Prospective observational cohort study of the association between thromboelastometry, coagulation and platelet parameters and bleeding in patients with haematological malignancies- The ATHENA Study.

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Summary

Previous studies have shown that total platelet count (TPC) predicts bleeding poorly in thrombocytopenic patients with haematological malignancies. This prospective cohort study evaluated whether rotational thromboelastometry (ROTEM), coagulation or other platelet parameters were more strongly associated with bleeding than TPC. Adults treated at two UK haematology centres for haematological malignancy were enrolled if they had thrombocytopenia (TPC ≤ $50x10^9$/L) at beginning of, or during treatment(ISRCTN81226121). TPC and bleeding symptoms were recorded daily for up to 30 days or until platelet count recovery, hospital discharge or death. Blood samples were tested thrice weekly using ROTEM, Platelet Function Analyser (PFA)-100®, coagulation and platelet cytometry assays. Bleeding symptoms and TPC from 49/50 enrolled participants who completed the study were recorded on 754/760 study days. Mean platelet volume and PFA-100® closure times were frequently inestimatable because of thrombocytopenia. TPC, absolute immature platelet number (AIPN) and ROTEM maximum clot firmness were significantly associated with bleeding day after blood sampling. Only AIPN was associated with bleeding after adjustment of test results for TPC (OR 0.27, 95% CI 0.08-0.93; P=0.038). In a predictive model, AIPN was superior to TPC at predicting bleeding. This study indicates that AIPN may be more clinically useful than TPC at predicting bleeding.

Key words: Haemorrhage; Haematological Malignancies; Transfusion; Rotational thromboelastometry; Thrombocytopenia

Introduction

Haematological malignancies constitute 8 to 9% of all new cancers diagnosed in the UK and US (CDC 2012, ONS 2012), and have increased in incidence by 10 to 14% in the last decade (Cancer Research UK 2013). The prevalence of haematological malignancies is also increasing because advances in intensive chemotherapy and haemopoietic stem cell transplantation have improved survival rates (Burnett, *et al* 2011, Fielding, *et al* 2007, Milligan, *et al* 2006). Despite these developments, both haematological malignancies and their treatment may lead to periods of severe thrombocytopenia (≤ 50 x 10^9/L) (Heddle, *et al* 2009, Stanworth, *et al* 2013, Wandt, *et al* 2012). During this thrombocytopenic period patients are at risk of severe and life-threatening bleeding (Heddle, *et al* 2009, Slichter, *et al* 2010).

In an attempt to minimise bleeding, most haematology treatment centres across the developed world transfuse prophylactic platelet components to patients with haematological malignancy, usually when the platelet count falls below 10 x10^9/L (BCSH 2003, National Blood Authority 2012, Schiffer, *et al* 2001). Platelets are currently the second most commonly issued allogeneic blood component (312,000 adult doses in UK last year) (Bolton-Maggs, *et al* 2013), of which up to 67% are issued to patients with haematological malignancies (Greeno, *et al* 2007, Pendry and Davies 2011). The majority of these are given to prevent rather than to treat bleeding (Estcourt, *et al* 2012). In contrast to other blood components, demand for platelets has increased by 24% over the last decade in the UK, and by 25% in the United States from 2006 to 2011 (Bolton-Maggs, *et al* 2013, Report of the US Department of Health and Human Services 2011, Taylor, *et al* 2010, Whitaker and Hinkins 2013). Platelet components are a costly and limited resource

(Williamson and Devine 2013) and are associated with significant risks to recipients (Blumberg, *et al* 2009, Heddle and Webert 2009, Popovsky and Moore 1985).

In current practice, the decision to transfuse platelet components prophylactically is primarily based on the platelet count determined from daily blood tests. However, previous studies have shown that the risk of bleeding the following day is the same over a wide range of platelet counts (Slichter, *et al* 2010, Stanworth, *et al* 2013). Bleeding remains prevalent in patients with haematological malignancies despite the use of platelet count to direct platelet prophylaxis (Slichter, *et al* 2010, Stanworth, *et al* 2013, Wandt, *et al* 2012). In some patient groups, bleeding occurred at a similar frequency and severity in patients receiving platelet prophylaxis directed by platelet count to those not on prophylaxis regimes (Stanworth, *et al* 2013). The most plausible explanations for these observations are either that the platelet count is an inadequate marker of platelet haemostatic function in this clinical setting, or that other defects in haemostasis contribute significantly to bleeding.

The aim of our study was to evaluate whether other laboratory markers of haemostasis showed a stronger association with bleeding than the platelet count in severely thrombocytopenic patients receiving treatment for haematological malignancies. In order for laboratory markers to be clinically useful in directing treatments to prevent bleeding, each marker must be detectible using rapid, robust and inexpensive laboratory tests. Therefore, we evaluated a panel of markers that can be detected using point-of care format ROTEM thromboelastometry

and Platelet Function Analyser (PFA)-100 devices or by using automated coagulation and haematology analyser tests that are in widespread use in other clinical settings.

Methods and Materials

The ATHENA study (ISRCTN81226121) was an observational cohort study of 50 consecutive adults (≥ 16 years) with haematological malignancies admitted for intensive chemotherapy or haemopoietic stem cell transplant at the Oxford or Bristol haematology centres between September 2010 and September 2012. Patients were eligible for the study if they were thrombocytopenic (total platelet count (TPC) ≤ $50x10^9$/L) at admission, or were expected to become thrombocytopenic for ≥ 5 days during inpatient treatment, and were able to comply with study monitoring. Patients were excluded from the study if they had any inherited disorder of haemostasis, if they required anti-thrombotic medication during the period of thrombocytopenia, or if they had a prior diagnosis of immune thrombocytopenia. Potential participants were approached at admission, checked for eligibility and invited to give written informed consent prior to study enrolment in accordance with UK Regional Ethics Committee approval 10/H0505/47.

All study participants were managed using standard institutional protocols for the duration of the study. This included prophylactic transfusion of 1 adult platelet unit on each day that a morning blood sample showed TPC ≤ 10 x 10^9/L. Additional platelet units were administered in response to bleeding of WHO Grade ≥2 and to prevent bleeding before invasive procedures. For each participant, the study observation period was from the first day of thrombocytopenia,

until platelet count recovery (unsupported TPC >50x10^9/L for >3 days), hospital discharge, death, or for 30 days if there was prolonged thrombocytopenia.

