Utility of a whole blood single platelet counting assay to monitor the effects of tirofiban in patients with acute coronary syndromes scheduled for coronary intervention

Anjan Siotia, Robert Buckland, Heather M. Judge, Padmini Sastry, Robert F. Storey
Cardiovascular Research Unit, University of Sheffield, Clinical Sciences Centre, Northern General Hospital, Sheffield, United Kingdom

Summary
This study aimed to establish the utility of a whole-blood single-platelet counting (WBSPC) assay, a measure of microaggregation, in monitoring the effects of tirofiban, comparing this with optical aggregometry (OA) and the Ultegra TRAP cartridge system (UTC), measures of macroaggregation. Fifty-nine patients with acute coronary syndrome scheduled for coronary angiography +/- angioplasty were studied. WBSPC assay (ADP 0.3–100 µM, Sysmex KX21 analyzer), OA (ADP 20 µM) and UTC were performed: before starting tirofiban; 30 min, 4 and 24 h after starting tirofiban; and 1 and 2 h after stopping tirofiban. Thirty minutes after starting tirofiban, there was substantial inhibition of platelet aggregation (40 ± 30%; WBSPC, 2 minutes after addition of ADP 30 µM) and this remained stable at 4 and 24 h. OA (86 ± 17%; inhibition of maximal aggregation, ADP 20 µM) and UTC (93 ± 7%) showed marked inhibition with less inter-individual variation. There was no significant correlation between OA and UTC results (R² = 0.006), but fair correlation between OA and WBSPC results (R² = 0.37). Greater inhibition of macroaggregation (OA and UTC) was seen compared to microaggregation (WBSPC) such that WBSPC was more discriminating in the therapeutic range when macroaggregation was often completely inhibited. A WBSPC assay of platelet microaggregation shows promise for monitoring GPIIb/IIIa antagonists.

Keywords
Platelets, tirofiban, whole blood single platelet count, micro-aggregation.

Introduction
Glycoprotein IIb/IIIa (GPIIb/IIIa) antagonists have been shown to reduce ischaemic events in patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI) (1–5). However, there is significant inter-individual variation in the degree of platelet inhibition obtained using standard dose regimens of GPIIb/IIIa antagonists and, in particular, an unpredictable response in patients with renal impairment (6). This raises important safety issues, particularly since patients receive a combination of aspirin and clopidogrel as standard therapy now, in addition to heparin, which may reduce the tolerability of GPIIb/IIIa antagonists in those patients who have the highest response in terms of platelet inhibition. Furthermore, patients with poor platelet inhibition response to GPIIb/IIIa antagonists may not receive the optimal dose, if they continue to be treated with standard dosing regimens. Using the Ultegra point-of-care device (7), evidence has been provided that lower levels of platelet inhibition are associated with higher risk of major adverse cardiac events (MACE) related to PCI (8). Many other studies (3, 9–13) have suggested that potent platelet inhibition may be a key determinant of the efficacy of GPIIb/IIIa antagonists in patients undergoing PCI. It has been suggested that the superiority of abciximab over tirofiban in the TARGET study was due to inadequate dosing of tirofiban with suboptimal inhibition of platelet aggregation in the first few hours of percutaneous coronary intervention (14). It may therefore be preferable to monitor the inhibition of platelet aggregation in patients receiving GPIIb/IIIa antagonists and adjust the dose to achieve a predefined optimal level of platelet inhibition.

Whilst the Ultegra analyser offers the potential of point-of-care monitoring and is currently the only assay shown to correlate with clinical outcomes in patients treated with GPIIb/IIIa antagonists, it is limited in its ability to discriminate platelet in-
hibitory effects at the higher end of scale as are achieved by current therapeutic regimens of GPIIb/IIIa antagonists (6). Thus, if one were to conduct a study exploring the safety and efficacy of higher levels of platelet inhibition to cover a PCI procedure, achieved by tailoring the dose according to assay results, then an assay such as Ultegra may not be very useful, as the standard dose of a GPIIb/IIIa antagonist would show most patients to have achieved near-maximal platelet inhibition. An assay of platelet microaggregation based on whole blood single platelet counting (WBSPC) is suitable for monitoring the therapeutic effects of GPIIb/IIIa antagonists and appears to define well the range of inhibitory effects of therapeutic regimens (6, 16).

We carried out this prospective clinical study to establish the utility of a WBSPC assay, in comparison with optical aggregometry and the Ultegra TRAP cartridge system, for monitoring the effects of tirofiban on platelet microaggregation in patients admitted to hospital with ACS and scheduled for coronary angiography and possible PCI.

