Thesis submitted in partial fulfilment of the requirements of the award of PhD by

Oxford Brookes University

January 2017

Does an Objective Laboratory Measurement of Platelet Quality Correlate with Clinical Efficacy?

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ABBREVIATIONS

ADP	Adenosine diphosphate
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
ATP:ADP	Adenosine triphosphate: adenosine diphosphate ratio
AWE	Automated water expulsion test
BSA	Body surface area
CAM	Cell adhesion molecule
CCI	Corrected count increments
CJD	Creutzfeldt Jakob disease
CMV	Cytomegalovirus
CPD	Citrate phosphate dextrose
CV	Co-efficient of variation
DLS	Dynamic light scattering
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESC	Extent shape change
FBC	Full blood count
fCCI	Fluorescent corrected count increment
FFP	Fresh frozen plasma
FIX	Fixed (measurement setting)
FSB	Fixed stir-bar (measurement setting)

GP	Glycoproteins
GP1b	Glycoprotein-1b
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDL3	High density lipoprotein-3
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSR	Hypotonic shock response test
HSC	Haematopoietic stem cell
HTLV	Human T cell lymphotrophic virus
iCCI	Impedance corrected count increment
LDH	Lactate dehydrogenase
MA	Maximum amplitude
MCF	Maximum clot firmness
MDS	Myelodisplasia
MPV	Mean platelet volume
MV	Microvesicles
NHFTR	Non-haemolytic febrile transfusion reaction
NHSBT	National health service blood and transplant service
NPA	No polynomial adjustment error
OBS	Owren's buffered saline
OD	Optical density
OUH	Oxford university hospitals

PAS	Platelet additive solution
PDW	Platelet distribution width
PF-4	Platelet factor-4
PMV	Platelet microvesicles
POC	Point of care
PRP	Platelet rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PSL	Platelet storage lesion
PTP	Post transfusion purpura
ROTEM	Rotation thrombelastometry
SD	Standard deviation
TA-GvHD	Transfusion associated graft versus host disease
TCA	Tricarboxylic acid cycle
TEG	Thromboelastography
TF	Tissue factor
TRALI	Transfusion related acute lung injury
UPS	Uninterrupted power supply
VAR	Variable (measurement setting)
vWF	von Willebrand factor
VSB	Variable stir-bar (measurement setting)
WBC	White blood cell count

ABSTRACT

Transfusion services have a weak evidence base compared to many other modern healthcare practices. Diversity of platelet concentrate quality and deterioration of function during ageing is poorly understood and difficult to measure. No single test is currently suitable to assess concentrate quality for product development purposes or to confirm suitability of individual concentrates for clinical use. Many of the studies in this field investigate the *in vitro* association between concentrate age and test results. Not all quality indicators are equally informative because a number of storage associated changes correct upon infusion. This study aims to evaluate novel platelet quality assessment techniques that may be of use in determining platelet concentrate quality.

The initial stages of the study involved development of reference methods and *in vitro* assessment of novel techniques for assessing platelet concentrate quality using out of date concentrates. Reference methods included swirl assessment, pH measurement, hypotonic shock response (HSR) and extent shape change (ESC) assays. Novel techniques investigated included the iZON impedance analyser, the ThromboLUX instrument, rotation thromboelastometry (ROTEM) and an automated, simplified version of the HSR test named the automated water expulsion (AWE) test.

After initial technical development of the assays, a study of the test results obtained throughout platelet concentrate ageing in the laboratory and under different storage conditions was undertaken. This was followed by a small, proof of concept clinical trial, in which *in vitro* test results of platelet concentrate samples obtained from the giving set after platelet transfusion were compared to measures of transfusion outcome in myelodisplasia (MDS) patients. Transfusion efficacy was assessed using self assessment bleeding questionnaires and 1 hour post-transfusion corrected count increment (CCI). Although the number of patients included in the trial was too low to reach any definite conclusions, the ThromboLUX, ROTEM and AWE tests all showed enough potential to warrant further investigation as part of a larger clinical trial in the future.

1 INTRODUCTION

1.1 Background

Platelets are small, anucleate disc shaped cells that are present in the peripheral blood at a concentration of between 150 and 400 x 10^9/L in normal individuals. One third of the platelets in the peripheral circulation are sequestered in the spleen. Megakaryocyte fragments are released into the bone marrow sinusoids and subsequently divide to produce platelets (Junt *et al*, 2007; Schwertz *et al*, 2010). Mature platelets retain mitochondria and can therefore produce energy aerobically. Although they do not have a nucleus, platelets possess an mRNA transcriptome and the necessary cellular machinery to translate this information into protein in a regulated fashion (Weyrich *et al*, 1998). The normal platelet lifespan is between 7 and 10 days *in vivo* and ageing, senescent cells undergo a form of programmed cell death that is not yet fully understood (Mason *et al*, 2007).

Platelets play a critical role in primary haemostasis. They become activated in the presence of tissue damage and form a 'primary haemostatic plug' that limits immediate blood loss. To achieve this, the platelets link to the damaged tissue and to other platelets, primarily via interactions with von Willebrand Factor and collagen. Upon activation, platelets undergo degranulation, releasing a variety of biologically active molecules into the circulation. The platelet activation process requires strict regulation, as it could otherwise lead to inappropriate haemostasis and potentially harmful vessel occlusion. Regulation is achieved via a balance of stimulatory and inhibitory molecules and physical processes. Inhibitory factors released by the intact endothelium include nitric oxide (NO), prostacyclin and CD39, which are all critical in maintaining haemostatic balance (Watson *et al*, 2005).

If platelet numbers or function becomes sufficiently impaired, spontaneous life threatening bleeding may take place. Patients may have congenital deficiencies in platelet function or may develop a

thrombocytopenia due to increased loss (for instance secondary to bleeding) or reduced platelet production (for instance due to chemotherapy) (Key *et al*, 2009). These patients may require platelet transfusion to treat or prevent bleeding episodes.

In addition to their well characterised function in primary haemostasis, platelets play a role in several other biological processes. Platelet dense-granules and α-granules contain molecules involved in other haemostatic pathways, leukocyte chemotaxis and activation, inflammation, vasoconstriction, vasopermeability and tissue regeneration (Burgers *et al*, 1993; Watson *et al*, 2005). Additional functions continue to be discovered and investigated. For instance platelets are involved in closure of the ductus arteriosus during neonatal development (Echtler *et al*, 2010) and in parasite clearance during malarial infection (McMoran *et al*, 2009).

However, the biochemical balance governing platelet activation is not always optimal and platelet dysfunction is implicated in a variety of pathologies. Platelets are involved in atherosclerotic plaque formation and in development of arterial clots, leading to myocardial infarction or stroke, upon plaque rupture (Watson *et al*, 2005). Immune system interactions can lead to pathological immune cell recruitment and inflammation (Theilmeier *et al*, 1999; Blair *et al*, 2009; Boilard *et al*, 2010). Platelets can promote neutrophil extracellular trap (NET) release during sepsis. NETs damage the vasculature and hepatic tissue (Clark *et al*, 2007). Bacteria mediated alterations to platelet surfaces can increase their thrombogeneity, contributing to disseminated intravascular coagulation (DIC) (Grewal *et al*, 2008). In addition, platelet tissue regeneration properties can aid development of the neovasculature in malignancy (Italiano *et al*, 2008; Klement *et al*, 2009).

1.1.1 Platelet concentrate production in the UK

Therapeutic platelet concentrates are prepared from donor blood and transfused to treat quantitative or qualitative platelet deficiencies. They only have a shelf life of up to seven days following collection from the donor. This short lifespan leads to wastage and logistical challenges

in platelet concentrate provision (Estcourt *et al*, 2016). In the UK, all platelet concentrates are produced using either the pooled buffy coat method or aphaeresis (Walters, 2016).

To prepare a pooled concentrate, four separate donations are used. Around 470mL of whole blood is collected from each donor into 63mL of citrate phosphate dextrose (CPD) anticoagulant. The donations are centrifuged and their red cell and plasma contents are removed to generate red cell concentrates and plasma derived products. The buffy coats remain in the original collection packs, which are connected together using a sterile connecting device. The platelets from all four donations are then passed through a leucodepletion filter into their final, breathable platelet concentrate bag along with a suitable suspension medium (Walters, 2016).

Apheresis collection of platelets is achieved using a separation instrument. The donor's blood is mixed with anticoagulant citrate dextrose (ACD) and drawn through a line into the machine, where it is gently centrifuged to separate the platelets (Walters, 2016). The remainder of the blood components are then returned to the donor's circulation. The total platelet yield from apheresis donation is generally much greater than that of traditional whole blood donation: it is common to harvest two or three complete therapeutic platelet doses per donation. Apheresis platelets currently account for 57% of the UKs platelet transfusions, with pooled buffy coat platelets making up the remainder (Hontoria del Hoyo, 2017).