During the observation period, participants underwent daily bleeding assessments and measurement of TPC. Further blood samples were collected thrice weekly for additional coagulation tests into Vacutainer® blood collection tubes (Becton Dickinson, Plymouth, UK) containing either 0.109 M buffered sodium citrate (for ROTEM, PFA-100®, prothrombin time (PT), activated partial thromboplastin time (aPTT) and Clauss fibrinogen (CF) tests) or 7.2 mg EDTA (for platelet parameters). The whole blood laboratory tests were performed within 2 hours of specimen collection. For the plasma coagulation tests, blood was centrifuged twice within 2 hours of specimen collection and plasma was stored at -80°C for later batched analysis at the Oxford Haemophilia and Thrombosis Centre.

Measurement of bleeding

Bleeding symptoms were recorded during a daily interview by a research nurse using a standardised questionnaire identical to that used in a previous randomized controlled trial of platelet transfusion policy in haematological malignancies (Stanworth, *et al* 2013). A computerised algorithm was used to classify bleeding symptoms according to modified World Health Organization (WHO) criteria (Supplementary figure 1) in which WHO grades 1-4 indicate bleeding with increasing severity (Stanworth, *et al* 2010, Stanworth, *et al* 2013).

Analysis of blood samples

Platelets were tested using a Sysmex XE2100 automated haematology analyser (Sysmex, Kobe, Japan) to determine the following platelet parameters- i.) Total platelet count (TPC); ii.) Mean platelet volume (MPV) (Kaito, *et al* 2005); iii.) Immature platelet fraction (IPF- the percentage of circulating platelets with above-threshold RNA) (Barsam, *et al* 2011, Bat, *et al* 2013, Briggs, *et al* 2006) and, iv.) Absolute immature platelet number (AIPN- the concentration of immature circulating platelets, calculated as IPF x TPC) (Barsam, *et al* 2011, Bat, *et al* 2013).

Thromboelastometry was performed on a ROTEM delta instrument (TEM International, Munich, Germany) with the EXTEM (thromboplastin initiated coagulation), INTEM (contact factor initiated coagulation) and FIBTEM (thromboplastin initiated coagulation with the platelet inhibitor cytochalasin D) activating reagents in accordance with the manufacturer's instructions (Ganter and Hofer 2008). For EXTEM and INTEM, the clot time (CT), clot formation time (CFT), maximum clot firmness (MCF) and maximal lysis (ML) were derived from the thromboelastometry traces. For FIBTEM only maximum clot firmness (MCF) and maximal lysis (ML) were derived from the thromboelastometry traces.

Platelet function was assessed by determining the PFA-100® closure times (Siemens Healthcare Diagnostics GmBH, Marburg, Germany) with the 150µm aperture Collagen/ADP (C-ADP) cartridge, and with the INNOVANCE® PFA P2Y (aperture 100µm, membrane coated with 20 µg Adenosine diphosphate (ADP), 5 ng prostaglandin E1 and 459 µg calcium chloride). The PFA-100® simulates platelet function at high shear stress.(Harrison 2005). Two types of ADP

cartridge were used to assess whether the smaller membrane aperture of the INNOVANCE® cartridge made the results less dependent on platelet count in thrombocytopenia. The PT, aPTT and Clauss Fibrinogen were determined using a STA-R Evolution® Expert Series Hemostasis System (Diagnostica Stago S.A.S., Asnières sur Seine, France) using reagents and methodology according to the manufacturer's instructions.

Statistical analysis

The sample size of 50 patients was pre-specified to enable inclusion of patients with different types of haematological malignancy and treatment. Statistical analysis was performed with STATA version 11.2 (StataCorp, Texas, USA). Parametric data were expressed as means and standard deviations [SDs] and non-parametric data as medians and interquartile ranges [IQRs]. Laboratory parameters were analysed as continuous variables. Associations between laboratory parameters and bleeding during the following 1 day and during the following 2 days after each blood sample were tested using a logistic regression model clustered on patient identity to account for repeated measures. Laboratory parameters other than the TPC were considered potentially predictive of bleeding if there was a statistically significant odds ratio ($P < 0.05$) for bleeding when adjusted for the TPC. Receiver operating characteristic (ROC) curves were derived for TPC and other potentially predictive laboratory parameters and were used to calculate, area under the curve (AUC) and 95% confidence intervals (CIs). Sensitivity, specificity, positive predictive values and negative predictive values were also calculated for various pre-specified thresholds of the TPC and any potential alternative parameter. No adjustment for

confounders such as underlying patient characteristics (e.g. type of treatment or haematological malignancy) was made because in current clinical practice, these variables are not considered when using TPC to guide administration of platelet transfusions.

Results

The 50 participants enrolled into the study had mean age 51.0 years (range 20-70) and 33 were male. The underlying diagnosis was leukaemia in 16/50 participants, lymphoma in 14/50, myeloma in 9/50 and other haematological disorders in 11/50. Treatment was with intensive chemotherapy for 4/50 participants, myeloablative allograft for 10/50, reduced intensity conditioning allograft for 23/50 and autograft for 13/50. One participant, who received induction chemotherapy for acute myeloid leukaemia, was reallocated to outpatient management after study enrolment and was withdrawn from the study. The remaining 49 participants were followed throughout their study observation periods, for a total of 760 days (Fig 1). Bleeding symptom data and the TPC on the preceding day were available for a total of 757 (99.6%) and 754 (99.2%) study days respectively.

