirin resistant compared to responding patients. When using the predefined cut-off values for PDW and miR-92a the combined PDW-miR92a score showed high specificity (93.1%) but poor sensitivity (19.8%) for aspirin resistance. By recalculation using new cut-off values identified in the validation cohort, a score with a specificity of 75% and a sensitivity of 54.9% was obtained.

Conclusion: Both PDW and plasma levels of miR-92a were confirmed to be significantly higher in aspirin resistant compared to responding patients in our validation cohort. We were, however, unable to confirm the high sensitivity of the combined PDW-miR92a-score previously published by our group in a pilot study.

1. Introduction

Peripheral arterial disease (PAD) is a major healthcare issue worldwide associated with increased cardiovascular (CV) mortality. In a recent study a 10 year all-cause mortality of 63% was found in patients with intermittent claudication, and a CV main cause of death accounted for 46% of these deaths [1]. Aspirin is the cornerstone in preventing thrombotic events in patient with CV disease. A recent meta-analysis, however, revealed that approximately 25% of patients have insufficient antiplatelet effect of the treatment [2]. Furthermore, the inability of aspirin to inhibit platelet function, a phenomenon called aspirin resistance, was found to be associated with a higher risk of recurrent CV events [3]. Existing laboratory tests to evaluate platelet function in patients using antiplatelet drugs show high intra- and inter-assay variation, and there is a lack of agreement between results obtained using the different tests [2,4,5]. The arachidonic acid stimulated aggregation test on Multiplate®Analyzer (ASPItest) and VerifyNow have both been suggested as a reliable methods in order to assess the antiplatelet effect of aspirin [6], and in the present study we chose the ASPItest as our reference method. However, the ASPItest has some disadvantages in relation to a routine clinical setting, as the test is time consuming, samples need to be analyzed within 30–120 min after blood sampling, and no standardization or quality controls exists.

In a recent pilot study we developed a simple laboratory test, a PDW-miR92a-score (formerly named PDW/miR-92a-score), for identification of patients with aspirin resistance [7]. The test consists of two measurements, platelet distribution width (PDW) and plasma levels of microRNA-92a (miR-92a), and patients were considered aspirin

Abbreviations: CV, cardiovascular; PDW, platelet distribution width; miR-92a, microRNA-92a; ASPItest, arachidonic acid stimulated platelet aggregation test; PAD, peripheral arterial disease; ROC, receiver operating characteristic curve; TLDA, TaqMan Low Density Arrays

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resistant if PDW ≥ 11.8 fL and miR-92a levels ≥4.5. PDW is widely available as part of the complete blood count profile (CBC) on automated hematological analyzers. Plasma samples for microRNA measurements may be prepared and stored at −80 °C (or − 20 °C for short term storage) for subsequent analysis [8,9]. Thus, the PDW-miR92a-score is applicable in a clinical routine setting, and when using the ASPItest with a cut-off of 30 U as reference for inadequate aspirin response [10,11], we found that the PDW-miR92a-score identified aspirin resistance in a claudication cohort with a sensitivity of 80% and a specificity of 97.5% [7].

In our pilot study miR-92a levels in plasma were quantified using TaqMan Low Density Arrays (TLDA), but for quantification of a single microRNA in a routine setting, single TaqMan assays would be the choice of methodology. In a recent study comparing different methodologies for microRNA quantification, we found poor correlation between miR-92a measurements using TLDA and single TaqMan assays [12], and thus the performance of the PDW-miR92a-score when using single TaqMan assays for miR-92a quantification needs to be investigated.

The present study aimed to validate the newly developed PDW-miR92a-score 1) in the pilot cohort using single TaqMan assays for microRNA quantification and 2) in a new and larger claudication cohort (validation cohort).

