Comparison of multiple electrode aggregometry with lumi-aggregometry for the diagnosis of patients with mild bleeding disorders

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Short title: Comparison of MEA and LTA

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Essentials

- There is a clinical need for new technologies to measure platelet function in whole blood
- Mild bleeding disorders were evaluated using multiple electrode aggregometry (MEA) and lumi-LTA
- MEA is relatively insensitive at detecting patients with mild platelet function and secretion defects
- More studies are required to investigate MEA in patients with defined set of platelet disorders

Abstract

Background: Multiple electrode aggregometry (MEA) measures changes in electrical impedance caused by platelet aggregation in whole blood. This approach is faster, more convenient and offers the advantage over light transmission aggregometry (LTA) of assessing platelet function in whole blood and reducing pre-analytical errors associated with preparation of platelet rich plasma (PRP). Several studies indicate the utility of this method in assessing platelet inhibition in individuals taking antiplatelet agents e.g. aspirin and clopidogrel.

Objective: Our current study sought to evaluate the ability of MEA in diagnosing patients with mild bleeding disorders by comparison with light transmission lumi-aggregometry (Lumi-LTA).

Methods: 40 healthy subjects and 109 patients with a clinical diagnosis of a mild bleeding disorder were recruited into the UK Genotyping and Phenotyping of Platelets study (GAPP, ISRCTN 77951167). MEA was performed on whole blood using one or two concentrations of ADP, PAR-1 peptide, arachidonic acid and collagen. Lumi-LTA was performed in PRP using several concentrations of ADP, adrenaline, arachidonic acid, collagen, PAR-1 peptide and ristocetin.

Results: Of 109 patients tested, 54 (49%) patients gave abnormal responses by lumi-LTA to one or more agonists. In contrast, only 16 (15%) patients were shown to have abnormal responses to one or more agonists by MEA.

Conclusions: In this study we showed that MEA is less sensitive in identifying patients with abnormal platelet function relative to lumi-LTA.

Keywords

Light transmission lumi-aggregometry, mild bleeding disorders, multiple electrode aggregometry, platelet aggregation, platelet function defects,

Introduction

Individuals with inherited platelet function disorders (PFD) comprise a heterogeneous group characterised by impaired platelet function and variable bleeding symptoms which in some cases can be life threatening. Characterisation of individual platelet function defects is crucial for optimal treatment and management as even frequent minor bleeding episodes can have a significant impact on quality of life. Diagnosis of severe forms of PFDs such as Glanzmann Thrombasthenia (GT) and Bernard-Soulier Syndrome (BSS) is less challenging because bleeding symptoms are not only usually identified early in life, but laboratory tests are often straightforward due to absence of aggregation and ristocetin induced agglutination, respectively [1, 2], and lack of expression of glycoproteins measured by flow cytometry [3]. In contrast, diagnosis of the milder forms of PFDs is complex and challenging. Bleeding symptoms may not manifest in early age due to absence of haemostatic challenges such as surgery, injury, and childbirth. Additionally, many PFDs exhibit unclear penetrance and symptoms are similar to other haemostatic disorders including type 1 Von Willebrand Disease (VWD) [4].

A number of tests are currently available to assess platelet function. The most commonly used is LTA which was first described by Born over 50 years ago [5]. The test measures the change in light transmission in real time when agonists are added to PRP or washed platelets. A typical panel of agonists includes ADP, collagen, arachidonic acid, adrenaline, PAR-1 peptide, U46619 and ristocetin [6]. The nature of the response is dependent on the agonist used, its concentration and the role of the feedback agonists ADP and thromboxane A_2 (TxA_2). During aggregation the platelet response may demonstrate an initial

increase in optical density as a result of shape change, followed by a primary, biphasic, sustained or reversible increase in light transmission [7]. The secretion of platelet ATP from the dense granules can be simultaneously monitored using luminescence by addition of luciferin–luciferase reagents. Measuring the secretion of dense granules is important in diagnosis of Hermansky-Pudlak syndrome (HPS) and other dense granule secretory and release disorders. However, a defect in ATP secretion cannot discriminate between abnormal granule formation or defects in platelet signaling, necessitating additional investigations such as electron microscopy, serotonin uptake and measurement of total platelet ATP/ADP content [4]. Although LTA is considered to be the gold standard method for the investigation of platelet function, the technique is time consuming, requires large blood volumes for preparation of PRP, needs to be performed on fresh samples and requires expertise for correct performance and interpretation [8]. Moreover, not all laboratories use lumi-LTA as a recent worldwide survey showed that 40.7% of laboratories use this for the diagnosis of inherited platelet function defects [9].