The participants had a median 3 days of bleeding of any WHO severity (IQR 0 to 6). A total of 65% of participants experienced at least one bleeding episode of WHO Grade I severity but 33% experienced at least one episode of bleeding of WHO Grade ≥2 (Fig 2a). The median interval between the start of thrombocytopenia and the first bleeding episode was 5 days (IQR 2 to 10 days; Fig 2b). The median total duration of thrombocytopenia was 11 days (IQR 8 to 16) for TPC ≤ $50x10^9$/L and 4 days (IQR 2 to 8) for TPC ≤ $20x10^9$/L (Figs 2c-d). The participants received a

total of 175 adult platelet units within 163 separate transfusion episodes during the study observation period, of which 124/175 (71%) were administered for prophylaxis, 34/175 (19%) in response to bleeding, and 17/175 (10%) to prevent bleeding during an invasive procedure. The type of platelet component was not specified. All platelet components were leucocyte-reduced, up to 80% were collected by apheresis, and common hospital practice was to transfuse ABO and RhD identical platelets. Two patients received HLA matched platelets because of a previous history of platelet refractoriness. The median 24 hour platelet count increment was $11x10^9$/L (IQR -5 to 45 $x10^9$/L).

A total of 157 red cell components were given within 100 separate transfusion episodes during the study observation period. Only 1 transfusion was given to treat bleeding. Median pre-transfusion haemoglobin was 81g/L (IQR 78 to 88g/L).

None of the participants received plasma transfusions during the study period.

Only 4 of the 49 participants did not receive antibiotics during the study period. Median number of days on antibiotics was 10 (IQR 6 to 13). Participants received antibiotics on 478/760 (63%) of study days. None of the participants were on anticoagulants or anti-platelet agents during the study period, as per the study protocol, and none of the participants received asparginase as part of their treatment.

Completion of laboratory tests

A total of 278 additional blood samples were obtained from the participants during the study. The TPC, IPF and AIPN platelet parameters and the PT, aPTT, CF and the ROTEM EXTEM, INTEM

and FIBTEM test results were determined for more than 98% of these blood samples. A summary of the laboratory results from the study cohort is shown in Table I. The PFA-100® test reached a closure time endpoint in only 9.3% of samples using the CADP cartridge and in 22.4% of samples using the INNOVANCE® PFA P2Y cartridge when the platelet count was ≤ 50 x 10^9/L. The MPV was not reported by the Sysmex XE2100 analyser in 63.8% of samples with platelet count ≤ 50 x 10^9/L. No further analyses of the PFA-100 closure times or MPV was performed.

Platelet parameters

There was a significant association between bleeding (any WHO grade) within 1 day of blood sampling and the TPC (OR 0.97 95% CI 0.95 to 0.99; P=0.008) and the AIPN (OR 0.77 95% CI 0.58 to .00; P=0.049), but not the IPF (Fig 3a). There was also a significant association between bleeding (WHO grade ≥2) within 1 day of blood sampling and the AIPN (OR 0.23 95% CI 0.08 to 0.93; P=0.007) and the TPC (OR 0.96 95% CI 0.94 to 0.99; P=0.012) but not IPF (Fig 3a). Data for the unadjusted TPC was calculated using all 754 study days that reported both the TPC and bleeding assessment data, whereas data for AIPN was only available for 277 days. The association between bleeding (WHO grade ≥2) within 1 day of blood sampling and AIPN remained statistically significant after adjustment of AIPN for TPC using logistic regression analysis clustered on patient id (OR 0.23 95% CI 0.08 to 0.93; P=0.038; Fig 3b). However, the association between bleeding (WHO grade ≥2) within 1 day of blood sampling and TPC was no longer significant after adjustment for AIPN (Fig 3c). Therefore the best 'model' in this analysis for WHO grade 2 or above bleeding is AIPN alone. The AIPN was not significantly associated

with bleeding (WHO grade ≥2) within 2 days of blood sampling after adjustment for TPC (Supplementary table I).

The presence of bleeding within 1 day of blood sampling within pre-specified TPC and AIPN categories were estimated with 95% CI (Figs 4a-b). For AIPN in the range 0 to 0.49 $x10^9$/L, the proportion of study days within 1 day of blood sampling in which there was bleeding of any WHO grade was 0.42 (95% CI 0.31 to 0.55). The AIPN showed a statistically significant relationship to the proportion of days with bleeding, with lower AIPNs leading to more frequent bleeding (P<0.001; Fig 4a). For TPC in the range 1 to 10 $x10^9$/L the proportion of study days within 1 day of blood sampling in which there was bleeding of any WHO grade was 0.34 (95% CI 0.20 to 0.47). There was a statistically significant trend between the proportion of days with bleeding and TPC (P=0.008; Fig 4b).

Thromboelastometry and plasma coagulation test results

There were significant associations between bleeding (any and ≥2 WHO grade) within 1 day of blood sampling and the EXTEM MCF (WHO grade 2 OR 0.93 95% CI 0.88 to 0.97; P = 0.003) and INTEM MCF (WHO grade 2 OR 0.92 95% CI 0.87 to 0.98; P=0.007), but not with the other ROTEM test results (Table II). The EXTEM MCF and INTEM MCF were also significantly associated with bleeding (any and ≥2 WHO grade) within 2 days of blood sampling (Supplementary table II).

Since the ROTEM thromboelastometry MCF is partly dependent on TPC, odds ratios were also calculated for the association between bleeding and MCF after adjustment for TPC. The adjusted odds ratios indicated that there was no longer a statistically significant association between either the EXTEM MCF or the INTEM MCF and bleeding (any and ≥2 WHO grade) within 1 day or within 2 days of blood sampling (Table II and supplementary table II). There were no significant associations between PT, aPTT or Clauss fibrinogen and bleeding (any and ≥ 2 WHO grade) within 1 day or within 2 days of blood sampling (Table II and supplementary table II).

Prediction of bleeding using TPC and AIPN

Since AIPN was the only platelet parameter or test result that was associated with bleeding in the study cohort independently of TPC, we plotted ROC curves to compare the sensitivity and specificity of TPC and AIPN as continuous variables for the prediction of bleeding within 1 day of blood sampling. The AIPN had higher values for AUC for bleeding of WHO grade ≥2 (AIPN AUC 0.75; 95% CI 0.66 to 0.84 versus TPC AUC 0.70; 95% CI 0.59 to 0.82, Fig 5) but the AUC were very similar for bleeding of any WHO grade (AIPN AUC 0.66; 95% CI 0.59 to 0.73 versus TPC AUC 0.66; 95% CI 0.59 to 0.74).