In the UK, aphaeresis platelet concentrates contain platelets suspended in the donor's plasma and pooled platelet concentrates contain platelets suspended in 30-50% plasma and 70-50% PAS (Walters, 2016). The PAS formulation used for standard pooled platelet concentrates is PAS-II, which is purchased as a product called SSP from MacoPharma UK Ltd (Twickenham, UK). MacoPharma UK Ltd also produce a PAS-III M product called SSP+ that is able to support platelet viability for up to 9 days (Hornsey *et al*, 2006). This is used to reconstitute apheresis platelets to create a specialist low plasma product for patients who have allergic reactions to plasma components. This blood product is referred to by the NHSBT as 'Platelets suspended in additive solution' to distinguish it from standard

pooled or apheresis platelet concentrates (Walters, 2016). The formulations of SSP and SSP+ are shown in Table 1.1.

Table 1.1: *SSP and SSP+ formulations*

SSP	**SSP+**
(Standard pooled platelet concentrates)	(Platelets suspended in additive solution)
• Sodium Chloride 6.75g/L • Sodium Acetate.3H2O 4.08g/L • Sodium Citrate.2H2O 2.94g/L • Water for Injection 1000 mL.	• Sodium Chloride 69.3 mmol/L • Sodium Acetate Trihydrate 10.8 mmol/L • Sodium Acetate 32.5 mmol/L • Sodium Phosphate 28.2 mmol/L • Potassium Chloride 5 mmol/L • Magnesium Chloride/Sulphate 1.5 mmol/L

1.1.2 Platelet concentrate use in the UK

The most frequent indication for platelet transfusion is haematological malignancy, which accounts for 67% of platelet concentrate usage in the UK (Estcourt *et al*, 2016). The majority of transfusions given to treat haematological malignancy are administered to patients with a diagnosis of acute myeloid leukaemia (AML - 29%), lymphoma (18%), MDS (11%), myeloma / plasma cell dyscrasia (9%), acute lymphocytic leukaemia (ALL – 6%) or aplastic anaemia (5%) (Birchall *et al*, 2011).

The British Committee for Standards in Haematology (BCSH) publishes guidelines on the therapeutic and prophylactic administration of platelet concentrates (Estcourt *et al*, 2016). These include a comprehensive list of recommended transfusion thresholds for different clinical conditions and circumstances which range from maintaining a platelet count at more than 100 x 10^9/L (for instance prior to neurosurgery) to withholding platelet transfusion regardless of count (e.g. for non-bleeding patients with chronic bone marrow failure or post autologous haematopoietic stem cell (HSC) transplant). They also cover the use of alternative treatments (such as tranexamic acid for reversal of anti-platelet therapy) and the selection of appropriate platelet products for specific patient subgroups (e.g. human leukocyte antigen (HLA) matched

platelets for patients with Glanzmann thrombasthenia). The transfusion thresholds most likely to be applied to patients with haematological malignancies are summarised in Table 1.2.

Table 1.2: *Summary of the main transfusion thresholds recommended by the BCSH guidelines that are likely to be relevant for patients with haematological malignancies*

Clinical Condition	Standard Threshold	Special Circumstances / Considerations
Not bleeding, reversible bone marrow failure (e.g. undergoing intense chemotherapy or HSC transplant)	Maintain platelet count above $10x10^9$/L	-Consider maintaining platelet count above $20x10^9$/L if there are additional risk factors for bleeding -Consider withholding prophylactic transfusions in post autologous HSC transplant patients who are otherwise well -These thresholds should be used as a guide for other (non-bone marrow failure) patients with chronic disease in the absence of bleeding or planned procedures
Not bleeding, chronic bone marrow failure (e.g. chronic disease or long term medication)	No routine prophylactic transfusion if patient is asymptomatic	-Patients with chronic bone marrow failure undergoing an intense course of treatment will likely need prophylactic transfusions -Chronic bleeding should be managed according to severity and it may be advised to initiate a prophylactic transfusion protocol for these patients when bleeding is resolved
Prior to procedures or surgery	Depends on type of procedure	-Not routinely required prior to bone marrow biopsy, peripheral insertion of central catheters, removal of tunnelled CVCs or cataract surgery -Raise count above $20x10^9$/L before venous central line insertion -Raise count above $30x10^9$/L before lumbar puncture -Raise count above $80x10^9$/L before epidural catheter insertion or removal -Raise count above $50x10^9$/L before major surgery -Raise count above $100x10^9$/L before neurosurgery or ophthalmic surgery involving the posterior of the eye -Raise count above $50x10^9$/L before percutaneous liver biopsy -Correct bleeding risk factors (anaemia, uraemia) prior to renal biopsy. Avoid platelet transfusion because it effectiveness is limited. Consider use of desmopressin or oestrogen as an alternative
Therapeutic platelet transfusion for bleeding	Depends on severity and location of bleeding	-Maintain count above $50x10^9$/L for severe bleeding -Maintain count above $100x10^9$/L for multiple trauma, brain injury or spontaneous intracerebral bleed -Maintain count above $30x10^9$/L for bleeding that is not severe or life threatening

1.1.3 Negative side effects of platelet transfusion

Platelet concentrate transfusion may have negative clinical side effects due to reactions of the recipient's immune system against the transfused material or by reactions of transfused immune elements against the recipient's own tissues. Non-haemolytic febrile transfusion reaction (NHFTR) occurs due to reactions between recipient allo-antibodies and transfused non-red cell elements or due to high cytokine content of the transfused component. Transfusion associated acute lung injury (TRALI) occurs when recipient neutrophils become inappropriately activated in the lungs in response to donor immune factors such as anti-white blood cell (WBC) antibodies in the product, leading to tissue damage. Transfusion associated graft versus host disease (TA-GvHD) occurs when a recipient-reactive donor lymphocyte population becomes established in the recipient. Post transfusion purpura (PTP) results from reactions between recipient allo-antibodies (usually of a human platelet antibody specificity) and donor platelets. However, the recipients own platelets are also destroyed by an unknown mechanism (Delaney *et al*, 2016).

Deficient platelet count incrementation after transfusion is referred to as platelet refractoriness. It is usually caused by recipient anti-HLA antibodies that react with antigens on donor platelets causing immunological clearance. The incidence of platelet refractoriness is reduced by platelet concentrate leucodepletion. In severe cases, HLA matched platelet concentrates may be required. Anaphylactic reactions to IgA can occur in in IgA deficient patients and may be life threatening (Delaney *et al*, 2016).

Blood component transfusion may transmit infection. All donations are tested for hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), syphilis and human T cell lymphotrophic virus (HTLV). Potential donors are excluded from giving blood if they are deemed to be at high risk of certain infections (e.g. high risk of Malaria as indicated by travel history). Additional tests, such as cytomegalovirus (CMV) typing, may be performed on selected donations to provide products suitable for vulnerable patients (Delaney *et al,* 2016). However, it is both

impractical and impossible to entirely exclude the possibility that a donation contains potentially infectious agents. Of particular concern to the UK Blood Transfusion Service is the prion disease, variant Creutzfeldt Jakob disease (CJD), for which no test is currently available (Blood Services Prion Working Group, 2010).

The risk of bacterial contamination and transmission is much greater with platelet concentrates than with red cell units. Unlike red cell concentrates, platelet concentrates cannot be stored in refrigerated conditions as this triggers clustering of the GP1b von Willebrand receptor complex and promotes rapid macrophage mediated clearance of transfused platelets (Hoffmeister *et al*, 2003a). A study of platelet and red cell transfusion in North America between 1998 and 2000 found that transfusion transmitted bacteremia occurred following transfusion of 9.98-10.64 / million platelet concentrates (for single donor and pooled products respectively), compared to 0.21 / million red cell concentrates (Kuehnert *et al*, 2001). Rates for fatal bacteraemia were 1.94-2.22 and 0.13 / million concentrates respectively.

Room temperature storage likely accounts for the high rate of bacterial contamination observed in platelet concentrates compared to red cell concentrates (Kuehnert *et al*, 2001). However, the National Health Service Blood and Transplant service (NHSBT) monitors platelet concentrates to detect bacterial infection, enabling identification and recall of contaminated units. Future advances in platelet concentrate preservation methodology may enable storage at $4^{o}C$ and consequential reduction in bacterial contamination (Hoffmeister *et al*, 2003b). Also, methods of pathogen inactivation have been found to limit bacterial growth in platelet concentrates without significantly compromising concentrate quality (Lin *et al*, 2004; Wagner *et al*, 2009).

1.1.4 Platelet quality

It is important to ensure that platelet concentrate transfusion is effective so that patients are not exposed to transfusion associated risks without any clinical benefit. Platelet concentrate quality is

thought to vary between individual units and transfusion of poor quality concentrates may not deliver the expected level of clinical benefit to the recipient. Between 10 and 30% of platelet transfusions have been reported to be ineffective (Maurer-Spurej *et al*, 2009; Sigle *et al*, 2012).