MEA has recently been developed for rapid assessment of platelet function. The device evaluates platelet responses in disposable ready-to-use test cuvettes, each containing two pairs of electrodes enabling two simultaneous measurements. During the test activated platelets adhere onto the sensor wires resulting in increased electrical resistance which is continuously monitored and reported as the area under the curve (AUC). As a whole blood method, MEA only requires small blood volumes making it possible to be used as a point of care test with standardized reagents. In addition, the use of whole blood offers the advantage of assessing platelet function under more physiological conditions and avoids the variables associated with the preparation of PRP [10]. Although MEA can also measure the

kinetic changes in aggregation over time including the parameters of lag time, slope and area under the curve and maximal aggregation, the traces are not as detailed as LTA which not only measures these but also includes shape change, primary (reversible) and secondary aggregation responses. In addition, MEA does not measure released ATP in parallel with aggregation. As MEA is becoming increasingly popular for measuring antiplatelet therapy and has been shown to be useful for detecting severe PFDs we assessed the potential utility of MEA for detecting mild PFDs by comparison with the current gold standard, lumi-LTA in participants recruited to the GAPP Study [11].

Materials and Methods

Participants

Between September 2013 and November 2015, a total of 149 individuals (109 patients and 40 controls) were evaluated and recruited into the GAPP study (www.birmingham.ac.uk/plateletgapp) from 9 UK Haemophilia Care Centres. All patients or their parents gave written informed consent in accordance with the GAPP project ethical approval (REC reference: 06/MRE07/36).

Reagents

ADP, ristocetin and adrenaline were purchased from Sigma (Poole, UK). Arachidonic acid and U46619 were purchased from Cayman Chemical Company (Michigan, USA). The PAR1 peptide (SFLLRN) was purchased from Severn Biotech (Kidderminster, UK). Collagen was purchased from Takeda (Austria) and luciferin luciferase reagent (Chrono-lume) was purchased from Chrono-log Corporation (Havertown, PA, USA). The reagents were dissolved in phosphate-buffered saline (PBS) at pH 7.4 and stored as frozen aliquots, thawed and

diluted in PBS when required and kept on ice. Collagen was stored as a concentrated stock at 1 mg/ml as supplied by the manufacturer at 4°C and diluted with the buffer provided.

Blood Sampling

Whole blood (40 ml from adults and 20 ml from children) was obtained and anticoagulated with one tenth volume of 0.109 mol/L buffered trisodium citrate in vacutainer plastic tubes (Becton Dickinson, Plymouth, UK). An additional 2-3 ml blood was anticoagulated with EDTA in vacutainer plastic tubes (Becton Dickinson, Plymouth, UK). The same volume of blood was collected from a healthy control at the same time in each Centre. Blood samples were immediately transported to the testing laboratory and assayed within 4 hours (by MEA) and within 6 hours (by lumi-LTA) from collection.

Blood sample preparation

PRP was prepared by centrifuging the citrated blood samples at 200 g for 20 min. PRP was transferred carefully into a 15 ml Falcon tube, capped and stored upright at room temperature. Platelet-poor plasma (PPP) was prepared by further centrifugation at 1,000 g for 10 min and transferred into a 15 ml Falcon tube, capped and stored upright at room temperature. About 2-3 ml of the buffy coat layer was also taken from the same tubes and stored at -80^{o}C for DNA analysis.

Whole blood platelet counting using the Sysmex XN-1000 analyser

EDTA blood samples from patients and controls were analysed using the XN-1000 whole blood counter (Sysmex UK, Milton Keynes). The XN-1000 is a state of the art whole blood analyser that is capable of rapidly producing a full blood differential cell count from 88µl of

human blood. The analyser has a set of unique platelet parameters including three platelet counts: platelet impedance (PLT-I), platelet optical (PLT-O) and platelet fluorescence (PLT-F) count, platelet mean volume (MPV) and the immature platelet fraction (IPF). Platelet counts, MPV and IPF were recorded for each patient and compared against reference ranges established from 40 healthy normal controls. Quality assurance of the instrument was checked by an internal quality control using commercial fixed samples (Xn check, Sysmex UK, Milton Keynes) with known cell counts and testing samples provided by an external quality assessment scheme (UKNEQAS, Watford, UK).

Platelet counting of PRP

An impedance analyser (Coulter Z2 Beckman Coulter, High Wycombe, Bucks, UK) was used to count the platelets within the PRP by diluting 5 µl of PRP into 10 ml of fresh Isoton II diluent (Beckman Coulter, Bucks, UK). The platelets were counted in triplicate and the mean was calculated and recorded.

Platelet aggregation in whole blood

Whole blood impedance aggregometry was performed on citrated blood using MEA (Roche Diagnostics, Munich, Germany) consisting of 5 reaction channels, an integrated computer, and guided automatic pipetting. The tests were performed in disposable ready-to-use test cells with 2 independent sensor units consisting of 2 silver-coated highly conductive copper electrodes (Roche Diagnostics, Munich, Germany). In each test cuvette, mixed whole citrated blood (300 µl) was immediately diluted, 1:1 vol/vol, with 0.9% saline solution and incubated at 37°C for 3 min before addition of 30 µl of each agonist. Platelets were stimulated with a range of agonists including ADP (10 and 2.5 µM), PAR-1 peptide (100 µM),

collagen (1 and 3 μg/mL) and arachidonic acid (0.5 mM). During the test, platelets adhere and aggregate upon the sensor electrode surfaces resulting in an increase in the electrical resistance (impedance) and the signal recorded and plotted as aggregometer trace. The total aggregation measured with this device is quantified as AUC or AU*min.