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of different TPC and AIPN thresholds for predicting bleeding are shown in table III. The TPC had poor PPV for bleeding (33.7% and 8.7% for bleeding of any and ≥2 WHO grade respectively)

with a TPC threshold of $10x10^9$/L, which is the threshold usually adopted to direct prophylactic platelet transfusion. The PPV of AIPN at a threshold of $0.5x10^9$/L was higher (42.3% and 17.3% for any and ≥2 WHO grade respectively) than the TPC threshold of $10x10^9$/L. The NPV of the TPC threshold of $10x10^9$/L (78.9% and 95.4% for bleeding of any and ≥2 WHO grade respectively) was similar to that of the AIPN threshold of $0.5x10^9$/L (77.7% and 95.5% for bleeding of any and ≥2 WHO grade respectively). AIPN thresholds of ≥$2x10^9$/L had 100% NPV for bleeding of WHO grade ≥2.

Discussion

To our knowledge this is the first study that has prospectively evaluated the associations between platelet parameters other than the TPC and other laboratory markers of haemostasis and bleeding the following day in thrombocytopenic patients undergoing treatment for haematological malignancy. It is a strength of our study that we enrolled participants with a wide range of underlying haematological diagnoses and treatments. Thus, our findings are likely to be applicable to patients with a broad range of haematological malignancies.

We have focussed on platelet parameters and other markers of haemostasis that can be detected using rapid, robust and inexpensive laboratory tests. To this end, we evaluated the platelet parameters MPV, IPF and AIPN which are determined by automated haematology analysers at the same time as the TPC in the full blood count test. We also evaluated platelet function determined using the point-of-care format PFA-100® device (Hayward, *et al* 2006) and coagulation factor and fibrinogen activity using the widely available PT, aPTT and Clauss

fibrinogen tests. Finally, we used the point-of-care format ROTEM thromboelastometry device to evaluate coagulation factor, fibrinogen and platelet function as well as severe fibrinolysis (Luddington 2005). ROTEM and similar technologies are used successfully in clinical settings such as cardiac surgery and liver surgery to detect coagulopathy, to direct appropriate treatments to prevent bleeding and to improve clinical outcomes (Álamo, *et al* 2013, Weber, *et al* 2012).

Our study has several important findings relevant to the prediction of bleeding in patients with thrombocytopenia during treatment for haematological malignancy. The first is that measurement of the platelet parameter MPV and platelet function by PFA-100® closure time is not feasible in this setting because these tests were not completed in a high proportion of study blood samples. PFA-100® closure times are highly dependent on TPC and may not reach endpoint in severely thrombocytopenic samples (Hayward, *et al* 2006). Similarly, MPV determined by automated haematology analysers with impedance endpoints requires analysis of large numbers of platelets in each blood sample, which may not be achieved in thrombocytopenic samples using standard instrument protocols (Latger-Cannard, *et al* 2012). Although our data do not exclude a role for the MPV and PFA-100 closure times in predicting bleeding, the inability to reliably detect these markers in our study precludes their use in this clinical setting.

For the remaining laboratory tests, in which a very high level of completion was achieved, we confirmed a significant association between low TPC and increased bleeding within 1 day, and

within 2 days of blood sampling. The study's power to detect a difference for the unadjusted TPC was strengthened by the use of data from all 754 study days, compared to only 277 days when adjusted for AIPN. One potential explanation for the poor association between bleeding and TPC in our study is that current clinical practice requires patients with TPC ≤ 10 $x10^9$/L to receive a platelet transfusion on the day of the blood sample. Therefore, the risks of bleeding because of thrombocytopenia after a blood sample with TPC ≤ 10 $x10^9$/L is likely to be lower than expected just by virtue of this intervention. Two recent randomised controlled trials have compared a therapeutic-only versus prophylactic platelet transfusion policy (Stanworth, *et al* 2013, Wandt, *etal* 2013)). Both studies showed that prophylactic platelet transfusions reduced bleeding rates overall, but the size of the effect differed between the two studies.

We also demonstrated a significant association between low ROTEM MCF and severe bleeding of WHO grade ≥2. The ROTEM MCF is a composite marker of the haemostatic function of both platelets and fibrinogen (Lang, *et al* 2009), and low ROTEM MCF values caused by hypofibrinogenaemia are associated with increased bleeding in settings such as cardiac surgery (Solomon, *et al* 2011). This is unlikely to hold true for low ROTEM MCF in haematological malignancy because there was no significant association between bleeding and the Clauss fibrinogen test result or the ROTEM FIBTEM MCF which also reflects functional fibrinogen (Solomon, *et al* 2011). The significant association between ROTEM MCF and bleeding was also no longer observed after adjustment for TPC. This suggests that low MCF in this setting is a direct effect of thrombocytopenia and that this marker has no additional value that the TPC. Our finding of no association between bleeding and the ROTEM CT, ROTEM CFT, PT and aPTT

test results suggests that coagulation factor defects do not contribute significantly to bleeding in haematological malignancy. Our finding of no association between the ROTEM ML test result and bleeding also suggests that severe defects in fibrinolysis are unlikely to contribute to bleeding. However, the ROTEM ML is insensitive to less severe fibrinolysis defects (Raza, *et al* 2013) so this coagulopathy cannot be ruled out from our data.

In contrast to the other laboratory markers of haemostasis evaluated in this study, low AIPN was associated strongly with increased bleeding both within 1 day and 2 days of blood sampling. There was also an approximately linear relationship between AIPN and the proportion of study days on which bleeding occurred in the study participants. We found that the association between AIPN and bleeding persisted after adjustment for variation in TPC. However, the association between TPC and bleeding lost statistical significance when adjusted for variation in AIPN, suggesting that the AIPN has a more direct association with bleeding.