2. Materials and methods

2.1. Pilot cohort

The pilot cohort has previously been described [7], and in short it consists of 50 patients with intermittent claudication enrolled at Lillebaelt Hospital during June and July 2014, using the same criteria as described for the validation cohort below. All laboratory measurements on the pilot cohort have previously been performed and described [7], and data on miR-92a measurements obtained using single Taqman assays have been provided as part of a study investigating the impact of preanalytical and analytical conditions when quantifying microRNA levels in plasma [12]. In the present study a recalculation of the performance of the PDW-miR92a-score using miR-92a measurement obtained by single TaqMan assays instead of TLDA was performed.

2.2. Validation cohort

A total of 209 patients with intermittent claudication were enrolled in the validation study during the period between Maj 2016 and December 2017 at Lillebaelt Hospital, Kolding, Denmark. Aspirin was prescribed as monotherapy with a standard dose of 75 mg per day, which they confirmed to have been taking daily for at least 10 days prior to blood sampling. Patients were excluded if they used any antiplatelet 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-20150191) and the Danish Data Protection Agency.

2.3. 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 all participants the first 3 mL of blood was collected into a K_2-EDTA containing tube (Becton-Dickinson), then 3 mL into a Hirudin containing tube (Roche Diagnostics International Ltd., Rotkreuz, Switzerland), 10 mL into a K_2-EDTA containing tube (Becton-Dickson) and finally 4 mL into a Li-Heparin containing tube (Becton-Dickinson). The samples were used for hematological analysis, aggregation analysis, miRNA analysis and biochemical analysis, respectively.

2.4. Hematological parameters

EDTA anticoagulated blood samples were analyzed using the fully automated Sysmex XN analyzer (Sysmex, Kobe, Japan). To minimize changes in cells size, all tests were performed within 30 min of blood sampling. The parameters obtained were platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), hematocrit (HCT), hemoglobin (HB), red blood cell count (RBC) and white blood cell count (WBC).

2.5. Platelet aggregation tests

Platelet aggregation in response to arachidonic acid (AA) 0.5 mM was measured by multiple electrode aggregometry (MEA) using Multiplate® Analyzer (Roche Diagnostics, Rotkreuz, Switzerland). All tests were performed in duplets within 30–120 min after blood sampling, using hirudin anticoagulated whole blood samples and the specifications supplied by the manufacturer [13]. 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 [10,11].

2.6. MicroRNA analysis

Platelet-poor-plasma (PPP) from 10 mL of EDTA anticoagulated whole blood was obtained by dual centrifugation at 3000*g* for 15 min (acceleration 5, brake 6, temperature 18 °C) using a Rotina 420R centrifuge (Hettich, Beverly, MA, USA). After each of the two centrifugation steps, the plasma phase was carefully transferred to a new tube, leaving approximately one mL of plasma on top of the buffy coat (first centrifugation) or in the bottom of the tube (second centrifugation). 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). MicroRNA was eluted using 30 μL of RNAse free water, and the samples were frozen at −80 °C.

cDNA synthesis was performed using TaqMan®MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and microRNA-specific stem-loop primers for miR-92a and cel-miR-39 (ThermoFisher assay-IDs 000431 and 000200). The reaction was performed with 2 μL of purified microRNA in a total volume of 15 μL, and the mixture was incubated at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min as recommended by the manufacturer.

Each qPCR contained 1.3 μL transcribed cDNA, 1 μL 20× TaqMan MicroRNA Assay and 10 μL 2× TaqMan Universal PCR Master Mix (both from Applied Biosystems, Foster City, CA) in a total volume of 20.3 μL. Each sample was processed in doublets in 40 cycles of 95 °C for 15 s and 60 °C for 60 s using the ABI Prism 7900HT, and the mean Ct-value was calculated. Ct-values were technically normalized using the exogenous added cel-miR-39, and the expression level calculated as $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{miR\text{-}92a} - Ct_{cel\text{-}miR\text{-}39}$). A detailed protocol is available at protocols.io (https://doi.org/10.17504/protocols.io.q9edz3e).

2.7. Biochemistry

Heparin plasma was analyzed for high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol (CHOL) and triglyceride (TG) using the fully automated Cobas 8000 platform (Roche Diagnostics International Ltd., Rotkreuz, Switzerland).