Platelet aggregation in platelet-rich plasma

Tests were carried out as described previously [7]. Aggregation studies were performed by using a dual Chrono-log lumiaggregometer (model ChronoLog 460 VS aggregometer, LabMedics, UK) in 400 μL mini cuvettes and stirred at 1200 rpm at 37°C. The 100% line was set using autologous platelet-poor plasma (PPP) and the 0% baseline established with native undiluted PRP. After one min platelets were stimulated with ADP (10, 30, and 100 μM), adrenaline (10, 30, and 100 μM), arachidonic acid (0.5, 1, and 1.5 mM), U46619 (1 and 3 μM), collagen (1 and 3 μg/mL), CRP (1, 3, and 10 μg/mL), PAR-1 peptide (10, 30 and 100 μM), and ristocetin (1.5 and 2 mg/mL). Platelet aggregation was monitored by measuring the change in optical density over 5 min after addition of the agonists and the maximal percentage of aggregation was recorded.

ATP secretion

ATP secretion from platelet dense granules was assessed simultaneously by lumi-LTA using the luciferase reagent (Chronolume). 30 μl of Luciferin reagent was added to the PRP and incubated for 1 minute. Platelets were then stimulated with the agonists listed above and monitored for 5 min followed by addition of 4 μl of an ATP standard (2 μM final concentration) to facilitate internal calibration. Secreted ATP levels were calculated by measuring the maximal amplitude of luminescence during the aggregation to a high dose of

PAR-1 peptide (100 μM) and comparing to the standard. A low cut-off point of 0.65 nmol ATP / 10^8 platelets was established from previously studied healthy volunteers.

Statistical analysis

All control results were run on IBM SPSS Statistics version 20 and Graph Pad Prism version 7.0 software to demonstrate the normal distribution and to obtain the mean and standard deviation values which were then used to determine the cut-off values. Abnormal results by Lumi-LTA were determined based on our previously published methodology that is based upon both the magnitude and time course of response [7, 11]. The time course, including reversibility is of particular importance. In contrast, because of the lack of these detailed kinetic changes detected by MEA, abnormal results were determined based on the cut off values calculated as the 5^{th} percentile of responses of normals to ADP 10 μM, collagen 1 μg/ml, collagen 3 μg/ml, PAR1 100 μM and arachidonic acid 0.5mM were 19.1, 21, 35.1, 42.3 and 19.7 (AUC), respectively

Results

Overall comparison between MEA and Lumi-LTA

We analysed a total of 109 patients (21 male and 88 female) with a median age 33 (range 3-73) and 40 healthy adult volunteers (18 - 57) recruited into the GAPP study and compared MEA results with lumi-LTA. The overall haematological parameters and ATP secretion value of patients and healthy individuals are shown in Table 1. Of 109 patients tested (Table 2), 54 (49%) patients gave abnormal responses by lumi-LTA to one or more agonists as defined using previous criteria [11]. In contrast, only 16 (15%) patients were shown to have abnormal responses to one or more agonists by MEA, as defined using the cut off values

obtained from healthy individuals. The overall agreement between the two instruments is shown in Table 3. 65/109 samples gave identical results by both tests, with the majority (52) giving normal responses. In contrast, there was disagreement in 44/109 samples. MEA detected an abnormality in 3 patients that were normal by lumi-LTA. However, 41 samples were normal by MEA but abnormal by lumi-LTA (figure 1). In 12 (11%) patients with mild thrombocytopenia as characterized by low platelet count (<150 $x10^9$/L) in whole blood (Table 2), abnormal aggregation responses were detected by lumi-LTA on 8 patients and by MEA on 6 patients. In 20 patients with secretion defects characterized by reduced ATP levels (Table 2), the lumi-LTA and MEA aggregation responses were abnormal in only 8 and 3 patients respectively. MEA is therefore less sensitive than lumi-LTA at detecting secretion defects which is not surprising in that MEA does not provide a direct readout of secretion.

Correlation between platelet counts and aggregation results by MEA and lumi-LTA

With MEA we found a significant but weak correlation between whole blood platelet counts and aggregation results in response to ADP 10µM (r=0.14, p= 0.016) but not with collagen 3 µg/ml (r= 0.072 p= 0.09), PAR-1 100µM (r= 0.043 p= 0.19) and arachidonic acid (r= 0.15 p= 0.096) (Supplementary figure 1). In contrast, with lumi-LTA, a weak correlation between the PRP counts and aggregation was only found in response to collagen 3µg/ml (r= 0.13 p= 0.02) but not with ADP 10µM (r=0.025, p= 0.33), PAR-1 100 (r= 0.026 p= 0.32) and arachidonic acid 0.5mM(r= 0.092 p= 0.22 (Supplementary figure 2).