Both the AIPN and IPF platelet parameters are determined by the Sysmex XE-2100 haematology analyser as part of the full blood count by detecting reticulated platelets which show increased staining with a fluorescent RNA dye (Briggs, *et al* 2004). Reticulated platelets are an immature platelet population that are newly synthesised in the bone marrow. Increasing circulating reticulated platelets is an early marker of thrombopoietic regeneration after chemotherapy or haemopoietic stem cell transplantation that precedes increases in the TPC (Briggs, *et al* 2006, Have, *et al* 2013). The strong association between low AIPN and increased bleeding in our study participants suggests that endogenous immature platelets are an important determinant of

haemostasis during treatment for haematological malignancies. This is consistent with previous reports that immature platelets have greater haemostatic function than mature or transfused platelets (Fager, *et al* 2010, Guthikonda, *et al* 2007). The IPF also provides a measure of circulating immature platelets, although expressed as a proportion of the total circulating platelet count rather than an absolute concentration. Consistent with this, transfusion of donor platelets significantly reduced the IPF in patients with haematological malignancy, but not the AIPN, at least in part because of a dilution effect from mature platelets in the donor units (Bat, *et al* 2013). Since platelet transfusion was common in our thrombocytopenic study population, it is unsurprising that the circulating immature platelet concentration expressed at the IPF failed to show a consistent association with bleeding.

The potential clinical utility of measuring the AIPN in thrombocytopenic patients with haematological malignancy was highlighted by our predictive model. This showed that for prediction of bleeding, the area under the ROC curve for AIPN was greater than for TPC. Moreover, the PPV of an AIPN threshold of 0.5 $x10^9$/L for bleeding was substantially higher than that of the TPC threshold of 10 $x10^9$/L which is currently used to direct prophylactic platelet transfusion, with similar NPV. We acknowledge that the small sample size in this exploratory study of multiple candidate laboratory markers is vulnerable to the identification of false positive associations. However, the AIPN is a biologically plausible marker of bleeding risk that requires further evaluation in larger scale observational studies.

Conflicts of Interest: PH is a consultant for Sysmex. There are no other conflicts of interest.

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Authorship : LE designed and executed the study, performed data analysis and wrote the manuscript. PH, AM and MM supervised laboratory analysis and data interpretation. GP performed bleeding assessments and coordinated data collection. SS, MM supervised study design and data interpretation. All authors contributed to the final manuscript.

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Figures

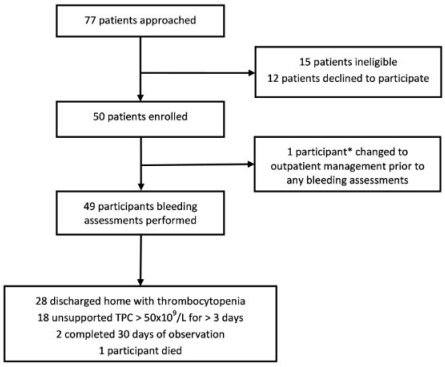


Fig 1: ATHENA study flow diagram.

* Patient with acute myeloid leukaemia being treated with induction chemotherapy

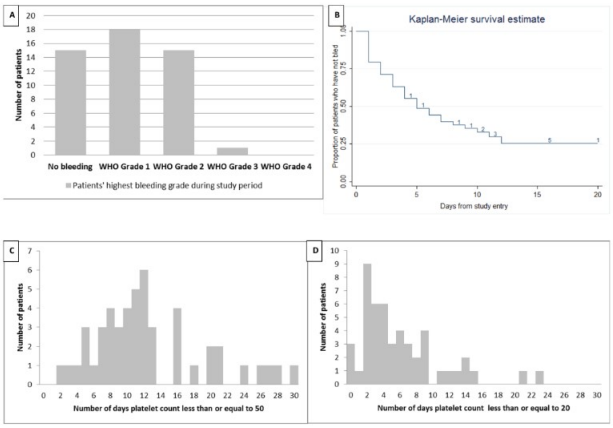
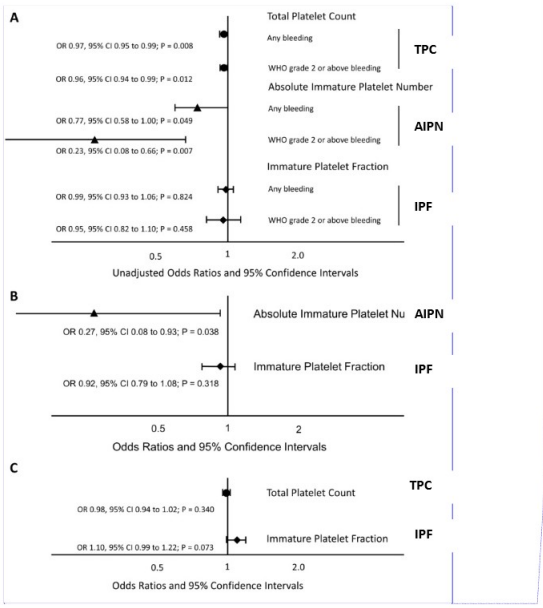


Fig 2. The characteristics of bleeding symptoms and total platelet count in the 49 study participants.

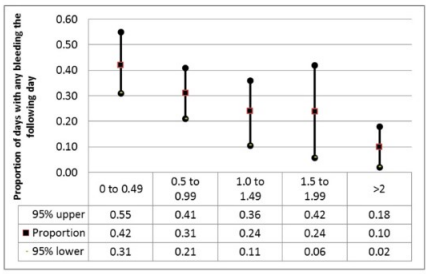
A: The highest WHO bleeding grade for each participant during the study observation period. **B:** Kaplan-Meier survival curve showing time from the start of thrombocytopenia (TPC ≤ 50 x 10^9/L to first bleeding episode in days. Numbers on graph represent censored patients (number of patients who had completed the study at that time point but had not bled. **C:** Distribution of the number of days the participants had TPC ≤ 50 x 10^9/L during the study period. **D:** Distribution of the number of days the participants had TPC ≤ 20 x10^9/L during the study period.



Comment [MA2]: Labels better like this? Not sure

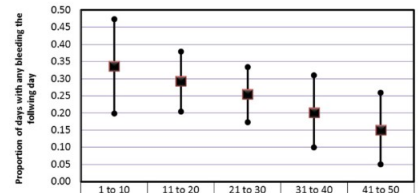
Figure 3. **Associations between platelet parameters and bleeding within 1 day of blood sampling. A:** Unadjusted odds ratios (OR) and 95% confidence intervals (CI) for bleeding of any WHO grade and for bleeding of WHO grade ≥2 in the day following blood sampling and total platelet count (TPC), immature platelet fraction (IPF) and absolute immature platelet number (AIPN). **B:** OR and 95% CI for bleeding of WHO grade ≥2 in the day following blood sampling and AIPN and IPF adjusted for TPC. **C:** OR and 95% CI for bleeding of WHO grade ≥2 in the day following blood sampling and TPC and IPF adjusted for AIPN.