Table 1
Baseline characteristics of participants in the validation cohort.

	Aspirin responder $n = 117$	Aspirin resistant $n = 92$	*P*-values
Clinical parameters			
Age, yrs. [IQR]	72 [66; 76]	70 [61; 75]	0.28
Female sex, %	41.0	40.2	1.00
BMI, kg/m^2 [IQR]	25.9 [23.9; 28.7]	26.2 [23.5; 30.1]	0.79
Current smoking, %	33.3	46.2	0.06
No. of cigarettes per day [IQR]	12 [6; 20]	10 [4; 15]	0.10
No. of alcoholic drinks per week [IQR]	4 [1; 10]	3 [0; 7]	0.12
Ankle-brachial index [IQR]*	0.69 [0.61; 0.90]	0.74 [0.58; 0.94]	0.56
Toe pressure, mm Hg [IQR]**	56 [50; 75]	65 [54; 79]	0.22
Maximum walking distance, m [IQR]	400 [200; 2000]	500 [175; 1250]	0.92
Diabetes, %	17.1	25.0	0.17
Aspirin dose ≥100 mg, n (%)***	5 (4.3)	2 (2.2)	0.47
Laboratory parameters			
WBC, x10^9/L [IQR]	7.5 [6.4; 8.6]	8.5 [7.6; 9.9]	**< 0.0001**
HB, mmol/L [IQR]	8.5 [7.9; 9.0]	9.1 [8.3; 9.5]	**0.0001**
RBC, x10^12/L [IQR]	4.5 [4.1; 4.7]	4.7 [4.4; 5.0]	**0.0006**
HCT, ratio [IQR]	0.41 [0.38; 0.42]	0.44 [0.40; 0.46]	**< 0.0001**
PLT, x10^9/L [IQR]	254 [210; 289]	268 [225; 306]	0.27
MPV, fL [IQR]	10.1 [9.6; 10.8]	10.6 [10.0; 11.2]	**0.007**
PDW, fL [IQR]	11.4 [10.3; 13.1]	12.3 [11.1; 13.7]	**0.003**
HDL, mmol/L [IQR]	1.5 [1.2; 1.9]	1.4 [1.2; 1.9]	0.20
LDL, mmol/L [IQR]	2.3 [1.8; 2.7]	2.0 [1.7; 2.7]	0.17
CHOL, mmol/L [IQR]	4.3 [3.7; 4.9]	4.1 [3.6; 4.6]	0.10
TG, mmol/L [IQR]	1.36 [0.97; 1.94]	1.34 [0.92; 1.87]	0.81
miRNA-92a, relative to cel-miR-39 [IQR]	2.2 [1.5; 3.6]	2.5 [1.9; 5.0]	**0.03**

Values of probability compare baseline characteristics in aspirin responding versus aspirin resistant patients classified according to results of the arachidonic acid stimulated platelet aggregation test (ASPItest). Continuous variables are presented as median values with interquartile ranges (IQR) (25% – 75%). Categorical variables are presented as percentage. *The ankle-brachial index was measurable in 98 and 71 of the responding and resistant patients, respectively. **For the remaining patients (19 responding and 21 resistant) the toe pressure is provided. *** The number of patients taking 100 mg or 150 mg aspirin per day is provided; all remaining patients were taking a standard dose of 75 mg per day. BMI, body mass index; WBC, white blood cell count; HB, hemoglobin; RBC, red blood cell count; HCT, hematocrit; PLT, platelet count; MPV, mean platelet volume; PDW, platelet distribution width; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CHOL, cholesterol; TG, triglyceride. P-values < 0.05 were considered significants and are shown in bold.

2.8. Statistical analysis

Statistical calculations were carried out using STATA statistical software (version 15.0) (StataCorp, Texas, USA). Groups of patients were compared using the two-sample Wilcoxon rank-sum test for the continuous variables and Fisher's exact for the categorical variables. Furthermore, Spearman's Rank correlation and nonparametric receiver operating characteristic curve analysis were used. *P*-values < 0.05 were considered significant.