Individual platelet concentrates are thought to deteriorate at different rates (reviewed in Shrivastava, 2009; Thon *et al*, 2008). If this is the case, some platelet concentrates that are still of good quality may be disposed of after seven days even though they would be clinically effective. Other platelet concentrates may deteriorate rapidly, becoming ineffective and potentially harmful to the patient, before the arbitrary seven day shelf life is reached. If these platelet concentrates could be reliably identified and discarded, it would prevent patient exposure to unnecessary transfusions. Patients who receive ineffective platelet concentrate transfusions usually require another transfusion, so preventing use of ineffective platelet concentrates should not greatly increase overall platelet concentrate demand. Also, in the longer term, good quality concentrates that are discarded when they reach their arbitrary expiry date could potentially be used.

Deterioration of platelet concentrates during storage is referred to as the platelet storage lesion (PSL). Changes include appearance of reactive platelet morphology, altered surface protein / lipid constitution and increased de-granulation due to *in vitro* platelet activation (Kunicki *et al*, 1975; Fratantoni *et al*, 1984; Botchway *et al*, 2000; Curvers *et al*, 2004). Increasing apoptosis and microvesiculation reduce platelet viability (Bode *et al*, 1991; Chang *et al*, 1993). Metabolic and storage induced deterioration cause biochemical changes (Murphy *et al*, 1975; Bellhouse *et al*, 1987). The combined effect of all these factors is estimated to reduce the functionality of stored platelets by around 30% after five days (Wenzel *et al*, 2012).

The degree of PSL observed *in vitro* is hypothesised to affect the lifespan and efficacy of transfused platelets *in vivo* (Apelseth *et al*, 2010; Holme *et al*, 1998; Mintz *et al*, 2005; Seghatchian *et al*, 1997). If true, high quality concentrates may lead to greater, longer lasting, normalisation of haemostatic function. This could enable reduced frequency of transfusion, reduced exposure to transfusion

associated risks and reduced platelet concentrate provision requirements. However, not all features of the PSL are of equal relevance to the clinical efficacy of platelets after transfusion. A number of storage associated changes, including metabolic function, aggregation potential and expression of activation related surface markers, have been found to correct upon addition of fresh plasma or upon re-infusion (Ishikawa *et al*, 1987; Berger *et al*, 1998; Rinder *et al*, 2003; Miyaji *et al*, 2004).

Better characterisation of the PSL and practically applicable methods for assessing it *in vitro* has the potential to prevent clinically effective platelet concentrates from being discarded simply because they have reached an arbitrary expiry date. It would also allow identification of concentrates that deteriorate more rapidly than average, becoming ineffective and possibly harmful to the patient before they have reached their expiry date.

1.2 Factors that Affect the PSL

1.2.1 Platelet deterioration

Paglia *et al* (2014) suggest that the PSL is not a single, progressive process but, rather, a series of shifts in platelet metabolism that results in distinct phases of platelet concentrate deterioration. Between days 0 and 3 they observed active glycolysis, pentose phosphate pathway activity and glutathione metabolism in stored apheresis platelet concentrates. From day 4 to day 6 the tricarboxilic acid cycle and purine metabolism were upregulated and from day 7 until day 10 the platelets entered a period of increased deterioration of their metabolism. In light of these observations, it may be too simplistic to expect any single measure of platelet quality to bear relevance through all stages of platelet concentrate deterioration.

Furthermore, the same team observed significant differences between the deterioration processes observed in apheresis units (as discussed above) and pooled buffy coat concentrates in a subsequent study (Paglia *et al*, 2015). A similar transition from active metabolism to metabolic decay pathways

was observed at day 4 in the buffy coat platelets. These concentrates, however, did not undergo a distinct shift from glycolysis to the tricarboxylic acid (TCA) cycle. Rather, they showed generally lower levels of activation and more gradual consumption of glucose. The two studies showed higher inter-bag variability between the apheresis concentrates than the buffy coat concentrates.

1.2.2 Platelet production methods

Other teams have also observed differences between apheresis and pooled buffy coat concentrates. Apheresis concentrates have been found to have greater haemostatic potential (Böck *et al*, 2002) and lower levels of activation as storage progresses (Ali, 2011). Quintero *et al* (2015) found apheresis platelets to be under less oxidative stress and, therefore, to have lower degrees of PSL changes. Although apheresis concentrates appear preferable in several respects, the buffy coat components of whole blood donations are more widely available and the blood service has a duty not to waste any part of these donations unnecessarily wherever possible. A better understanding of the differences between the two components may enable optimisation of production and storage methods that raises the quality of pooled buffy coat concentrates to a level comparable with those produced by apheresis (Vassallo *et al*, 2006).

1.2.3 Platelet additive solutions

For several reasons it may be desirable to replace plasma as a storage medium for platelets with an artificial platelet additive solution (PAS). Firstly, use of PAS has been found to reduce incidence of allergic reactions such as transfusion related acute lung injury (TRALI) by reducing the concentration of white cell cytokines in the storage media (Azuma *et al*, 2009; Heddle *et al*, 1994; Van der Meer, 2016). Secondly, careful manipulation of the platelet storage environment can be used to reduce the PSL by, for example, reducing glucose metabolism to limit production of lactic acid and consequentially reduction of pH (van der Meer, 2016). Finally, use of PAS has a potential application in supporting pathogen reduction, which is particularly relevant in platelet transfusion due to the

high rate of bacterial contamination observed due to the room temperature storage required for preservation of platelet viability (Kuehnert *et al*, 2001; Osman *et al*, 2016).

A variety of different PAS formulations have been studied and some are in routine use (van der Meer, 2016). Different additive solutions have different effects on the PSL. The potential to extend platelet shelf life by finding the optimal balance of contents and specifically targeting pro-PSL signalling pathways is a matter of considerable scientific interest (Leitner *et al*, 2016; Schubert *et al*, 2010). A number of compounds involved in lipid metabolism have been investigated recently and found to be potentially beneficial. These include high density lipoprotein-3 (HDL3) and apolipoprotein A1 (Pienimaeki-Roemer *et al*, 2014), anandamide (Zhuang *et al*, 2014) and L-carnitine (Deyhim *et al*, 2015).

1.2.4 Platelet storage

Following collection and processing, the conditions under which platelet concentrates are stored is also critical to maintaining their optimal quality. Firstly, all platelet concentrates require storage at room temperature because refrigeration leads to enhanced clearance of transfused platelets, possibly due to clustering of the glycoprotein-1b (GP1b) von Willebrand factor (vWF) receptor complex (Hoffmeister *et al*, 2003a). The pH of platelet concentrates decrease during storage with the accumulation of lactic acid due to glucose metabolism. This decrease is more rapid in the presence of anaerobic glucose metabolism (glycolysis), which results in greater lactic acid build up than aerobic metabolism (oxidative phosphorylation) due to its relative inefficiency (Murphy *et al*, 1975). Oxygen permeable containers are used to reduce the rate of pH decrease and prolong the lifespan of clinically useful platelets for transfusion (Murphy *et al*, 1975). Finally, an agitator is used to provide constant movement of the platelets within the concentrate throughout storage. This movement ensures that all platelets in the concentrate regularly come into contact with the oxygen permeable surface and prevents the development of localised anoxic areas (Torres *et al*, 2016).

1.2.5 Individual donor variability

A degree of variability is observed between the results of *in vitro* test results obtained for different platelet concentrate units even when they are matched for age, collection method and storage factors (AuBuchon *et al*, 2005; Carvalho *et al*, 2006; Seghatchian, 2006a). The extent to which this variability reflects differences between the physiological make up of different donors, and the significance of any such differences to recipients, is not yet clear. Differences in platelet aggregation and platelet production of the critical haemostatic proteins tissue factor (TF) and tissue factor pathway inhibitor have been observed in the presence of oestrogen, suggesting a qualitative difference in platelets from male and female donors (Jayachandran *et al*, 2005a; Jayachandran *et al*, 2005b). Qualitative platelet differences have been linked to underlying disease (e.g. diabetes) in the donor (Stratmann *et al*, 2005; Stringer *et al*, 2009) and a variety of metabolites, such as caffeine metabolites and fatty acid metabolites, have been found to affect either recovery or survival (Zimring *et al*, 2016). Recipient specific factors, including age, gender, body weight, medication and medical conditions, have also been found to influence the recovery and survival of transfused platelets (Slichter *et al*, 2005).

1.3 Determining Transfusion Efficacy

Radio-labelling of platelets prior to infusion enables very accurate measurement of recovery and survival and is the gold standard method for assessing transfusion efficacy. However, assessment of *In vivo* platelet concentrate efficacy has also historically been achieved using platelet count increments, which are easier, cheaper and less invasive to measure.