A



	0 to 0.49	0.5 to 0.99	1.0 to 1.49	1.5 to 1.99	>2
95% upper	0.55	0.41	0.36	0.42	0.18
Proportion	0.42	0.31	0.24	0.24	0.10
95% lower	0.31	0.21	0.11	0.06	0.02

B



	1 to 10	11 to 20	21 to 30	31 to 40	41 to 50
95% upper	0.47	0.38	0.33	0.31	0.26
Proportion	0.34	0.29	0.25	0.20	0.15
95% lower	0.20	0.20	0.17	0.10	0.05

Figure 4: Proportions of the study days after blood sampling in which there was bleeding of any WHO grade grouped by platelet parameter result. A: Absolute immature platelet number (AIPN) showing a statistically significant trend between increasing proportions of days with bleeding and lower AIPN (P<0.001). B: Total platelet count (TPC) showing a statistically significant trend between increasing proportions of days with bleeding and lower TPC (P=0.008) (Created using data from all 754 study days when both TPC and bleeding assessment data known). Data were adjusted for repeated measures and are expressed as proportions with 95% confidence intervals.

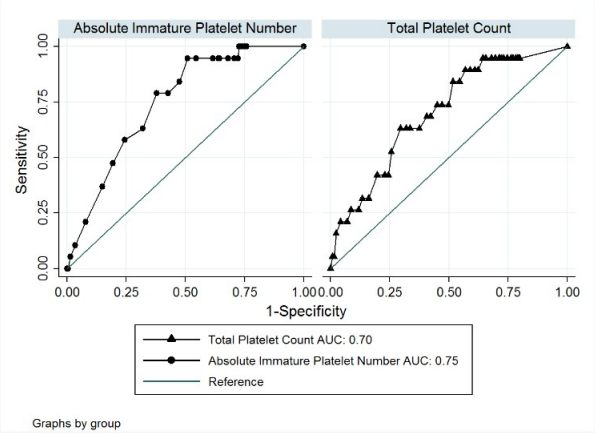


Fig 5. Receiver operator characteristic curves for thresholds of absolute immature platelet number and total platelet count (TPC) for prediction of bleeding of any WHO grade in the day following blood sampling. ROC curve for total TPC created using data from all 754 study days when both TPC and bleeding assessment data are known. AUC = area under curve.

Rotational Thromboelastography					
	Median	**95% CI of median**	**IQR**	**Range**	**Reference interval**
EXTEM CT (s)	59	57 to 61	51 to 70	14 to 329	42 to 74
EXTEM CFT (s)	166	144 to 187	86 to 283	27 to 1474	46 to 148
EXTEM MCF (mm)	45	43 to 46	37 to 52	19 to 73	49 to 71
EXTEM ML (%)	3	2 to 3	1 to 6	0 to 15	0 to 18
INTEM CT (s)	156	154 to 159	144 to 170	19 to 289	137 to 246
INTEM CFT (s)	162	148 to 191	87 to 295	31 to 2513	40 to 100
INTEM MCF (mm)	43	41 to 45	35 to 51	20 to 71	52 to 72
INTEM ML (%)	2	1 to 2	0 to 4	0 to 14	0 to 12
FIBTEM MCF (mm)	23	22 to 24	18 to 29	4 to 51	9 to 25
FIBTEM ML (%)	0	0 to 0	0 to 1	0 to 22	-
Platelet Parameters					
	Median	**95% CI of median**	**IQR**	**Range**	**Reference interval**
TPC (x10^9/L)	26	25 to 28	15 to 48	2 to 380	140 to 400
IPF (%)	4.9	4.4 to 5.2	2.8 to 7.3	0 to 32.7	0.9 to 5.2
AIPN (x10^9/L)	1.12	0.96 to 1.3	0.65 to 2.49	0 to 27.0	2.7 to 12.5
Plasma coagulation tests					
	Median	**95% CI of median**	**IQR**	**Range**	**Reference interval**
PT (s)	13.7	13.2 to 14.1	11.3 to 15.3	10.1 to 67.1	13 to 16
aPTT (s)	31.4	30.6 to 32.4	27.4 to 34.5	19.5 to 67	26 to 36
CF (g/L)	4.3	4.0 to 4.5	3.3 to 5.5	1.29 to >15.0	1.4 to 4.0

Table I: Summary of laboratory parameters from 49 study participants from a total of 276 blood samples obtained during the study observation period. CT = clot time; CFT = clot formation time; MCF = maximum clot firmness; ML = % maximal lysis; TPC = total platelet count; IPF = immature platelet fraction; AIPN = absolute immature platelet number; PT-prothrombin time; aPTT- activated partial thromboplastic time; CF-Clauss fibrinogen.