Analysis and identification of patients with various platelet function defects

Functional defects identified by lumi-LTA were classified into 4 main groups (Gi defect, secretion defects, COX-like defect and unclassified defects) according to their pattern of responses to specific agonists as previously described [11]. As shown in figure 2, MEA only detected 4 out of 17 patients with Gi defects and 2 out of 20 with secretion defects. MEA only detected 4/7 patients with a COX-like defect and none/4 patients with unclassified defects by lumi-LTA. Finally MEA gave abnormal responses to 3 samples out of 6 with multiple platelet defects.

Correlation between MEA and lumi-LTA with identical concentrations of agonists

To give a fairer comparison of the two tests, identical concentrations of the agonists ADP, collagen and PAR1 peptide were compared by both tests (with no ATP measurements included) and confirmed that MEA was still unable to detect some abnormalities detected by lumi-LTA 16 (15%) versus 41 (38%). Overall kappa statistics demonstrated only a fair agreement between MEA and lumi-LTA in response to ADP (k = 0.26, CI = 0.11 to 0.40) and collagen (k = 0.28, CI = 0.05 to 0.52) and a poor agreement in response to PAR1 (k = 0.07, Cl = -0.17 to 0.32) (Figure 3).

Discussion

MEA performs platelet aggregation in whole blood and offers the advantage of assessing platelet function with more physiological conditions in small blood volumes and without sample processing [10]. As MEA is popular for measuring antiplatelet therapy and has been shown to be useful for detecting severe PFDs, we assessed the potential utility of MEA for detecting mild PFDs [12-15].

Comparison of the overall results in 109 patients with mild PFDs (Table 3) showed a moderate agreement between MEA and lumi-LTA with the diagnosis being concordant in 60% of cases. The underlying genetic causes of these cases are yet to be confirmed by sequencing. Although MEA therefore gave a negative predictive value of 56%, in healthy individuals there was good agreement between MEA and lumi-LTA with no difference in response between identical concentrations of the agonists ADP, collagen and PAR-1 peptide (Figure 3). Our findings support a recent study by Seyfert et al., demonstrating equivalent results of MEA and lumi-LTA in healthy samples [16]. In patients' samples however, we observed significant differences between the two techniques with a fair to poor agreement between MEA and lumi-LTA with different agonists when overall results were analysed using kappa statistics.

As MEA dilutes the whole blood 1:1 with saline before testing we also investigated the influence of platelet counts (within normal ranges) on both MEA and lumi-LTA results. With MEA a linear increase in aggregation responses with higher counts was only observed with ADP but not collagen, PAR-1 or arachidonic acid. In contrast with lumi-LTA this was only observed with collagen but not ADP, PAR-1 or arachidonic acid (See supplementary figures). Previous studies with lumi-LTA have found no correlation between platelet count and aggregation results [7, 16-18]. Seyfert et al. also found a significant correlation between platelet count and platelet aggregation using MEA induced by ADP and collagen, but not by arachidonic acid [16]. Mengistu et al. and Stissing et al. also demonstrated the influence of platelet count on whole blood aggregometry towards low platelet concentrations [19, 20]. Two more studies also reported correlations between platelet count and extent of platelet aggregation on MEA [18, 21]. However, the latter results are difficult to interpret as the

counts were adjusted either by addition of PPP [21] which is known to inhibit platelet aggregation [17], or with dilution with Tyrode's buffer [18].

We found that MEA also detected more patients classified with COX-1 like defects than those with Gi and secretion defects. This suggests that MEA may be more sensitive in response to defects in the thromboxane pathway. However, the lack of sensitivity of MEA in detecting Gi defects may also be related to the higher ADP concentration (10 µM) used in this study which is slightly higher than the standard recommended concentration (6.5 µM). Interestingly, we have also observed that MEA showed high consistency in detecting abnormalities in patients with dual defects. This findings support the earlier reports suggest that MEA is reliable in detecting more severe forms of platelet defects such as GT [12, 13].

Measurement of secreted ATP levels is an additional but important tool in identifying patients with platelet secretion defects [22]. Indeed the Chronolog whole blood aggregometer can also be used in conjunction with ATP secretion [23]. Moreover, it is well known that individuals with reduced dense granule secretion can often demonstrate normal aggregation responses [11]. In this study MEA and lumi-LTA gave normal responses on 17(85%) and 12(60%) patients respectively that were found to have low secreted ATP levels. This is probably unsurprising given that MEA does not directly measure ATP secretion and the aggregation responses are probably less sensitive at detecting the feedback loop from released ATP/ADP from the dense granules.