1.3.1 Laboratory measures of efficacy

One hour post-transfusion increments are used to assess recovery and 24 hour increments are used to assess survival (AuBuchon *et al*, 2005). In research for publication, these increments are usually

corrected for the patient's body weight and the number of platelets transfused (Balduini *et al*, 2001; Elfath *et al*, 1999). However, CCIs are not always reliable predictors of bleeding due to analytical shortfalls, variable platelet reactability, variability in other elements of the haemostatic process and possibly additional, as yet unknown, factors. Time intervals between platelet concentrate transfusions have also been measured as an indicator of efficacy, although the reliability of this parameter as an outcome measure is determined by the extent to which platelet concentrate usage is informed by transfusion guidelines (Slichter *et al*, 2005). It is worth noting that platelet function deteriorates rapidly *ex vivo*, necessitating testing within as little as 2 hours of sampling (Watson *et al*, 2005). Several platelet disorders affect both the functionality and number of platelets produced (Margaglione, 2005).

1.3.2 Clinical bleeding assessment

Measurement of platelet concentrate transfusion outcome could potentially be improved by assessing the degree of clinical bleeding (Stanworth *et al*, 2006). A variety of assessment methods have been used to this end, including that described by the World Health Organisation (WHO, 1979) and a modified version of the WHO criteria employed by the strategies for transfusion of platelets (SToP) study (Heddle *et al*, 2009). Clinical bleeding assessment would enable evaluation of platelet functionality in addition to incrementation. In addition, *in vitro* platelet function testing, performed on post platelet concentrate transfusion specimens drawn from the patient, may be of use in establishing the efficacy of transfused platelets. Suggested methods include thromboelastometry (Apelseth *et al*, 2010), thrombin generation measurement and assessment of whole blood platelet adhesion and clot formation capacity under flow conditions (Cauwenberghs *et al*, 2006).

1.4 Basic Laboratory Characterisation of Platelet Concentrate Quality

A simple, easily applicable test of platelet concentrate quality would be highly useful to ensure that patients are not transfused with sub-standard products, to enable evidence based development of collection, processing and storage techniques and to aid the best possible management of this limited and valuable resource. There is currently no single test available that can be used to assess platelet concentrate quality. Those tests that are available all have drawbacks in terms of sensitivity, reproducibility and / or applicability in a routine screening environment.

1.4.1 Current tests

The NHSBT determines the volume, WBC count and total platelet yield of each platelet concentrate prior to release (Walters, 2016). The pH of platelet concentrates that remain within the NHSBT stock at the time of expiry is also measured and is expected to be between 6.4 and 7.4. A large number of additional tests, including some of those used to investigate platelet function in patients, have been applied to assessment of the PSL on a research basis. Many of these are too cumbersome to be routinely used for a high volume of tests. In addition, the clinical relevance of PSL assessment methods is often uncertain as many indicators of PSL development can revert to normal following transfusion (Rinder *et al*, 2003).

1.4.2 Platelet count and mean platelet volume

The NHSBT requires the platelet count of each concentrate to be >240 x 10^9 platelets / concentrate in order to achieve a therapeutic dose (Walters, 2016). However, if platelet concentration is too high, metabolic processes may result in accelerated quality deterioration (Murphy *et al*, 1975). Increased mean platelet volume (MPV) in clinical samples may indicate high platelet turnover or the presence of a platelet disorder (Briggs *et al*, 2004; Watson *et al*, 2005). The MPV and the distribution of platelet sizes (platelet distribution width – PDW) may change during platelet concentrate storage in response to alterations of the platelets within the concentrate. It is difficult to predict the effect of

storage on these platelet indices. Aggregation of platelets can cause an increase in MPV and microvesiculation can cause a decrease in the MPV. Simultaneous occurrence of both of these changes would result in an increase in the PDW, although the MPV may remain unaltered (Seghatchian *et al*, 1997).

The full blood count (FBC) is a standardised panel of test relating to the quantity of different types of common blood cells and their basic features such as size and granularity. It is usually performed on a dedicated analyser that reports all parameters for each sample tested. FBC results produced by different, mainstream analysers are generally considered to be comparable. Platelet count and MPV are both included in the standard FBC test panel.

Impedance platelet counting methods are widely used, inexpensive and easily incorporated into high throughput systems. They detect and count cells as they move between two chambers of the system under the influence of an electrical charge. Impedance counts may be inaccurate in certain circumstances, for instance in the presence of fragmented / microcytic red cells or large platelets, which can prevent accurate separation of the two populations (Sandhaus *et al*, 2002).

Fluorescent platelet counts may be more accurate than impedance counts in specific conditions. The fluorescent platelet count performed on Sysmex XN series automated analysers (as utilised in this study) uses an oxazine dye that attaches to the platelet RNA, enabling the test to distinguish platelets from other blood cells with better specificity and sensitivity, producing a more accurate count.

1.4.3 Morphology

The majority of platelets in a normal individual who is not bleeding are in a quiescent state and have a regular, discoid shape. Upon activation, platelets undergo actin remodelling which causes significant morphological alteration (Watson *et al*, 2005). The platelets become spherical and then assume a dendritic form (they develop spiny protrusions called filipodia). The morphology observed in platelet concentrates generally indicates increasing activation as the concentrate ages.

Platelet concentrates composed mainly of disk shaped platelets produce a characteristic light scattering pattern that is visible to the naked eye. This phenomenon is known as swirling and can be assessed inexpensively, easily and non-invasively in the transfusion laboratory (Bertolini *et al*, 1994). Swirl assessment comprises a simple visual observation and is therefore highly subjective. When good quality, discoid platelets are observed under a bright light, swirling patterns can be seen in the solution. These swirls disappear as the proportion of discoid platelets decreases and the quality of the concentrate deteriorates. Loss of swirling indicates an activation associated change in platelet morphology. The sensitivity and specificity of platelet concentrate swirling assessment are not optimal and it is a totally subjective technique. However, its ease of use and low cost makes it an appealing test that could possibly be incorporated into practice as a screening method.

The Kunicki platelet score (Kunicki *et al*, 1975) is obtained by examining platelet morphology using phase contrast microscopy. Each cell is assigned to one of the following categories: discoid, spherical, dendritic or spread. Examples of these four different platelet morphology categories are shown in Figure 1.1. A multiplication factor of four, two, one or zero is applied to the percentage of platelets counted within each of these categories respectively. Scores of over 200 suggest acceptable platelet concentrate quality and extremely high or low morphology scores are thought to correlate with good and poor platelet concentrate quality respectively (Kunicki *et al*, 1975).

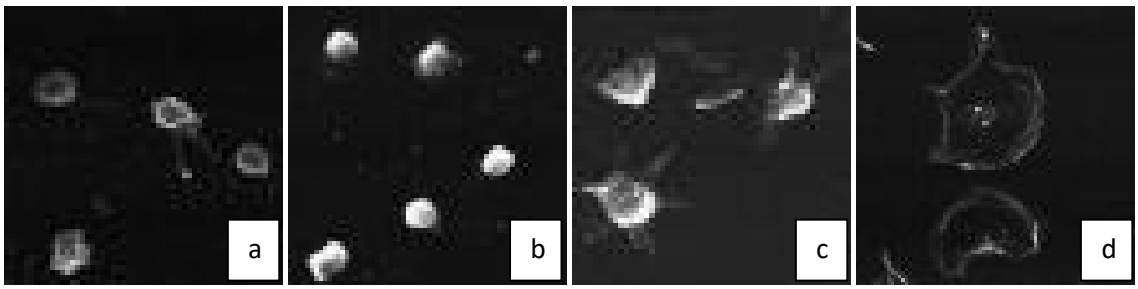

Figure 1.1: *Discoid (a), spherical (b), dendritic (c) and spread (d) platelets (taken from Chien et al, 2010)*

However, correlation between platelet concentrate quality and retention of discoid shape is uncertain. Initial studies showed the two variables to be related (Holme *et al*, 1998; Kunicki *et al*, 1975; Murphy *et al*, 1970) but these results are not universally reproducible (Mintz *et al*, 2005). This discrepancy is likely due to the ability of platelets to reverse the age related shape changes under certain conditions (Mintz *et al*, 2005; Rinder *et al*, 2003). In this case, the capacity to assume discoid morphology under optimal conditions (e.g. following transfusion) is likely to be a better indicator of platelet quality than the morphology score at any one, fixed point in time (Maurer-Spurej *et al*, 2006; Mintz *et al*, 2005).

The extent shape change (ESC) assay measures a similar variable to the Kunicki score. It utilises an aggregometer to spectrophotometrically detect changes in the amount of light transmitted through a platelet sample. The platelets are treated with ethylenediaminetetraacetic acid (EDTA) to prevent aggregation and then activated with adenosine diphosphate (ADP), which causes them to assume a spherical conformation and increases the optical density (OD) of the solution. Larger reductions in light transmission equate to higher levels of activation, greater shape change and a higher percentage of discoid platelets in the platelet concentrate under examination (Holme *et al*, 1998).