Comment [MA3]: Have adjusted spacing so table fits on 1 page , also below

Any bleeding						
	Unadjusted Odds Ratio			Odds Ratio adjusted for TPC		
	Odds ratio	95% CI	P value	Odds ratio	95% CI	P value
EXTEM CT	1.00	0.99 to 1.01	0.632	1.00	0.99 to 1.01	0.791
EXTEM CFT	1.00	1.00 to 1.00	0.242	1.00	1.00 to 1.00	0.628
EXTEM MCF	0.96	0.93 to 0.99	0.009**	0.97	0.92 to 1.02	0.207
EXTEM ML	0.93	0.85 to 1.02	0.105	0.97	0.88 to 1.06	0.487
INTEM CT	1.00	0.99 to 1.01	0.464	1.00	0.98 to 1.01	0.369
INTEM CFT	1.00	1.00 to 1.00	0.179	1.00	1.00 to 1.00	0.442
INTEM MCF	0.95	0.92 to 0.99	0.006**	0.96	0.91 to 1.01	0.125
INTEM ML	0.92	0.82 to 1.04	0.173	0.96	0.86 to 1.09	0.557
FIBTEM MCF	1.00	0.96 to 1.05	0.844	1.01	0.97 to 1.06	0.591
FIBTEM ML	0.98	0.89 to 1.08	0.667	0.98	0.89 to 1.09	0.774
PT	1.03	0.95 to 1.11	0.517			
aPTT	1.04	0.98 to 1.10	0.218			
CF	1.12	0.91 to 1.38	0.287			
WHO Grade ≥2 bleeding						
EXTEM CT	1.00	0.98 to 1.01	0.835	1.00	0.98 to 1.02	0.976
EXTEM CFT	1.00	1.00 to 1.00	0.232	1.00	1.00 to 1.00	0.912
EXTEM MCF	0.93	0.88 to 0.97	0.003**	0.95	0.89 to 1.00	0.066
EXTEM ML	0.79	0.62 to 1.02	0.069	0.85	0.65 to 1.11	0.234
INTEM CT	1.01	1.00 to 1.03	0.135	1.01	0.99 to 1.03	0.206
INTEM CFT	1.00	1.00 to 1.00	0.104	1.00	1.00 to 1.00	0.424
INTEM MCF	0.93	0.87 to 0.98	0.007**	0.94	0.87 to 1.01	0.090
INTEM ML	0.69	0.47 to 1.01	0.058	0.75	0.51 to 1.10	0.143
FIBTEM MCF	0.98	0.91 to 1.07	0.705	0.99	0.91 to 1.08	0.861
FIBTEM ML	0.87	0.71 to 1.07	0.187	0.87	0.69 to 1.08	0.209
PT	0.96	0.77 to 1.20	0.706			
aPTT	1.00	0.91 to 1.10	0.995			
CF	1.05	0.82 to 1.36	0.689			

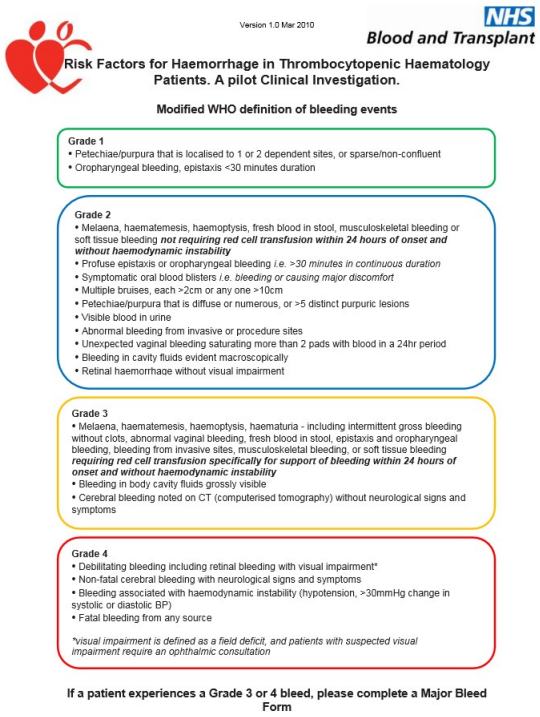
Table II: Association between ROTEM parameters and plasma coagulation test results and bleeding with 1 day of blood sampling. The associations between ROTEM parameters and bleeding are presented as unadjusted odds ratios and as odds ratios adjusted for TPC. The plasma coagulation test (PT, aPTT and CF) results are presented as odds rations unadjusted for TPC. All odds ratios account for repeated measures. CT = clot time; CFT = clot formation time; MCF = maximum clot firmness; ML = maximum lysis; PT-prothrombin time; aPTT-activated partial thromboplastin time; CF-Clauss fibrinogen.* = P < 0.05; ** = P < 0.01.

	Any Bleeding			
	Sensitivity	Specificity	PPV	NPV

TPC ≤ 10	20.3%	88.1%	33.7%	78.9%
TPC ≤ 20	50.6%	66.5%	30.9%	82.0%
TPC ≤ 30	75.6%	44.2%	28.6%	86.0%
TPC ≤ 40	84.3%	34.0%	27.4%	88.0%
TPC ≤ 50	90.1%	24.6%	26.1%	89.4%
AIPN <0.5	31.0%	85.1%	42.3%	77.7%
AIPN <1	66.2%	57.7%	35.6%	82.9%
AIPN <1.5	83.1%	38.3%	32.2%	86.5%
AIPN <2	90.1%	30.3%	31.4%	89.7%
AIPN <2.5	90.1%	25.9%	30.0%	88.1%
WHO Grade 2 or above Bleeding				
TPC ≤ 10	23.1%	86.7%	8.7%	95.4%
TPC ≤ 20	53.8%	63.5%	7.4%	96.2%
TPC ≤ 30	76.9%	40.6%	6.6%	97.0%
TPC ≤ 40	87.2%	30.8%	6.4%	97.8%
TPC ≤ 50	89.7%	21.8%	5.9%	97.5%
AIPN <0.5	47.4%	83.0%	17.3%	95.5%
AIPN <1	84.2%	54.2%	12.1%	97.9%
AIPN <1.5	94.7%	34.8%	9.8%	98.9%
AIPN <2	100.0%	26.9%	9.3%	100.0%
AIPN <2.5	100.0%	23.3%	8.9%	100.0%

Table III: Predictive value of total platelet count (TPC) and absolute immature platelet number (AIPN) for any bleeding within 1 day of blood sampling. Data are presented for sensitivity, specificity, positive predictive Value (PPV) and negative predictive value (NPV) of different threshold values for TPC and AIPN.

Supplementary information



Version 1.0 Mar 2010

NHS Blood and Transplant

Risk Factors for Haemorrhage in Thrombocytopenic Haematology Patients. A pilot Clinical Investigation.