3. Results

3.1. Clinical and laboratory characteristics

Clinical and laboratory characteristics of participants in the validation cohort are depicted in Table 1. According to our cut-off value for the ASPItest, 92 patients (44%) showed poor aspirin response and were classified as aspirin resistant whereas the remaining 117 patients showed the intended response to aspirin.

WBC, HB, RBC, HCT, MPV, PDW and plasma levels of miR-92a were all significantly higher in the aspirin resistant group compared to the responding group. The two patient groups showed no significant differences in age, sex, BMI, smoking status and volume, alcohol usage, maximum walking distance, ankle-brachial index, diabetes diagnosis or lipid-status (Table 1).

3.2. Platelet distribution width

PDW differed significantly between aspirin responding and aspirin resistant patients in both the pilot ($p = .03$) and the validation cohort ($p = .003$), Table 2, Fig. 1A and Fig. 2A. In the validation cohort spearman's rank correlation analysis showed a weak (rho = 0.22) but significant ($p = .001$) correlation between the ASPItest and PDW. In the pilot cohort the correlation coefficient was rho = 0.33 ($p = .02$).

3.3. MicroRNA levels

In the validation cohort the normalized plasma levels of miR-92a differed significantly between patients with sufficient versus insufficient aspirin response ($p = .03$), Table 2 and Fig. 2 (B and C). Spearman correlation analysis showed a weak (rho = 0.23) but significant ($p < .001$) correlation between the ASPItest and miR-92a level.

In the pilot cohort miR-92a levels was also found to differ significantly between the two patient groups when analyzed using TLDA ($p = .02$), but when analyzed using single TaqMan assays no significant difference was observed, Table 2 and Fig. 1 (B and C).

3.4. Performance of the PDW-miR92a-score

In our pilot-study the PDW-miR92a-score identified aspirin resistance with a sensitivity of 80% and a specificity of 97.5% using the cut-off values PDW ≥ 11.8 fL and a normalized plasma level of miR-92a ≥ 4.5 [7].

When reanalyzing microRNA samples from the pilot study using single TaqMan assays instead of TLDA, the score was found to have a sensitivity of 90.0% and a specificity of 77.5%.

In the validation study, still using the same cut-off values as in the pilot study and single TaqMan assays, the PDW-miR92a-score showed a sensitivity of 19.8% and a specificity of 93.1%.

3.5. Optimization of the PDW-miR92a-score in the validation cohort

By receiver operating characteristic (ROC) curve analysis (Fig. 3) we

Table 2
Summary of PDW, miR-92a and ASPItest results from the pilot and validation studies.

	Aspirin responder	Aspirin resistant	P-value
Pilot cohort	*n = 40*	*n* = 10	
PDW, fL [IQR]*	11.6 [10.7; 12.8]	12.6 [12.3; 12.8]	**0.03**
miR-92a (TLDA) [IQR]*	3.6 [2.9; 5.0]	5.3 [4.6; 7.1]	**0.02**
miR-92a (single TaqMan assay) [IQR]**	6.1 [4.2; 11.4]	7.1 [5.5; 11.8]	0.42
ASPItest, U [IQR]*	22 [20; 25]	49 [38; 53]	
Validation cohort	*n = 117*	n = 92	
PDW, fL [IQR]	11.4 [10.3; 13.1]	12.3 [11.1; 13.7]	**0.003**
miR-92a (single TaqMan assay) [IQR]	2.2 [1.5; 3.6]	2.5 [1.9; 5.0]	**0.03**
ASPItest, U [IQR]	22 [18; 25]	44 [38; 57]	

MicroRNA levels were normalized to the spiked-in cel-miR-39. Data are presented as median values with interquartile ranges (IQR) (25% - 75%). PDW, platelet distribution width; TLDA, TaqMan Low Density Array; ASPItest, arachidonic acid stimulated aggregation test.
*results previously published in our pilot study [7], **measurements from a previous study comparing different miRNA platforms [12], and here used to validate single TaqMan assays in relation to the PDW-miR92a-score. P-values < 0.05 were considered significants and are shown in bold.

found the optimal cut-off values in the validation cohort to be PDW ≥ 11.4 fL and a plasma miR-92a level ≥ 1.93. Using these cut-off values the PDW-miR92a-score discriminated aspirin responding and aspirin resistant patients with a sensitivity of 54.9% and a specificity of 75.9%.