1.4.4 Biochemistry

The biochemistry observed within platelet concentrates changes throughout storage. This occurs due to both the normal ageing processes undergone by the platelets and artificially induced activation and apoptosis triggered or exacerbated by processing and storage. Some of these biochemical changes may be responsible for the reduction of platelet concentrate efficacy observed as the PSL develops and may, therefore, provide an effective measure of its extent. In addition, targeting these factors may enable development of less damaging processing and storage techniques and a means for their validation and monitoring.

Exposure to hypotonic conditions leads to an osmotic increase in platelet water content, which can be detected by an associated decrease in refractive index using an aggregometer as described for the ESC assay (Holme *et al*, 1998). This water influx can be corrected by metabolically healthy platelets with intact membranes, providing an indicator of platelet quality – the hypotonic shock response (HSR). ESC and HSR measurements have been found to correlate with clinical efficacy but again, only at extreme levels (Holme *et al*, 1998; Shrivastava, 2009).

Exposure to a low pH environment induces the shape changes associated with platelet activation and quality deterioration within a platelet concentrate (Murphy *et al*, 1970). Morphological change is dramatic and irreversible at pH levels of <6.0 and is associated with poor *in vivo* recovery and survival of platelets (Murphy *et al*, 1970). Measurement of oxygen, carbon dioxide, bicarbonate, lactate, glucose consumption, adenosine triphosphate: adenosine diphosphate (ATP:ADP) ratio and intracellular calcium levels (which are controlled by ATP dependant mechanisms) have been used to assess platelet respiration in platelet concentrates. However, only pH and ATP:ADP ratio have been found to correlate with *in vivo* platelet viability and these measures only show a relationship at extreme levels (reviewed in Shrivastava, 2009).

Although some of the traditional platelet concentrate quality measurements described above have shown correlation with platelet recovery and survival *in vivo*, they are not sensitive enough to be of great use in the routine assessment of platelet concentrate quality. Measurements of the pH, ATP:ADP ratio, morphological status and HSR reflect the extremes of the platelet quality scale but usually remain within the normal range for the entire shelf-life of modern concentrates (Cardigan *et al*, 2008; Dumont *et al*, 2002; Dumont *et al*, 2006; Levin *et al*, 2008; Seghatchian, 2006b).

1.5 Assessment of Functional Markers

Platelets must be able to participate in four separate functional processes in order for effective primary haemostasis to be maintained. They must be able to adhere to exposed collagen at the site of tissue damage, become activated and undergo degranulation, aggregate with other platelets to form a primary platelet plug and provide a suitable (negatively charged) phospholipid surface to support thrombin generation (Watson *et al*, 2005). Assessment of any of these functions could potentially provide insight into the quality of the platelet concentrate under investigation.

1.5.1 Surface glycoproteins

A number of cell surface glycoproteins (GP) are essential for normal platelet function and are involved in adhesion, activation and granule release and aggregation (Watson *et al*, 2005). Genetic abnormalities that affect cell receptors critical to platelet function can cause rare heritable disorders of primary haemostasis e.g. Glanzmann thrombasthenia, Bernard Soulier disease or collagen receptor deficiency, which result from $\alpha IIb\beta_3$, GpIb and GPIa/IIa or GpVI receptor abnormalities respectively (Margaglione, 2005). Flow cytometry can be used to demonstrate a lack or reduction of the affected glycoprotein on the platelet surface. It can also be used to quantify the ability of platelets to express markers of activation, such as P-selectin and pro-coagulant phospholipids and to release their granules (Watson *et al*, 2005).

The expression, concentration and responsiveness of some GPs are altered during PC storage. $\alpha IIb\beta_3$ is the most densely expressed GP on the platelet surface. The $\alpha IIb\beta_3$ protein has fibrinogen and vWF binding sites, which are exposed following conformational change that occurs when platelets are activated (Watson *et al*, 2005; Metcalf *et al*, 1997). Although $\alpha IIb\beta_3$ levels during PC storage have traditionally been thought to remain consistent (Metcalf *et al*, 1997), recent studies have observed a rise in gene expression of this receptor. Thon *et al* (2007) found GPIIIa mRNA to

be detectable and translationally active throughout the 10 day study period. However, the ability of $\alpha IIb\beta_3$ to bind fibrinogen upon stimulation of activation has been found to deteriorate as the PCs age (Curvers *et al*, 2004).

The GPIb-V-IX complex mediates platelet interactions with vWF and thrombin (Vicente *et al*, 1990; Lozano *et al*, 1997). During PC storage, GPIb expression decreases due to metalloprotease mediated proteolytic cleavage (Metcalf *et al*, 1997; Michelson *et al*, 1988; Bergmeier *et al*, 2003). GPIb expression is observed to become completely absent on a proportion of platelets present in the concentrate (Michelson *et al*, 1988). A simultaneous increase in concentration of glycocalicin: the cleaved extracellular section of GPIbα, is observed in the supernatant. This is found to correlate with surface P-selectin expression, supernatant concentration of soluble P-selectin, Kunicki scores and biochemical variables (Kostelijk *et al*, 2000). Reduction of GPIb expression is accompanied by a diminished reactivity of the platelets to thrombin (Lozano *et al*, 1997).

The GPV component of the GPIb-V-IX complex has a role in regulating platelet response to thrombin (Ramakrishnan *et al*, 2001). Levels of soluble GPV have been found to correlate with a variety of different PC quality indicators, including ESC, HSR, lactate concentration, glucose concentration, P-selectin expression and CD63 expression (Javela *et al*, 2005). GPVI is thought to function in association with the GPIb-V-IX complex, as disruption of either one of these glycoproteins has been found prevent aggregation when specific stimulation is applied to the other (Michelson, 2007). GPVI functions as a collagen receptor and is required for normal platelet activation to occur (Michelson, 2007; Poole *et al*, 1997).

1.5.2 Assessment of platelet activation responses

Defects affecting the storage pool of platelet granule contents can prevent effective secondary aggregation, which should occur as an amplification effect of primary platelet activation (Margaglione, 2005). Release of both α-granules and dense-granules is associated with increasing

activation status and has been observed as PCs age. Measuring the ratio of ATP:ADP in platelet rich plasma (PRP) can aid detection of storage pool deficiency in disorders such as Hermansky-Pudlak syndrome (Iannello *et al*, 2003).

ATP is present at a fairly consistent concentration within the cytoplasm of all cells and functions as a cellular energy currency. ADP acts as a biological signalling molecule involved in platelet function. It is stored in high concentrations in the dense granules of healthy platelets and is released upon activation. An increase in the PRP ATP:ADP ratio indicates a depleted pool of ADP to participate in platelet function. Dense-granule depletion has been demonstrated in PCs via increase of the ATP:ADP ratio (Rao *et al*, 1981; de Korte *et al*, 1990). ADP is preferentially degraded to AMP and hypoxanthine as it is actively expelled from the platelets, along with the other granule contents, into the supernatant. This observation has been confirmed by other groups (Botchway *et al*, 2000).

A number of other analytes are known to be contained within the platelet granules and can be detected, at increasing levels, in the PC supernatant as the concentrate ages. As an example, Rao *et al* (1981) found that platelet factor-4 (PF-4) concentration within the supernatant increased as the PC aged and in response to processing. This reflects release of platelet α-granules, where PF-4 is stored in fresh platelets.

Another marker of granule release is increased expression of transmembrane proteins that are stored within the granules of resting platelets. This protein group includes P-selectin (CD62P), which is found on the internal α-granule membrane of resting platelets and CD63, which is stored on the dense granule membrane (Larson *et al*, 1989; Stenberg *et al*, 1985; Berman *et al*, 1986; Nishibori *et al*, 1993; Israels *et al*, 2005). Upon platelet activation, the granules migrate to the cell surface, expelling their contents into the surrounding environment, and the granule membrane fuses with the cell membrane. This results in the translocation of P-selectin to the platelet surface.

CD63 functions as a signal transducer that is involved in initiating the cytoskeleton remodelling process that enables platelet spreading to occur (Israels *et al*, 2005). At present, the exact pathways involved have not been fully elucidated and the usefulness of CD63 as a PC quality marker may benefit from further investigation.

P-selectin is a cell adhesion molecule (CAM) expressed on activated platelets and endothelial cells (Larson *et al*, 1989; Stenberg *et al*, 1985; Berman *et al*, 1986). When expressed on the cell surface it is pro-thrombotic and enhances fibrin deposition (Palabrica *et al*, 1992; Mayadas *et al*, 1993; Hartwell *et al*, 1998). P-selectin glycoprotein ligand-1 (PSGL-1) is expressed on a wide range of leukocytes and is involved in their extravasion during inflammation and incorporation into platelet aggregates during clot formation (Sako *et al*, 1993; Vachino *et al*, 1995).