Materials and methods

Patients
This was a single centre study based at the Northern General Hospital, Sheffield, UK. Ethical approval was obtained from the North Sheffield Research Ethics Committee. Following informed consent, 67 patients with ACS who were scheduled for coronary angiography and, where appropriate, PCI, were enrolled for a study of platelet aggregation monitoring while receiving tirofiban (Aggrastat). All these patients had either a raised serum troponin level or a TIMI risk score ≥ 3. None were on a GPIIb/IIIa antagonist at the time of enrolment. Patients who had a contraindication to GPIIb/IIIa antagonist, such as active bleeding, recent stroke, platelet count < 140 x 10^9/ml or renal failure with acratinine > 200 µM, were excluded from the study. Baseline characteristics of all patients were recorded including co-medications, cardiac history and concurrent illnesses. Serum biochemistry and full blood counts were performed at baseline, as per normal practice.

Study protocol
tirofiban was administered at the dose of 0.4 µg/kg/min for 30 min followed by 0.1 µg/kg/min. Nine ml venous blood was taken and anticoagulated with 1 ml hirudin (recombinant desulphathirudin in saline, final concentration 50 µg/ml) in polystyrene tubes at the following time points: 1) Baseline sample, before starting tirofiban; 2) At 30 minutes, following the intravenous loading dose of tirofiban; 3) At four hours after onset of tirofiban infusion; 4) At the time of PCI in those patients proceeding to PCI (in nearly all cases, this coincided with time point 3 as tirofiban was commenced in the morning prior to PCI in the afternoon and these time points are therefore presented together); 5–7) Whenever possible (in patients proceeding to PCI and continuing tirofiban), prior to termination of the infusion the next day, and at one hour and two hours after termination of infusion. The blood was incubated at 37°C prior to performing platelet function tests.

Platelet function tests

The following platelet function tests were performed:

- (1) Whole blood single platelet counting assay using ADP 0.3, 1, 3, 10, 30, and 100 µM as agonist, stirring at 1,000 rpm for 4 min at 37°C prior to fixing [in saline with 4.6 mM sodium EDTA, 4.5 mM Na₂HPO₄, 1.6 mM KH₂PO₄ and 0.16% (w/v) formaldehyde, pH 7.4] and counting using a Sysmex KX21 haematology analyzer. The total single platelet count was obtained using EDTA 4 mM instead of ADP Percentage aggregation was calculated as follows: [(EDTA count – ADP count) / (EDTA count) x 100].

- (2) Whole blood single platelet counting assay using ADP 30 µM as agonist, duplicate samples, stirring at 1,000 rpm for 2 min prior to fixing and counting using a Sysmex KX21 haematology analyzer. The 2 minute analysis was done, because it reduces the influence of clopidogrel on the measured platelet inhibition since a major effect of clopidogrel is to induce reversibility of platelet aggregation. Also, we have previously reported studies comparing this method with other assays (6).

- (3) Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by centrifugation. Optical aggregometry was performed (with a Biodata PAP-4D) using ADP 5 and 20 µM as agonist (the concentrations of ADP used in PRP and whole blood are not equivalent in view of the concentrating effect of haematocrit in whole blood) and recording aggregation for 4 min. Both maximum and final aggregation were recorded. Maximum aggregation values were used for subsequent comparison with other assays. This value was used because it is not so much affected by the antitplatelet effects of clopidogrel.

- (4) In some patients (n = 39), blood was also analysed using the Ultegra device with TRAP cartridges, according to the manufacturer’s instructions. Aliquots of hirudin-anticoagulated blood were placed in plain glass Vacutainer tubes for analysis using the Ultegra device.

PCI and co-medications

PCI was performed according to institutional standards. All the patients were on tirofiban for at least four hours prior to balloon inflation. All patients were on aspirin 300 mg once daily and clopidogrel 75 mg once daily. The majority of patients (n = 54) were at steady state levels of inhibition following initiation of clopidogrel more than 48 hours previously (clopidogrel 300 mg loading dose then 75 mg daily), and all patients had received a loading dose of clopidogrel (300 mg) before the start of the study. Heparin was used during the procedure to keep the activated clotting time (ACT) > 200 s. All patients were on other medications including statins, β-blockers, ACE-inhibitors, nitrates, and calcium channel blockers.