Modified WHO definition of bleeding events

Grade 1
- Petechiae/purpura that is localised to 1 or 2 dependent sites, or sparse/non-confluent
- Oropharyngeal bleeding, epistaxis <30 minutes duration

Grade 2
- Melaena, haematemesis, haemoptysis, fresh blood in stool, musculoskeletal bleeding or soft tissue bleeding ***not requiring red cell transfusion within 24 hours of onset and without haemodynamic instability***
- Profuse epistaxis or oropharyngeal bleeding *i.e. >30 minutes in continuous duration*
- Symptomatic oral blood blisters *i.e. bleeding or causing major discomfort*
- Multiple bruises, each >2cm or any one >10cm
- Petechiae/purpura that is diffuse or numerous, or >5 distinct purpuric lesions
- Visible blood in urine
- Abnormal bleeding from invasive or procedure sites
- Unexpected vaginal bleeding saturating more than 2 pads with blood in a 24hr period
- Bleeding in cavity fluids evident macroscopically
- Retinal haemorrhage without visual impairment

Grade 3
- Melaena, haematemesis, haemoptysis, haematuria - including intermittent gross bleeding without clots, abnormal vaginal bleeding, fresh blood in stool, epistaxis and oropharyngeal bleeding, bleeding from invasive sites, musculoskeletal bleeding, or soft tissue bleeding ***requiring red cell transfusion specifically for support of bleeding within 24 hours of onset and without haemodynamic instability***
- Bleeding in body cavity fluids grossly visible
- Cerebral bleeding noted on CT (computerised tomography) without neurological signs and symptoms

Grade 4
- Debilitating bleeding including retinal bleeding with visual impairment*
- Non-fatal cerebral bleeding with neurological signs and symptoms
- Bleeding associated with haemodynamic instability (hypotension, >30mmHg change in systolic or diastolic BP)
- Fatal bleeding from any source

**visual impairment is defined as a field deficit, and patients with suspected visual impairment require an ophthalmic consultation*

If a patient experiences a Grade 3 or 4 bleed, please complete a Major Bleed Form

Supplementary Figure 1: Modified World Health Organisation grading for bleeding symptoms

WHO Grade 2 or above bleeding									
	Unadjusted Odds Ratio			Odds Ratio adjusted for TPC			Odds Ratio adjusted for AIPN		
	Odds ratio	95% CI	P value	Odds ratio	95% CI	P value	Odds ratio	95% CI	P value
TPC	0.96	0.94 to 0.99	0.014*	-	-	-	0.96	0.92 to 1.00	0.079
IPF	0.99	0.89 to 1.09	0.808	0.97	0.87 to 1.08	0.557	1.09	0.97 to 1.22	0.133
AIPN	0.45	0.24 to 0.86	0.016*	0.60	0.32 to 1.12	0.106	-	-	-

SupplementaryTable I: Associations between platelet parameters and bleeding within 2 days of blood sampling. Associations are expressed as unadjusted odds ratios and as odds ratios adjusted for either TPC or AIPN. All odds ratios account for repeated measures. TPC = total platelet count (when platelet count ≤50x10 ^{9}/L); AIPN = absolute immature platelet number; IPF = immature platelet fraction. * = P < 0.05; ** = P < 0.01.

Any bleeding						
	Unadjusted Odds Ratio			Odds Ratio adjusted for TPC		
	Odds ratio	95% CI	P value	Odds ratio	95% CI	P value
EXTEM CT	1.00	0.99 to 1.01	0.367	1.00	0.99 to 1.01	0.510
EXTEM CFT	1.00	1.00 to 1.00	0.458	1.00	1.00 to 1.00	0.872
EXTEM MCF	0.96	0.93 to 0.99	0.009**	0.98	0.93 to 1.03	0.451
EXTEM ML	0.94	0.87 to 1.01	0.096	0.98	0.90 to 1.06	0.541
INTEM CT	1.00	0.99 to 1.01	0.407	0.99	0.98 to 1.01	0.345
INTEM CFT	1.00	1.00 to 1.00	0.375	1.00	1.00 to 1.00	0.989
INTEM MCF	0.96	0.93 to 0.99	0.004**	0.97	0.92 to 1.02	0.248
INTEM ML	0.95	0.85 to 1.05	0.287	0.99	0.90 to 1.10	0.875
FIBTEM MCF	1.02	0.98 to 1.06	0.364	1.03	0.98 to 1.07	0.213
FIBTEM ML	0.97	0.88 to 1.06	0.446	0.97	0.87 to 1.07	0.520
PT	1.02	0.94 to 1.10	0.684			
aPTT	1.05	0.99 to 1.12	0.082			
CF	1.19	0.96 to 1.48	0.113			
WHO Grade 2 or above bleeding						
EXTEM CT	0.99	0.97 to 1.02	0.576	1.00	0.97 to 1.02	0.682
EXTEM CFT	1.00	1.00 to 1.00	0.300	1.00	1.00 to 1.00	0.835
EXTEM MCF	0.93	0.89 to 0.97	0.001**	0.95	0.90 to 1.00	0.041*
EXTEM ML	0.85	0.72 to 1.00	0.060	0.91	0.77 to 1.08	0.286
INTEM CT	1.00	0.98 to 1.03	0.828	1.00	0.98 to 1.03	0.912
INTEM CFT	1.00	1.00 to 1.00	0.140	1.00	1.00 to 1.00	0.603
INTEM MCF	0.93	0.88 to 0.97	0.001**	0.94	0.88 to 1.00	0.057
INTEM ML	0.83	0.65 to 1.06	0.134	0.89	0.70 to 1.14	0.365
FIBTEM MCF	0.99	0.93 to 1.06	0.743	1.00	0.92 to 1.07	0.913
FIBTEM ML	1.03	0.89 to 1.19	0.709	1.03	0.87 to 1.22	0.722
PT	0.94	0.77 to 1.14	0.500			
aPTT	1.05	0.99 to 1.10	0.104			
CF	1.05	0.82 to 1.34	0.726			

Supplementary Table II: Association between ROTEM and other coagulation parameters results and bleeding within 2 days of blood sampling. The associations between ROTEM parameters and bleeding are presented as unadjusted odds ratios and as odds ratios adjusted for TPC. The plasma coagulation test (PT, aPTT and CF) results are presented as odds ratios unadjusted for TPC. All odds ratios account for repeated measures. CT = clot time; CFT = clot formation time; MCF = maximum clot firmness; ML = maximum lysis; PT-prothrombin time; aPTT-activated partial thromboplastin time; CF-Clauss fibrinogen.* = P < 0.05; ** = P < 0.01.