Hirudin is the anticoagulant of choice for MEA recommended by the manufacturer [10]. Previous studies have shown that different anticoagulants can affect platelet function activity [24]. In this study we only used citrated blood for both techniques in order to have a similar comparison with identical levels of free calcium levels. Furthermore, a study using

citrated blood from healthy individuals showed no significant difference between MEA and LTA even at higher citrate concentrations [16]. Storage time between blood collection and sample processing is a crucial factor when testing platelet activity particularly on whole blood aggregometry. To avoid variation of results due to time delays, MEA was always tested during PRP/PPP preparation for LTA to ensure that the analysis was always completed rapidly within 4 hours [16, 25].

Study limitations

Our study was performed using only trisodium citrate anticoagulant in blood samples tested by LTA and MEA. The study was also performed on individuals with suspected mild platelet defects with a bleeding history. It would be of interest to fully investigate the utility of MEA in patients with a defined set of pre-diagnosed platelet function disorders. ADP was also used at a higher concentration of 10 μM above the recommended concentration of 6.5 □M for MEA and may have contributed to the lack of sensitivity observed.

Conclusions

Overall, MEA demonstrates a lack of sensitivity in identifying patients with mild bleeding associated with abnormal platelet function and platelet secretion defects. More studies are required to further evaluate the role for MEA in the diagnosis of bleeding disorders.

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Authorship

Contribution: R.A. Performed research, analyzed data, and wrote the paper; S.D. performed research and analyzed data; S.P.W. designed the research and contributed to writing the paper; N.V.M. designed the research and contributed to writing the paper; and P.H. designed the research, analyzed data and contributed to writing the paper.

Disclosure of conflict of interest

The authors report no declarations of interest

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Tables

Table 1 Baseline characteristics of healthy individuals (n=40) and patients (n=109).

	Healthy individuals	Patients
Platelet count in Whole Blood (x10^9/L)	234.5 ± 54	237.9 ± 52.5
	(150 - 363)	(101 - 360)
Platelet count in PRP (x 10^8/L)	3.27 ± 1.25	3.61 ± 0.95
	(1.2 - 7.68)	(1.6 - 6.08)
Mean Platelet Volume (fL)	11.7 ± 1.1	11.3 ± 1.2
	(8.3 - 13.4)	(9.2 - 14.0)
ATP secretion level (nmol/1x10^8 platelets)	1.1 ± 0.33	1.02 ± 0.43
	(0.64 - 2.21)	(0.19 - 2.2)

Data are presented as absolute numbers (percentages) or means (standard deviations)

Table 2 Comparison between MEA and lumi-LTA on overall results, patients with thrombocytopenia and patients with reduced ATP levels

	No of patients	MEA	Lumi-LTA
Overall results (normal:abnormal)(%)	109	93:16 (85:15)	55:54 (51:49)
Patients with thrombocytopenia (%)	12	6 (50)	8 (67)
Patients with reduced ATP secretion (%)	20	3 (15)	8 (40)

Table 3 Contingency table showing the overall agreement between the MEA and lumi-LTA in patient samples (n=109)

		Lumi-LTA		
		Abnormal	Normal	
MEA	Abnormal	13	3	PPV=81%
	Normal	41	52	NPV=56%
		Sensitivity=24%	Specificity=95%	

Figure legends

Figure 1 Platelet function assessed by MEA (AUC) and lumi-LTA (%) in samples from healthy subjects (n=40) and patients (n=109) after stimulation with, ADP 10 µM, Collagen 3 µg/ml, PAR-1 peptide 100 µM and Arachidonic Acid 0.5 mM (healthy subjects= 19 and patients =36). The horizontal black lines indicate cut-offs determined as 5^{th} percentile of the corresponding measurement in healthy volunteers. In the patient groups: closed circles indicate individuals with an abnormality detected by lumi-LTA, and closed squares indicate an abnormality detected by MEA.

Figure 2 Bar charts show the number of patients with various platelet function defects detected by lumi-LTA (black) and MEA (grey) The response to each agonist was considered to be abnormal (on MEA) when it falls below the cut off value that was set from healthy individuals and (on lumi-LTA) as defined using previously published criteria [11].

Figure 3 Correlation between MEA (AUC) and lumi-LTA (%) in samples from healthy subjects (n=40, open circles) and patients (n=109, closed circles) after stimulation with, ADP 10µM, Collagen 3µg/ml and PAR-1 peptide 100. The vertical black lines indicate the cut-offs value of MEA determined as 5^{th} percentile of the corresponding measurement in healthy subjects. The horizontal black lines indicate cut-offs value of lumi-LTA previously established in the GAPP program using samples from healthy subjects [11].