Statistical analysis

The results are presented as % inhibition calculated by the following formula: [(baseline aggregation or PAU – measured aggregation or PAU)/baseline aggregation or PAU] x 100. Data were analysed as mean and standard deviation. Correlations between assays were performed using Microsoft Excel.
Results

Study population
Sixty-seven patients who were admitted with a diagnosis of ACS and were scheduled for coronary angiography, with a view to PCI if appropriate, were enrolled in the study. Of these, 63 patients proceeded with tirofiban infusion following baseline platelet function tests and recording of demographic data. Mean age of the patients was 62 years (range 42–80). Forty-six were males and 17 females. Of the 63 patients, two were treated diabetics, 29 were hypertensive, and 50 had hypercholesterolaemia. Forty-eight patients had raised troponin levels at the time of enrolment into the study. Four patients did not have further blood samples taken due to withdrawal of consent or early transfer to the cardiac catheterization suite for angiography, leaving 59 patients suitable for final analysis. Of these 59 patients, 37 underwent PCI and continued tirofiban infusion following the procedure. The remaining 22 patients had their tirofiban infusion stopped following the angiogram and hence did not undergo further platelet function tests for subsequent time points.

Whole blood single platelet counting assay 1
Figure 1 shows the results of the first whole blood microaggregation assay, assessing the inhibition of platelet aggregation in response to a range of ADP concentrations at 4 min after addition of tirofiban. The mean combined data shows a rapid onset of action of tirofiban with steady-state inhibition of aggregation seen following the 30 min loading infusion (mean inhibition 51 ± 33%, ADP 30 µM), which remained stable at 4 and 24 hours. There was marked variability in the baseline aggregation results prior to administration of tirofiban (79 ± 19%, ADP 30 µM). Aggregation induced by the lower concentrations of ADP was more substantially inhibited than aggregation induced by the higher concentrations, such that a concentration of ADP 3 µM appeared most favourable for detecting the effects of lower levels of tirofiban following termination of infusion. There was substantial and progressive recovery of aggregation at one and two hours following termination of infusion, particularly in response to the higher concentrations of ADP.

Whole blood single platelet counting assay 2
Results for the second whole blood microaggregation assay, assessing the effects of ADP 30 µM at 2 min after addition of ADP, were, not surprisingly, similar to results of the first assay. The mean percentage inhibition ± standard deviation for different timepoints for 30 µM ADP is shown in Figure 2. As with the other two methodologies, there was a wide range of variation in baseline samples (WBSPC 30 µM ADP, 2 min 87.5 ± 15.3%; OA, 20 µM ADP Max. 45.1 ± 18.2%; Ultegra 175 ± 50.4 PAU), and there was substantial inter-individual variation in the levels of inhibition seen during tirofiban infusion.

There was excellent agreement between duplicate samples indicating the reproducibility of the assay (R² = 0.98) (for a total of 212 duplicate samples). The mean difference between the duplicate samples was 2.6 ± 2.7% (range 0–22).

Optical aggregometry
As with the whole blood single platelet counting assay, optical aggregometry demonstrated attainment of steady state inhibition of platelet aggregation at the end of the 30 min loading infusion of tirofiban (86 ± 17%; inhibition of maximal aggregation with ADP 20 µM) and stable inhibition throughout the course of the infusion (Fig. 2). Levels of steady-state inhibition of platelet aggregation (80–100%) mirrored those seen in clinical trials. Recovery of platelet aggregation occurred rapidly after termination of infusion.

Ultegra TRAP cartridge measurements
Again, the Ultegra TRAP cartridge system showed attainment of steady state inhibition of platelet aggregation at the end of the 30 min loading infusion of tirofiban (93 ± 7%), and stable inhibition throughout the course of the infusion (Fig. 2). Most measurements (96%) showed more than 80% inhibition compared to baseline.
Comparison between different assays
The levels of mean inhibition seen with optical aggregometry and Ultegra assays were the same whilst the WBSPC assays showed lesser levels of inhibition, as expected. However, correlation of the individual results yielded a different picture. There was some correlation between the results obtained from WBSPC and optical aggregometry (Fig. 3) ($R^2 = 0.37$), allowing for the relationship between microaggregation and macroaggregation (15). It was possible for substantial microaggregation to occur even when final levels of macroaggregation were completely suppressed as can be seen by the spread of data from WBSPC compared to that from OA. Direct correlation appears to show poor relationships between the results obtained with the Ultegra TRAP cartridge system and either optical aggregometry (Fig. 4, $R^2 = 0.006$) or WBSPC (Fig. 5, $R^2 = 0.0016$).

Discussion
Inhibition of platelet aggregation has resulted in one of the most important therapeutic advances in the history of cardiovascular medicine (17). Although GPIIb/IIIa antagonists are the most effective inhibitors of platelet aggregation available at the present time, the evidence available on these drugs is mixed. Most studies have shown a beneficial effect but a few are neutral and a few have shown harm (18). There is sufficient evidence now to suggest that the clinical efficacy of these drugs is closely related to the level of platelet inhibition. Clinical trials of GPIIb/IIIa antagonists that have shown clinical benefit have aimed for more than 80% inhibition of platelet aggregation as determined by optical aggregometry, although the optimal level for an individual patient is not known. Failure to achieve this high level of inhibition is associated with lack of protection from ischaemic events and this may explain the findings of some of the studies that showed negative results with GPIIb/IIIa antagonists. The IMPACT-II trial (19), which used a low dose of eptifibatide and achieved a receptor blockade of 50% only (20), showed a benefit of 18% relative risk reduction, as opposed to the ESPRIT trial (21), which used a much higher dose of eptifibatide that resulted in a 35% relative risk reduction. The potential importance of monitoring platelet inhibition in patients receiving GPIIb/IIIa antagonists was demonstrated in the GOLD study (8), which used the Ultegra point-of-care system (7).