4. Discussion

Among the 209 patients with intermittent claudication included in this validation study, 92 (44%) were aspirin resistant according to the ASPItest with a cut-off of 30 U. Resistant patients had significantly higher PDW and plasma miR-92a levels as compared to aspirin responding patients, which is consistent with the findings in our pilot study [7]. Nevertheless, we were unable to confirm the high performance of the combined PDW-miR92a-score found in the pilot study.

A striking difference between our pilot study compared to the validation study was the prevalence of aspirin resistance, which was more than twice as high in the validation cohort. In consistence with our findings in the validation cohort, a recent study found the prevalence of aspirin resistance in PAD patients to be 45% [14]. In the validation study only 4.3% of the aspirin responding patients were taking > 75 mg of aspirin per day, whereas in the pilot study as many as 15% of the responding patients were using a higher dose of aspirin, and of these 10% were taking 150 mg aspirin per day. It has been reported, that in some patients aspirin resistance may be overcome by increasing the aspirin dose [15]. Thus, the usage of a higher aspirin dose in relatively more patients in the pilot compared to the validation study might be

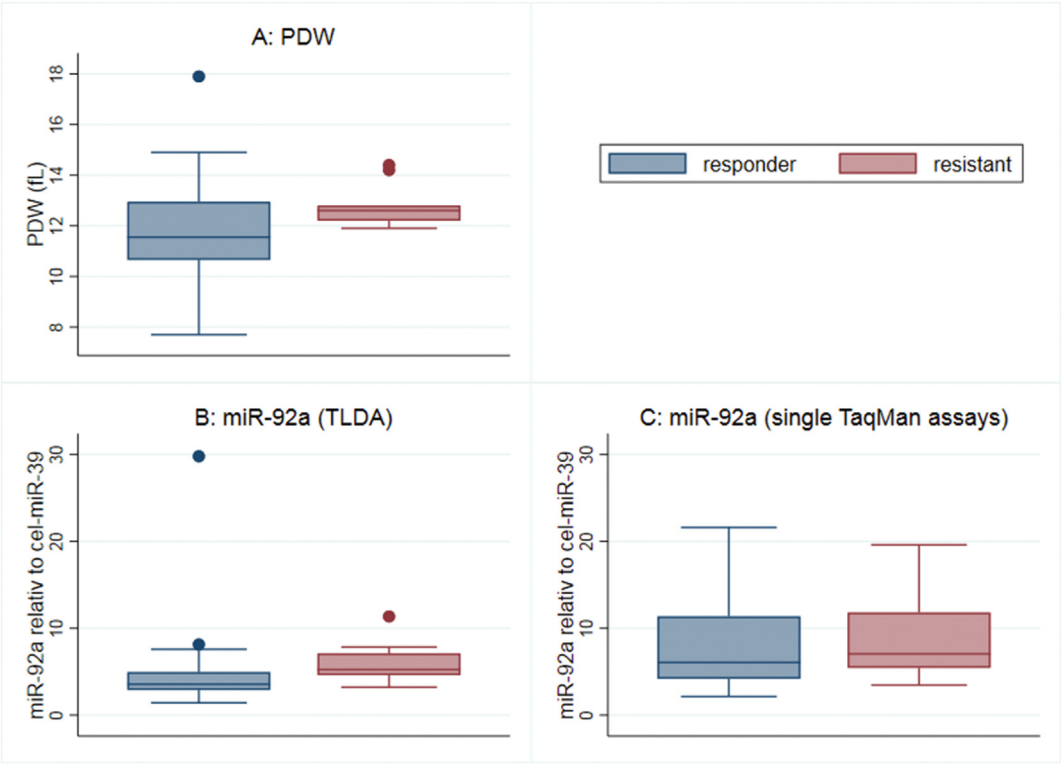

Fig. 1. Distribution of PDW and miR-92a levels in the pilot cohort*. For aspirin responding and aspirin resistant patients of the pilot study, the boxplots shows the distribution of A) PDW, B) miR-92a levels quantified using TLDA and C) miR-92a levels quantified using single TaqMan assays. Plasma levels of miR-92a were normalized to the spiked-in cel-miR-39.
ASPItest, arachidonic acid stimulated aggregation test; PDW, platelet distribution width; TLDA, TaqMan Low Density Array. *Data has previously been published [7,12].

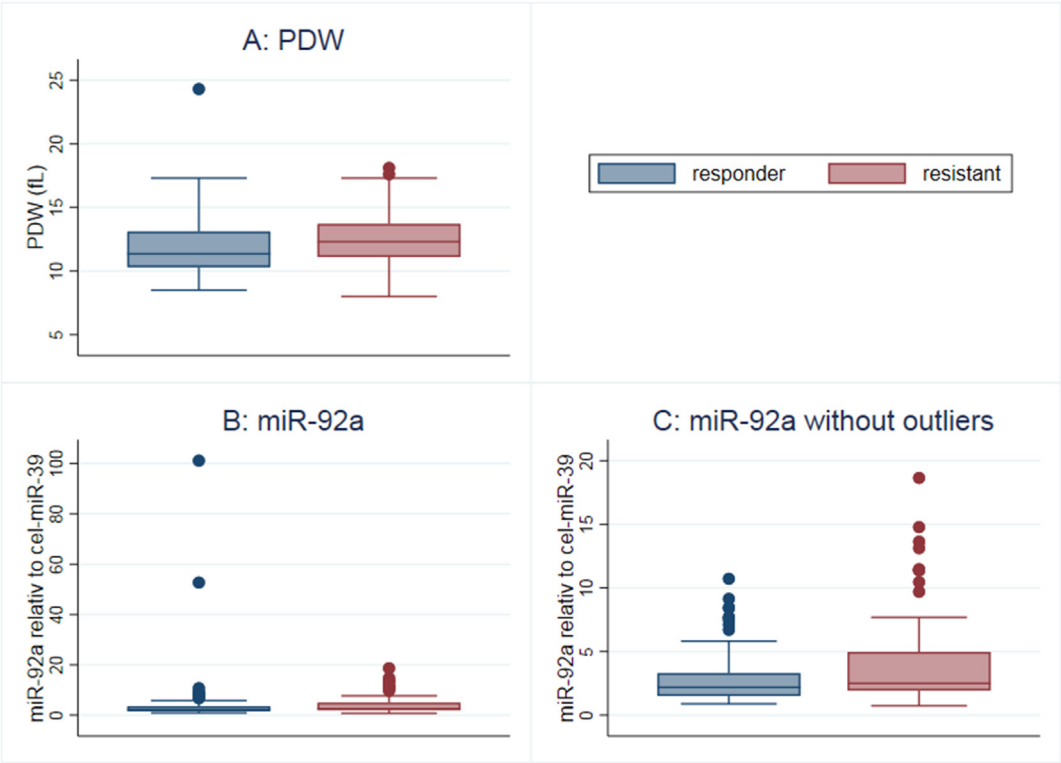

Fig. 2. Distribution of PDW and miR-92a levels in the validation cohort. The box-plots show the distribution of A) PDW and B) miR-92a levels in aspirin responding (n = 117) and aspirin resistant (n = 92) patients. Plasma levels of miR-92a were normalized to the spiked-in cel-miR-39. In plot C) the two very high microRNA measurements in the responding group has been removed to visualize the different distribution of miR-92a in aspirin responding versus resistant patients.
ASPItest, arachidonic acid stimulated aggregation test; PDW, platelet distribution width.