Figure 1

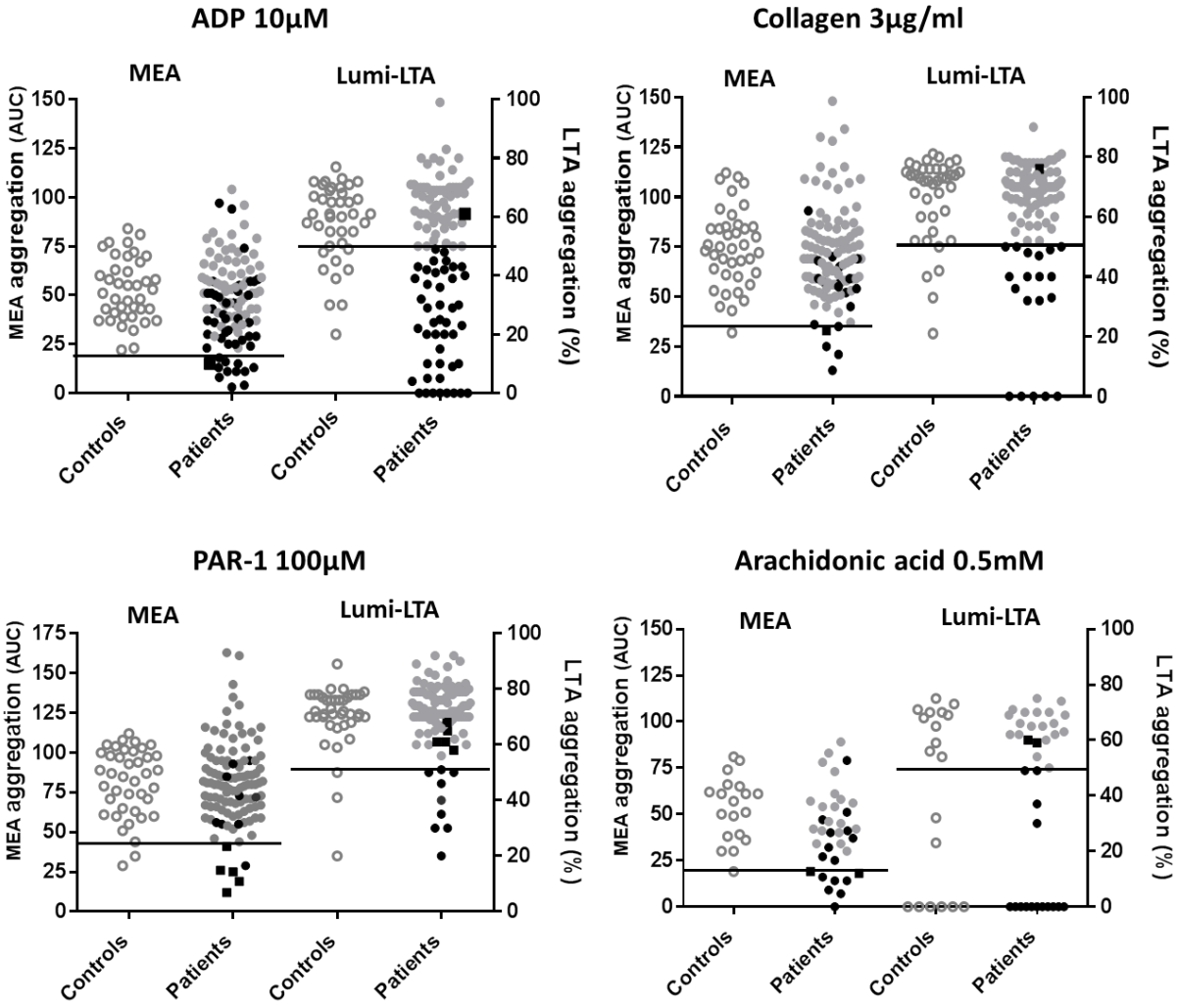

ADP 10µM
MEA
Lumi-LTA
MEA aggregation (AUC)
LTA aggregation (%)
Controls
Patients
Controls
Patients
Collagen 3µg/ml
MEA
Lumi-LTA
MEA aggregation (AUC)
LTA aggregation (%)
Controls
Patients
Controls
Patients
PAR-1 100µM
MEA
Lumi-LTA
MEA aggregation (AUC)
LTA aggregation (%)
Controls
Patients
Controls
Patients
Arachidonic acid 0.5mM
MEA
Lumi-LTA
MEA aggregation (AUC)
LTA aggregation (%)
Controls
Patients
Controls
Patients