The relationship between inhibition of platelet microaggregation and platelet macroaggregation, and hence the relationship between the results of optical aggregometry and WBSPC, has been demonstrated previously (6, 14) and shows fair correlation between the two methods, indicating that WBSPC may provide a reliable alternative to optical aggregometry, the standard method of assessing the effects of antplatelet therapies. Generally, 80%
inhibition of ADP-induced macroaggregation by GPIIb/IIIa antagonists yields 20–50% inhibition of microaggregation, the exact relationship depending mainly on the antagonist being studied and the in vitro anticoagulant used. Thus, as inhibition of macroaggregation approaches 100%, inhibition of microaggregation reaches the middle range of inhibition, as clearly demonstrated in this study. Trials of GPIIb/IIIa antagonists have generally aimed to achieve 80–100% inhibition of ADP-induced macroaggregation (as measured by optical aggregometry), explaining why the microaggregation assay in this study showed mean values of inhibition of aggregation in the middle range following GPIIb/IIIa antagonist administration. This allowed the assay to discriminate most effectively between different degrees of inhibition in different patients. The assay was also highly reproducible and, in the form used for comparisons here, like the Ultegra, gives results in 5–10 min compared to 30 min for optical aggregometry. This study, therefore, indicates that the whole blood macroaggregation assay has good potential for monitoring the effects of GPIIb/IIIa antagonists and warrants further study to determine its clinical role in optimising GPIIb/IIIa antagonist therapy.

Our study showed that the Ultegra TRAP cartridge system and optical aggregometry show clustering of results in a narrow range of inhibition following initiation of tirofiban as well as poor correlation between each other. The poor correlation between Ultegra and OA give a different picture to the data presented in Figure 2, which shows almost overlapping curves for the two assays. However, the overlapping curves only indicate that the average inhibition (for all patients taken together) at different time-points as measured by these two assays were high and nearly the same as each other. On the other hand, the correlation figure (Fig. 4) shows that the individual patient results of Ultegra and OA did not correlate well. This finding is contrary to other studies that have shown good correlation between Ultegra and, not only OA, but also the GP IIb/IIIa receptor binding assay (22). The likely explanation for this is that previous studies have included subtherapeutic concentrations of GPIIb/IIIa antagonist in the correlation determination, and we have found that this enhances the correlation (unpublished data). However, our primary interest is to assess which method, if any, is suitable for guiding the dose of GPIIb/IIIa antagonist for individual patients following a standard therapeutic dose, and our results raise serious concerns about which assay may be most appropriate. The answer to this must, therefore, lie in further clinical studies that assess the safety and efficacy of using a particular assay to guide the dose of antagonist. However, the OA and the Ultegra assays may both be useful for looking at residual inhibition after termination of infusion, for example in patients going for urgent surgery. In this study, we have shown that the WBSPC assay is also sensitive to lower levels of platelet inhibition by these drugs when a low concentration of ADP (such as 3 µM) is incorporated into the assay alongside higher concentrations.

In addition to these three methods of platelet function monitoring, flow cytometry can be used to measure platelet reactivity, circulating activated platelets, platelet-platelet aggregates, leukocyte-platelet aggregates, procoagulant platelet-derived microparticles, and calcium flux (23). It has been used for direct measurement of the binding of abciximab and the direct determination of the extent of blockade of the GP IIb/IIIa receptors on the individual platelet (24). In contrast, WBSPC determines the overall aggregatory function of the mixture of all platelets in the sample. To our knowledge, there are no studies comparing these two methods of platelet function monitoring. We have not used flow cytometry in our study as it is time-consuming, and the running and purchase costs are far greater than any of the technologies tested.

This study establishes the potential of a WBSPC assay in monitoring the level of platelet inhibition in patients receiving tirofiban and provides a basis for further studies of up titration of tirofiban dose during PCI according to the results of this assay. This study was not designed either to draw clinical conclusions or establish the superiority of one assay over another. Further studies to fully establish the role of the WBSPC assay in optimising GPIIb/IIIa antagonist therapy and to assess its usefulness in predicting clinical outcomes are warranted.

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