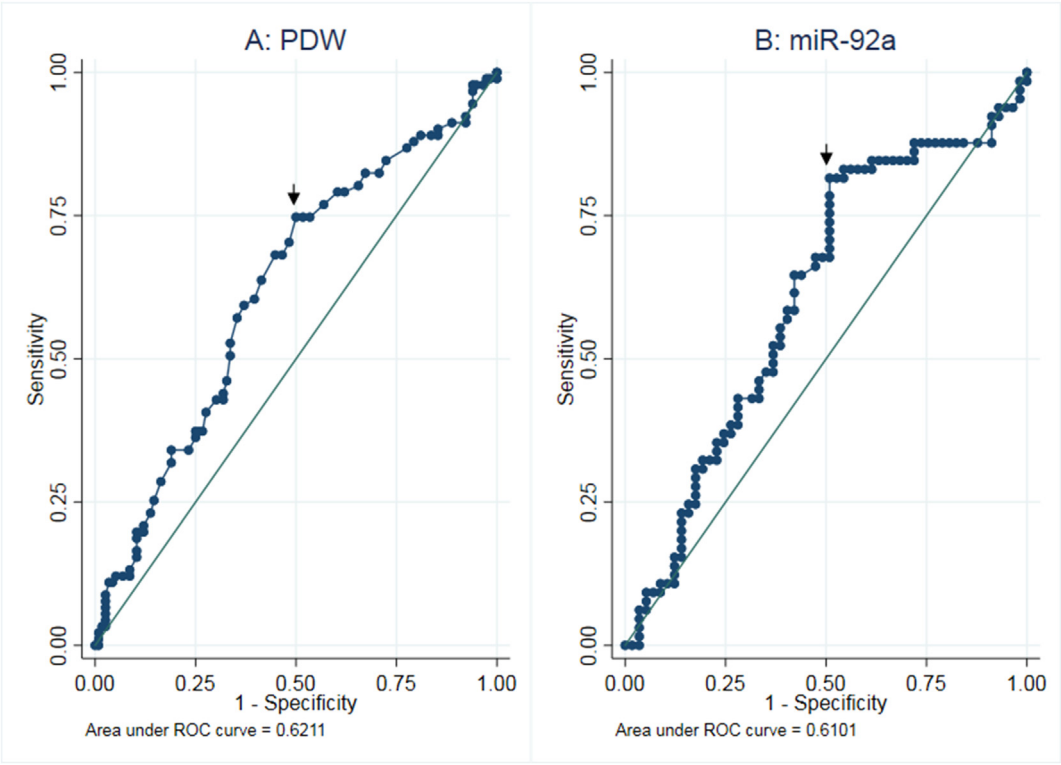

Fig. 3. ROC-curves. Using the ASPItest with a cut-off of 30 U as reference method for aspirin resistance, ROC-curve analysis for PDW (A) was calculated and the optimal cut-off value was found to be PDW ≥ 11.4 fL. In patients with a PDW ≥ 11.4 fL the ROC-curve analysis for miR-92a (B) showed that the optimal cut-off was a miR-92a level ≥ 1.93. The two cut-off values are indicated on the ROC-curves by arrows.
ASPItest, arachidonic acid stimulated aggregation test; ROC-curve, receiver operating characteristic curve; PDW, platelet distribution width.

part of the explanation why the prevalence of aspirin resistance was lower in the pilot study. In addition, in the small sized pilot cohort ($n = 50$) even small changes in the number of resistant patients would have significantly impacted the calculated prevalence.

PDW was found to be significantly higher in the aspirin resistant compared to the aspirin responding patients, which is in line with results published by Kim YG et al. [16]. The distribution of PDW among aspirin responding patients in the pilot and validation cohorts was very similar (Table 2). Also, in the aspirin resistant patients the median PDW values was similar in the two cohorts, but the range of PDW was larger among resistant patients in the validation cohort. Therefore, whereas it in the pilot study we were able to select a cut-off value for PDW which included all resistant patients in the high PDW category, this was not possible in the validation study without also including most of the responding patients.