Figure 2

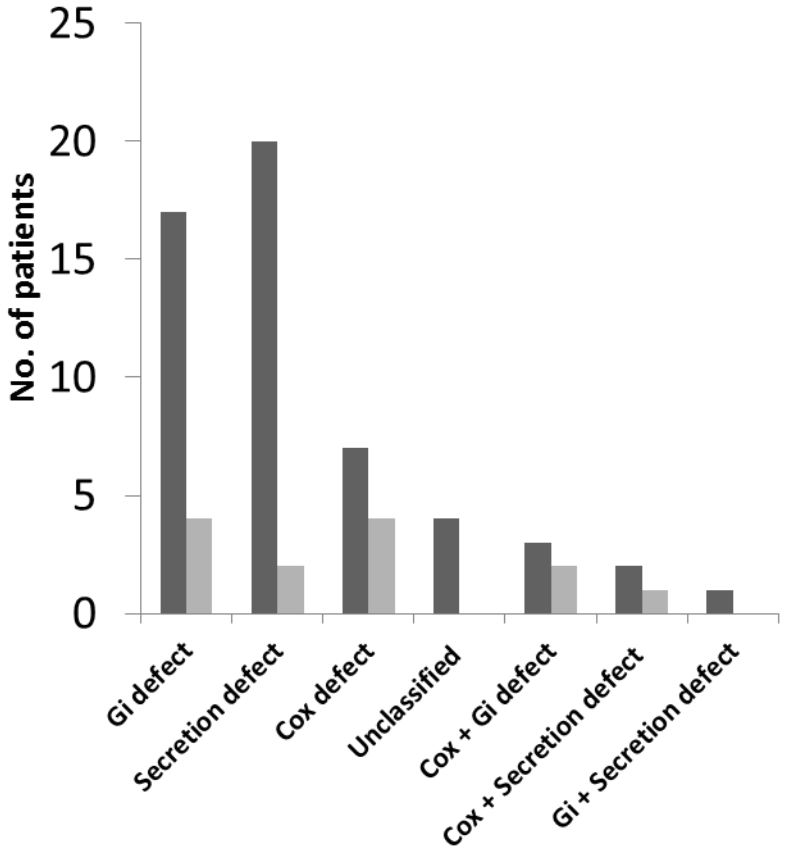
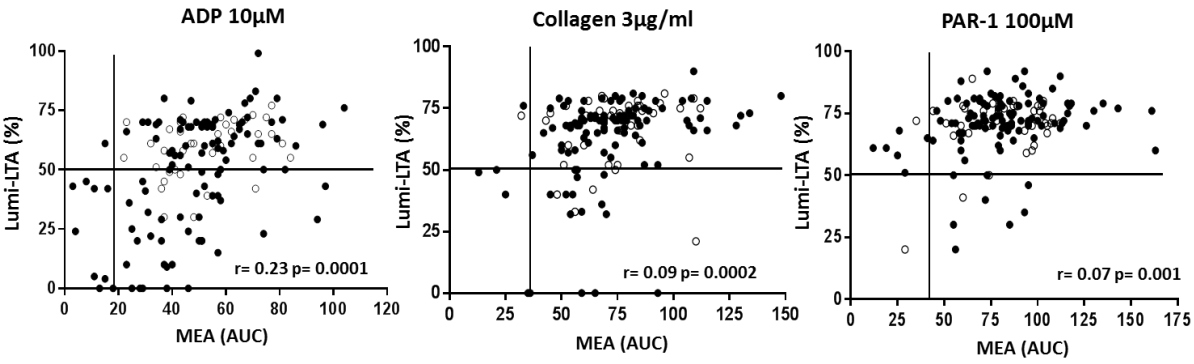
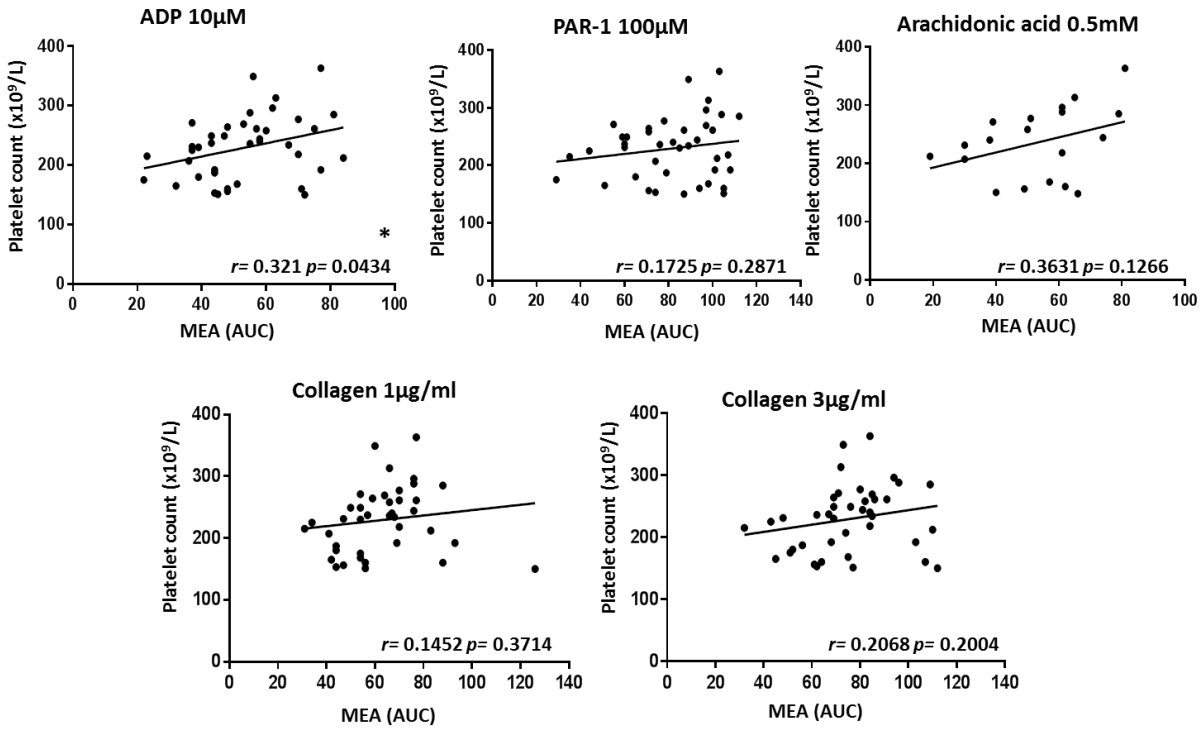