P-selectin is proteolytically cleaved and shed from surface of platelets (Michelson *et al*, 1996). Measurements by flow cytometry can be used to detect either platelet bound P-selectin or soluble P-selectin. Although P-selectin levels may give an indication of *in vitro* PC activation status, P-selectin positive platelets have been found to lose this marker, whilst retaining functionality, following transfusion (Berger *et al*, 1998). These factors limit the usefulness of P-selectin as a marker of PC quality (Holme *et al,* 1997; Bergmeier *et al*, 2003; Goodrich *et al*, 2006).

1.5.3 Reduction in responsiveness

Aggregation mediated changes in light transmission can be measured spectrophotometrically to assess platelet response to agonists such as collagen, ADP, adrenaline, arachadonic acid and ristocetin (Watson *et al*, 2005). More recently, it has become possible to measure platelet aggregation using an automated Sysmex coagulometer (Lawrie *et al*, 2014). Platelet aggregation response to ADP and collagen exposure has been found to decrease significantly after PC storage (Botchway *et al*, 2000; Akay *et al*, 2007). As storage time increased, the potential for thrombin and ADP stimulated Ca^{2+} release deteriorated and activation using the PAR1 thrombin receptor

agonist, SFLLRN, generated reduced P-selectin expression and fibrinogen binding (Curvers *et al*, 2004). The ability of platelets to bind to collagen under flow conditions and respond to this stimulus, in terms of Ca^{2+} release, was found to be significantly reduced after eight days of storage (Curvers *et al*, 2004).

However, platelet aggregation responses are maintained in stored platelets, at levels consistent with those observed using fresh platelets, if certain combinations of stimulatory agonists are used (DiMinno *et al*, 1982). Simultaneous activation via multiple stimulatory pathways is more representative of the initiation of haemostasis *in vivo* and suggests that the poor efficacy observed *in vitro* may be misleading. Re-suspension of stored platelets in fresh or fresh frozen plasma has been found to lead to normalisation of aggregation and $\alpha IIb\beta_3$ up-regulation upon activation (Ishikawa *et al*, 1987; Rinder *et al*, 2003). Comparison of aggregometry results from pre-transfusion PC samples and specimens drawn from patients following PC transfusion also indicate normalisation of the platelet aggregation response (Miyaji *et al*, 2004).

1.5.4 Anionic phospholipid detection with annexin V

In resting platelets, the anionic phospholipid content of the cell membrane is preferentially present on the internal surface. Upon platelet activation, increased intra-cellular calcium prompts externalisation of negatively charged, procoagulant membrane constituents such as phosphatidylserine (PS). This redistribution of charge enables coagulation factors to bind to the platelet surface and increases the efficiency of thrombin generation (Hemker *et al*, 2002). PS exposure can be measured by flow cytometry using the calcium binding protein annexin V or the opsonin lactadherin as probes (Metcalf *et al*, 1997; Albanyan *et al*, 2009). Both probes measure the degree of platelet activation and / or apoptosis, although lactadherin has been found to be more sensitive to low levels of PS expression (Albanyan *et al*, 2009).

Surface expression of PS is evident during apoptosis as well as platelet activation. This shared feature complicates distinction between activation and apoptosis in platelets compared to cells that only express PS during apoptosis (reviewed in Jackson *et al*, 2010). Experiments confirm these processes are separately initiated and regulated (Schoenwaelder *et al*, 2009; Leytin *et al*, 2007). Further investigation of these processes and the differences between them is called for.

1.5.5 Methods that replicate high shear environments

A major drawback of many platelet test methods is that they do not replicate the *in vivo* circulatory conditions, which have a significant effect on primary haemostasis. The flow of blood through the vessels at pressure creates a high shear environment that influences the actions of some haemostatic elements. For instance, vWF circulates in a globular conformation but, upon binding to collagen and under the influence of shear forces, unwinds to reveal additional GP1bα (platelet binding) sites, dramatically increasing its avidity (Auton *et al*, 2010; Barg *et al*, 2007). The PFA-100 platelet function analyser addresses this problem by mimicking the high shear conditions found *in vivo*. This method determines the ability of whole blood to form a thrombus under high shear conditions and upon contact with collagen and either adrenaline or ADP (Fressinaud *et al*, 1998). It is of use in assessing bleeding tendency in congenital disorders of primary haemostasis and may be helpful in monitoring response to anti-platelet therapies (Fressinaud *et al*, 1998; Marshall *et al*, 1997; Wuillemin *et al*, 2002). PFA analysis has been used to demonstrate weak but statistically significant reductions in aperture occlusion using modified collagen/adrenaline/ADP containing cartridges and addition of donor red cells (Beck, 2002).

1.5.6 Global haemostatic assessment

Another potential shortcoming of laboratory assessment of haemostatic function is that it rarely measures the combined effects of all elements of the haemostatic system and their interactions. Viscoelastic coagulometry techniques such as thromboelastography (TEG) and thromboelastometry

(ROTEM) measure clot formation features in whole blood (reviewed in Ganter *et al*, 2008). Resistance to rotation of a sample cup (TEG) or of a pin placed inside the sample cup (ROTEM) grows as a clot forms and is related to the clot size and strength. These point of care (POC) techniques allow rapid, holistic investigation of the combined ability of primary and secondary haemostatic mechanisms to form an effective clot. It also allows characterisation of clot breakdown features that may help put clot formation data into context and aid clinical bleeding / clotting risk assessment. These global assessment methods incorporate the contribution of all elements of the blood (platelets, red cells, white cells and plasma proteins) to generate a result. This may invalidate their use in separated blood components such as platelet concentrates as the red cells and white cells are absent. However, their potential to assess a wide range of platelet function indicators and ease of application make them a potentially useful tool for assessing platelet concentrate quality.

The EXTEM test on the ROTEM uses an extrinsic coagulation pathway activator to initiate clotting of the sample via TF / factor VII interaction. The ROTEM FIBTEM test uses the same reagents with the addition of cytochalasin-D, which inhibits actin polymerisation to prevent platelet shape change and subsequent activation. The clot formation observed during FIBTEM tests is due to fibrin production without the added involvement of platelets (Nielsen *et al*, 2000). The platelet contribution to the clot can then be determined by comparison with untreated reactions. Use of cytochalasin D in viscoelastic coagulometry testing has shown platelet activity to vary independently of platelet count in a rabbit model (Nielsen *et al*, 2000).

There have been some doubts expressed regarding the sensitivity of ROTEM or TEG assays without further optimisation to poor platelet concentrate quality (Arbaeen *et al*, 2016; Bontekoe *et al*, 2014; McNulty *et al*, 1997). However, a number of studies have used thromboelastography or thromboelastometry as a measure of platelet concentrate quality under various circumstances, without any apparent justification or assessment of the methods relevance (Bynum *et al*, 2016; Ostrowski *et al*, 2011; Ponschab *et al*, 2015; Svendsen *et al*, 2007).

1.5.7 Thrombin generation

The ability of the platelet phospholipid surface to support thrombin generation can be measured using a calibrated automated thrombogram (CAT) assay (Hemker *et al*, 2002). This is a semi-automated method that enables measurement of the total thrombin production within a sample over time. The timing and magnitude of thrombin generation observed enables identification of hypo or hypercoagulability that may be of future use in monitoring this aspect of PC quality (Hemker *et al*, 2002).

1.6 Microvesicles

Small, membrane bound microvesicles (MV) are present in the blood. MV are formed when membrane encapsulated fragments bud off of parent cells. The origin of particular populations can be determined by analysis of their surface antigens, for instance, platelet MVs (PMV) carry platelet specific cell markers such as $\alpha IIb\beta_3$, GP Ib and P-selectin (Heijnen *et al*, 1999; Horstman *et al*, 2004). The largest population of MV in the blood are those shed from platelets and they have been found to have a pro-coagulant function, which is evident from day one post collection (Curvers *et al*, 2004; Keuren *et al*, 2006; Wolf *et al*, 1967).

PMV are present in platelet concentrates and rise in concentration throughout storage as the platelets age (Bode *et al*, 1991; Simak *et al*, 2006). Their release is increased during platelet activation and apoptosis (Tan *et al*, 2005). PMVs provide a source of coagulation promoting (negatively charged) phospholipids that may partially compensate for poor platelet viability in ageing platelet concentrates (Chang *et al*, 1993; Xiao *et al*, 2000). In addition, PMVs have been found to express activated protein C resistant factor Va (Magdeleyns *et al*, 2007). They are able to bind to fibrinogen, vWF and collagen under flow conditions (Keuren *et al*, 2007) and have been observed to reduce micro-vascular bleeding time following infusion into thrombocytopenic rabbits (McGill *et al*, 1987).