When developing the PDW-miR92a-score in the pilot study we used TLDA for microRNA quantification but since it is more applicable to use single TaqMan assays when only a single microRNA is to be quantified, we investigated whether the choice of PCR methodology would impact the performance of the score. Though the same probes and primers were used in the TLDA and single TaqMan assays, the obtained miRNA-92a levels were found to differ between the two methods. The observed level difference may partly be explained by the fact that the TLDA protocol includes a preamplification step which beside the additional pipetting for the preamplification reaction itself, will enhance variations due to microRNA purification and pipetting of samples and reagents in the cDNA synthesis. Furthermore, the efficiency of the qPCR-reaction may be influenced by the large difference in reaction volume used, 1 μL for TLDA and 20.3 μL for the single TaqMan assays. Other studies have also described discrepancies between results obtained by TLDA and single TaqMan assays [17–19]. In the pilot study we found that the PDW-miR92a-score could identify aspirin resistance with a sensitivity of 80% and a specificity of 97.5% [7], and when miR-92a was quantified using single TaqMan assays, we observe a 10% increase in sensitivity and a 20% decrease in specificity. Clopidogrel is another antiplatelet drug, which have been shown to be a good alternative to aspirin [20] and more effective in reducing cardiovascular events in atherosclerotic patients with the same level of safety [21]. Thus, sensitivity of the score may be more important than specificity, and we concluded that single TaqMan assays can be used for quantification of miR-92a in relation to the PDW-miR92a-score.

The levels of miR-92a appear to be generally higher in the pilot compared to the validation study. Nevertheless, when analyzed by single TaqMan assays the mean Ct-value for miR-92a was 24.3 in the pilot study and 24.6 in the validation study. For the spiked-in cel-miR-39 the mean Ct-value was 27.1 in the pilot study and 26.1 in the validation study ($\Delta Ct = 1{\sim}2$ fold change). Thus, the observed difference in normalized miR-92a levels between the two studies may largely be explained by the fact, that a new dilution of our highly concentrated cel-miR-39 stock solution was used when purifying microRNA in the validation study. This issue was overcome, in relation to the PDW-miR92a-score, by choosing another cut-off value for miR-92a in the validation study, but it may have been avoided by using a commercial cel-miR-39 spike-in kit.

In the validation study we found the same high specificity of the PDW-miR92a-score as seen in the pilot study, but the sensitivity was low in the present study. When ROC-curve analysis was used to adjust the cut-off values for PDW and miR-92a in the validation cohort, the sensitivity improved, but not enough to make the PDW-miR92a-score useful in a clinical setting. Since the specificity of the score found in the pilot cohort was higher when miR-92a levels were analyzed using TLDA, one might speculate whether the sensitivity of the PDW-miR92a-score found in the validation study could have been improved by using TLDA for microRNA analysis. However, due to the distribution of PDW in the validation cohort, as discussed above, we could not confirm the previously found high sensitivity of the combined PDW-miR92a-score.

The current study underscores the importance of performing independent validation of previous research findings as a general scientific principle. This has been addressed in a recent review confirming that model development studies often are relatively small regarding the challenges posed by specifying the form of a prediction model and deciding on which predictors to include and might face an inherent risk of overfitting using standard estimation methods [22].

The strengths of the present validation study include the thorough description of patient cohorts and methods. Patients in the pilot and validation cohorts were enrolled at the same hospital department and the diagnosis of intermittent claudication and the interview of patients at enrollment were performed by the same staffs using the same inclusion and exclusion criteria. Furthermore, all blood samples were analyzed at the same laboratory, and the definition of aspirin resistance was identical in the two studies. The data set was very similar in the validation compared to the pilot study and thus suitable for the assessment for reproducibility. A limitation was that we did not include a laboratory measurement of compliance, and thus the two cohorts may include patients not taking the prescribed antiplatelet drug.

In conclusion, though both PDW and plasma levels of miR-92a were confirmed to be significantly higher in aspirin resistant compared to aspirin responding patients in our validation study, we were unable to confirm the high sensitivity of the combined PDW-miR92a-score previously published by our group in a pilot study.

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Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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