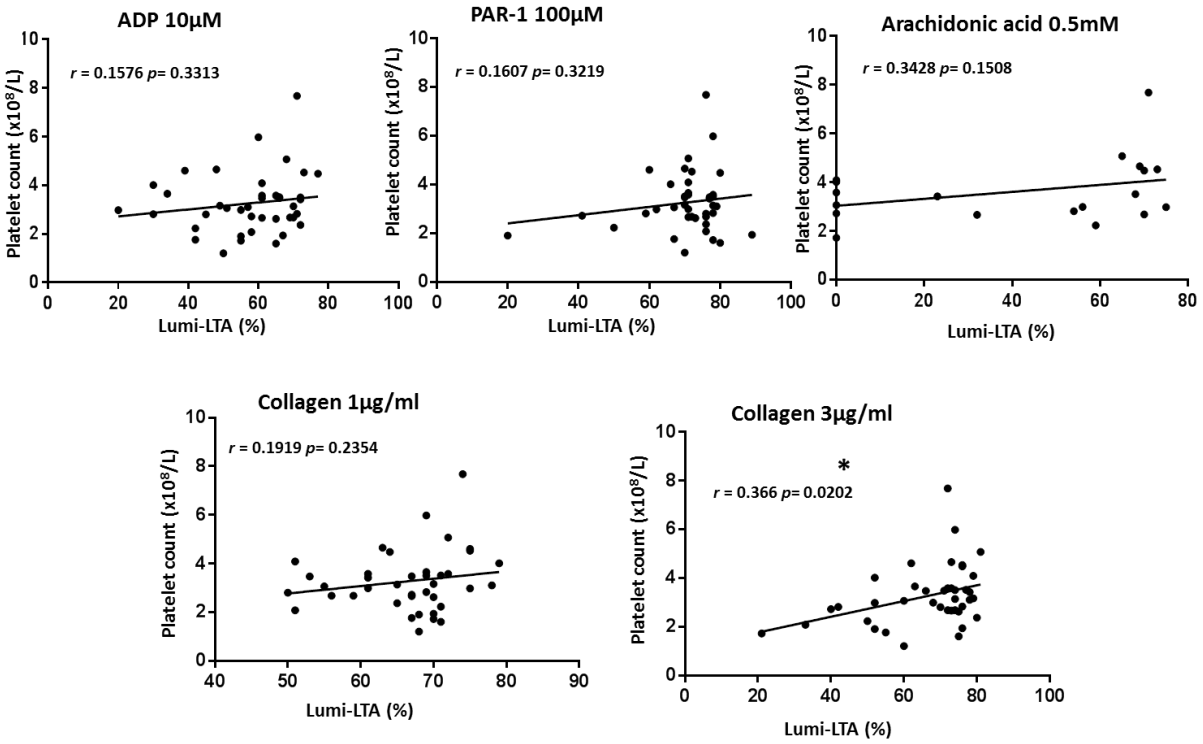
Figure 3



Supplementary figures



Supplementary figure 1 Correlation between MEA (AUC) and whole blood platelet count (x10^9/L) in samples from healthy subjects (n=40) after stimulation with ADP 10µM, Collagen 3µg/ml, Collagen 1µg/ml, PAR-1 activating peptide 100µM and Arachidonic Acid 0.5mM.



Supplementary figure 2 Correlation between Lumi-LTA (aggregation %) and PRP count (x10^8/L) in samples from healthy subjects (n=40) after stimulation with ADP 10µM, Collagen 3µg/ml, Collagen 1µg/ml, PAR-1 activating peptide 100µM and Arachidonic Acid 0.5mM.