However, PMVs are also associated with inflammation and alteration of immune function, which may lead to pathological consequences post-transfusion (Simak *et al*, 2006; Sprague *et al*, 2008). Increases in PMV numbers are thought to correspond to a decrease in platelet concentrate quality as the capacity of platelets that have undergone microvesiculation to activate normally during clot formation is diminished. Analysis of MV is difficult due to their small size and incompletely characterised biological features (Harrison *et al*, 2010; Zwicker *et al*, 2009). However, a number of techniques for MV analysis are now available or under development.

1.6.1 Functionality based measurement

Functionality based assays have largely focussed on the pro-coagulant features of negatively charged phospholipid or TF bearing MV (Harrison *et al*, 2010). Enzyme linked immunosorbent assay (ELISA) or other capture based methods can be combined with functional assays or scaled up to assess multiple cell surface markers at once but give no information about the size distributions of the populations under investigation (Harrison *et al*, 2010; Lal *et al*, 2008). These methods limit assessment to populations of MV that are specifically targeted according to their functions or surface molecules and may overlook clinically significant MVs with different characteristics.

1.6.2 Flow cytometry

Flow cytometry has been extensively applied to MV analysis and the necessary equipment is widely available. Multicolour analysis enables simultaneous assessment of multiple surface antigens, potentially relating to multiple MV populations and use of calibration beads allows MV quantitation. The major drawback of flow cytometry based methods is the lower size limit for detection. MV of less than about 500 - 300nm (diameter) cannot be reliably distinguished from background noise using forward and side scatter alone (Harrison *et al*, 2010). Use of fluorescence can improve resolution of smaller MV but limits analysis to targeted populations. However, even

though large, detectable MVs form the minority, flow cytometry MV measurements appear to show correlation with disease (Harrison *et al*, 2010).

1.6.3 Imaging and tracking of microvesicles

Imaging techniques such as electron microscopy (EM) or atomic force microscopy (AFM) give morphological information and can be used to analyse a wide size range of MVs but are labour intensive and require specialist equipment and skilled operators (Harrison *et al*, 2010). Whilst EM is able to visualise all MV present in a sample, AFM requires the MV under analysis to be immobilised on a mica surface and is, therefore, limited to assessment of targeted populations. More user friendly techniques specifically designed for tracking MV, such as the nanoparticle tracking analysis (NTA) instrument (Nanosight Ltd, 2009), have made MV analysis widely accessible and provide rapid measurement of size distribution and concentration.

1.6.4 Microvesicle impedance methods

Impedance methods are widely used for cell counting and have recently been adapted for MV enumeration. They can be combined with flow cytometric techniques to improve resolution of small MV (Zwicker *et al*, 2009). Also, there is potential in the application of impedance counting, sizing and electrophoretic characterisation as a stand-alone method.

The iZON nanoparticle analyser produced by iZON Science Ltd is an impedance instrument that can measure the size and charge of small cells and particles such as MV. Measurement of MV is difficult, as very fine resolution is required to detect the smaller particles. Reduced pore size improves resolution but renders the instrument very prone to blockage by larger particles. The iZON analyser tackles this problem by enabling the user to vary the pore size to remove blockages during operation (Sowerby *et al*, 2007; Willmott *et al*, 2008). iZON Science Ltd claim that this technology enables the user to accurately quantitate, size and characterise particles of between 50nm and 2µm diameter (iZON Science Ltd, 2008).

1.6.5 ThromboLUX instrument

Dynamic light scattering (DLS) measures the Doppler shift of light scattered by MV suspended in solution. The degree of Doppler shift is proportional to the Brownian motion of the particles, which is, in turn, related to their size. Large, irregularly shaped particles move more slowly than small, regularly shaped ones (Maurer-Spurej *et al*, 2007). As MV are theoretically uniformly spherical, DLS results are solely related to particle size (Xu *et al*, 2011).

The ThromboLUX instrument measures the size and quantity of platelets and PMV by DLS and the response of these variables to temperature change (Maurer-Spurej *et al*, 2006; Maurer-Spurej *et al*, 2007; Maurer-Spurej *et al*, 2009; Xu *et al*, 2011). Large, uniform sized platelets that can reverse temperature-induced changes and low MV numbers are associated with higher quality (fresher) platelet concentrates. A 'platelet quality score' in the range of 0-40 is generated. Scores above 14 indicate satisfactory concentrate quality. The ThromboLUX score has been found to correlate with 1 hour and 24 hour corrected count increments, representing platelet recovery and platelet survival respectively (Maurer-Spurej *et al*, 2009).

The ThromboLUX instrument has undergone extensive beta testing at multiple international sites over recent years. The ThromboLUX score has been found to deteriorate with platelet concentrate age between tests carried out at days two and ten after platelet concentrate production (Middleburg *et al*, 2013). Its potential to measure MV and aggregate populations in platelet concentrates has also been demonstrated (Labrie *et al*, 2013). However, Kraemer *et al* (2015) did not find any correlation between the ThromboLUX score and the HSR or ESC and Raczat *et al* (2014) found it to be insensitive to inhibition of platelets with a number of anticoagulants, including EDTA.

1.7 Summary

Platelets are a vital component of haemostasis and platelet transfusion is a life-saving procedure. Provision of safe, high quality platelet concentrates can be challenging and improvement and optimisation of platelet concentrate production techniques would be beneficial. Increased shelf-life of platelet concentrates would reduce wastage and identification of poor quality concentrates would prevent exposure of patients to unnecessary clinical risk. An inexpensive, quick and easy to use laboratory test would be a useful tool to aid in achieving all of these goals.

Platelets are functionally complex cells that are not yet completely understood, either in terms of their function or their storage characteristics. A wide range of tests have been applied to platelet concentrates in an attempt to comprehensively characterise the PSL but many of them give unclear or conflicting results or do not correlate with observed clinical outcomes. In addition, most of these tests are not suitable for routine application. However, a small number of techniques show some potential for routine application and may provide relevant clinical information. A selection of suitable methods will be chosen for investigation as part of this project.

1.8 Aims of the Study

Platelet concentrate quality and functional deterioration during ageing is poorly understood and difficult to measure. No single test can be used to assess concentrate quality. The aim of this project is to investigate novel laboratory approaches to this problem, including the ThromboLUX platelet concentrate analyser, the iZON system, thromboelastometry and automation of existing tests.

Following completion of the *in vitro* stages of the project, a proof of concept clinical study was undertaken. The aim of this study was to trial a method for assessing the ability of a panel of laboratory tests to predict transfusion outcome, as measured using platelet count increments, resolution / prevention of bleeding and adverse affects suffered by the patient. Tests were carried out on the remains of the platelet concentrate following transfusion.

The specific project aims are set out in the list below:

1. Select a panel of *in vitro* tests that have the potential to improve the assessment of platelet concentrate quality
2. Set up and optimise the selected reference and novel tests in the laboratory
3. Apply all reference and novel test methods to a sample of platelet concentrates throughout the ageing process to investigate the pattern of test results that are given over the entire concentrate lifespan and gain information relating to platelet concentrate ageing
4. Further assess the most promising novel methods, alongside appropriate reference tests, in a small, proof of concept clinical trial

2 METHODS AND PRELIMINARY TEST DEVELOPMENT

2.1 Samples and Platelet Concentrates

Platelet rich plasma samples and platelet concentrate samples from a variety of sources were used for this study. Due to the limited availability of these samples, there are times when the selection of test samples used for a specific set of investigations were not optimal or the design of an experiment was modified to gain the maximum benefit from the specimens available at that time. It would have been preferable to be more consistent with regards to the type of testing undertaken with each novel method but this was an evolving project, carried out over a long period of time and tests were undertaken as new methods were developed or selected for inclusion.

Platelet concentrate samples were available from several different sources. Initially, 25 expired platelet concentrates were obtained, over a period of about a year, from the clinical transfusion laboratory at OUH Trust. 23 of these expired at the end of day five after collection and the other two expired at the end of day seven (due to an NHSBT rule change at this time). They were, therefore, available for inclusion in the study from the beginning of day six or day eight respectively. 12 were pooled platelet concentrates and 13 were apheresis concentrates. FBC, iZON and ThromboLUX testing (including precision assessments) were carried out on all 25 concentrates. ESC and HSR testing was carried out on four of these concentrates and ROTEM testing was carried out on five of them. Samples from two of these concentrates were stored at inappropriate temperatures and then tested using the HSR, ESC, ROTEM and ThromboLUX methods.

In each case, the whole platelet concentrate was stored in an air conditioned room in which the temperature was maintained and monitored at 22^{o}C. It was kept in the original, breathable platelet bag and agitated on a platelet agitator. To draw samples for analysis, sterilised scissors were used to cut off the end of the outflow line. A small amount of concentrate was passed through the line to waste in order to flush it before samples were collected, although care was taken not to use lines

with visible debris or to leave any of the concentrate trapped in the line when resealing due to concerns that platelets trapped in the line would not undergo adequate agitation or oxygen exchange. Lines were re-sealed after sample collection with a heal sealer, enabling the whole process to be repeated from scratch for subsequent sampling events.

A series of platelet concentrate sample swapping experiments was undertaken to investigate the potential for different ThromboLUX results due to variations in platelet concentrate processing practices in different countries. For this section of the study, an assortment of different platelet concentrate samples from various locations and with diverse quality features was obtained from a number of different international centres. Although the storage of these concentrates was not controlled in house by the study team, the required conditions were specified and controlled by the participating laboratories.

Further to these preliminary experiments, ten fresh, pooled platelet concentrates were purchased over the course of a year from the NHSBT for comprehensive *in vitro* characterisation as they aged. They were produced using standard NHSBT methods in the Brentwood NHSBT processing centre and shipped on the day of pooling (the day after donation) to OUH Trust. Shipping was via a two hour temperature controlled service as routinely recommended for platelet transport. Platelet count, MPV, HSR, ESC, pH, swirl assessment, ROTEM testing and ThromboLUX analysis were carried out on all ten of these concentrates. The AWE test was developed during this phase of the study and, therefore, was only performed on the final four concentrates. Sampling and storage of platelet concentrates during this phase of the study was as for the expired concentrates discussed above.

The final source of platelet concentrate specimens were samples obtained during the clinical trial. These samples consisted of the residual platelets left in the bag and the giving set following platelet transfusion. The volume was, therefore, limited and the platelet storage conditions in the period immediately prior to sample collection was relatively uncontrolled. The residual concentrate was harvested into an airtight specimen pot and stored at room temperature without agitation. For these

reasons, testing was always completed no more than two hours after collection unless specifically stated otherwise (e.g. for 'aged' samples) in the text.

In addition to four of the fresh platelet concentrates purchased from the NHSBT and the six clinical trial samples, three PRP samples were used in the early development of the AWE test. They were prepared from whole blood collected into 3.2% buffered trisodium citrate using a minimal stasis technique to avoid venepuncture related platelet activation. The first tube taken during each venepuncture was discarded, all tubes were suitably filled and samples were checked to ensure no other signs of inferior quality (e.g. gross haemolysis, blood clots) were apparent. Immediately following collection, specimens were left undisturbed on the bench for 15 minutes at room temperature to allow platelets to revert to a resting state. They were then centrifuged at 150g for 10 minutes. The PRP from all samples was harvested and, when multiple samples were available from a single venepuncture, pooled immediately after centrifugation. When PPP was also required, the residual sample was then centrifuged at 4500g for 7 minutes to enable the harvesting of the PPP. Analysis for all samples labelled as 'fresh' or 'day 1' was completed within 3 hours of venepuncture. 'Aged' PRP samples were stored in capped, airtight containers at room temperature unless specifically stated otherwise.

2.2 Ethical Approval

Study specific ethical approval was not required for the *in vitro* part of the project. It was agreed at a departmental level, after discussions with the OUH Trust Research and Development Department that use of anonymised samples from healthy volunteers for small scale technical development of laboratory tests and as normal controls did not require ethical approval. Informed consent is obtained prior to blood donation and donors are notified that their tissue may be used for transfusion related research rather than transfused directly to a patient. All Platelet Concentrates are

anonymised by the NHSBT according to their normal protocol before they are supplied to the laboratory. No patient samples were used during this stage in the study.

An ethical approval application was made for the clinical trial stage of the project and full approval was granted in April 2015. The application reference details are as follows:

Study title:	*The evaluation of novel platelet concentrate quality tests and their ability to predict response to platelet transfusion*
REC reference:	*15/SC/0155*
IRAS project ID:	*131997*

2.3 Reference Test Methods

A limited panel of platelet concentrate quality reference tests were chosen for use in this study. Tests previously found to correlate with post-transfusion platelet function and those that covered as many different aspects of platelet function as possible were selected. Very complex or expensive tests were deemed to be beyond the scope of the study, which focused on analytical methods applicable to routine clinical practice.

Swirl assessment is difficult to standardise and can be affected by environmental factors such as light quality. However, it is quick, simple, inexpensive and has been found in the past to relate to post-transfusion platelet function (Bertolini *et al*, 1994) so its inclusion in the study appeared worthwhile. Both pH measurement and FBC (including platelet count and MPV) were freely available in our laboratory and were included to ensure concentrates complied with NHSBT expectations. These tests were also used to exclude the presence of very poor platelet quality. The HSR and ESC tests were selected as a more sensitive measure of platelet quality that could be performed using equipment that was readily available in our setting. As some of the only widely applicable platelet quality tests known to relate to post transfusion efficacy, these aggregation based methods were particularly desirable. The Kunicki score was also investigated due to its potential correlation with transfusion outcome but, ultimately, could not be performed using the equipment available.

2.3.1 Swirl

Assessment of 'swirl' within the platelet concentrates was carried out visually according to methods demonstrated by NHSBT staff. The concentrates were held in front of a bright light source (e.g. a 50 or 100 watt bulb) and moved around by hand as the contents were examined. 'Swirl' was apparent as a pattern of streaming light reflective swirls, most easily visualised in the parts of the bag where manipulation resulted in a thin layer of liquid. Results were recorded as positive or negative and no grading system was applied. No calibration or internal quality control of this method was possible.

2.3.2 pH

The pH of all concentrates was measured using a Hanna HI221 pH meter that was validated and maintained for routine diagnostic use in the Oxford University Hospitals (OUH) NHS Foundation Trust Haemophilia laboratory. A two point calibration was performed before each use, alternating between using pH 4 and pH 7 calibrators on one occasion and pH 7 and pH 10 calibrators on the next occasion. The probe was placed in the sample and the pH was displayed on the meter.

2.3.3 Platelet count and volume

During the early stages of this study, platelet count and MPV were determined using a Sysmex KX21 (Milton Keynes, UK) FBC analyser. This automated cell counter was validated, maintained and quality controlled daily for routine diagnostic platelet count measurement in the OUH Haemophilia laboratory and used as an impedance technique to count platelets. During the final, clinical stage of the study, Sysmex XN10 analysers were used to determine platelet count and volume for all patient and platelet concentrate samples. In addition to impedance counts (using the same technology to the KX21 analyser described previously) these analysers were able to report a fluorescent platelet count. All reagents were supplied by Sysmex and quality control samples were processed every four hours. For both analysers, the suitably mixed aliquot of sample was presented for the test probe and a button was pressed to initiate measurement. The results were displayed on the analyser software and then printed out.

2.3.4 Kunicki morphology score

The Kunicki morphology score is a useful reference method due to its correlation with clinical efficacy of platelet concentrate transfusion. An adaption of the method utilised by Maurer-Spurej *et al* (2009) that used light microscopy was initially trialled but the resolution of platelets was poor. A phase contrast system located in the Transfusion Department at the John Radcliffe Hospital was then trialled but the resolution of this microscope was also too poor to achieve acceptable quality results. In addition, neither of these systems had any capacity for image capture, making it difficult to record and confirm the results obtained using this highly subjective method. Unfortunately, in house measurements of the Kunicki Morphology Score were not possible but measurements have been performed by study collaborators at LightIntegra in Vancouver on occasion and are referred to in the section 3.5 below.

2.3.5 ESC and HSR assays

ESC and HSR assays were set up on a Helena Biosciences (Gateshead, UK) AggRAM aggregometer based on the methods described by Holme *et al* (1998).Both tests require the platelet concentrate sample to be diluted in platelet free plasma (Cryocheck normal reference plasma, Precision BioLogic, Canada) to give a count of around 300 x 10^9 platelets / L. All diluted samples tested within this project had platelet counts within the range of 270 – 330 x 10^9 / L. An aliquot of the normal plasma used to prepare the dilutions was read as a calibrator in every channel used for testing prior to running a platelet sample. This step was required by the preset measurement programmes in the software. The PRP OD value was used as part of the manual calculation for the ESC test but it did not, ultimately, contribute to the manually calculated HSR result.

For the HSR, two 250µL diluted samples were placed into the aggregometer, which measured the OD of the samples at 650nm for the course of the test. After measuring the samples for one minute to detect spontaneous platelet aggregation, 125µL of Owren's buffered saline (OBS) (Siemens, Frimley) was added to the control sample and 125µL of water was added to the test sample. Decreases in the

optical densities of both solutions were observed but the decrease in the test sample was higher when intact platelets were present as the platelets took up water by osmosis (see Figure 2.1 for an example). The extent by which the OD of the test sample corrected to the same level as the OD of the control sample in the four minutes after the addition of the water / saline was used to calculate the % HSR result using the following equation:

HSR = (Y4 x 100%) / (Y – S)

Where Y is the difference between the starting OD and the OD of the maximum water response, Y4 is the difference between the maximum water response OD and the OD as measured four minutes after maximum response and S is the measured OD change in the saline control sample.

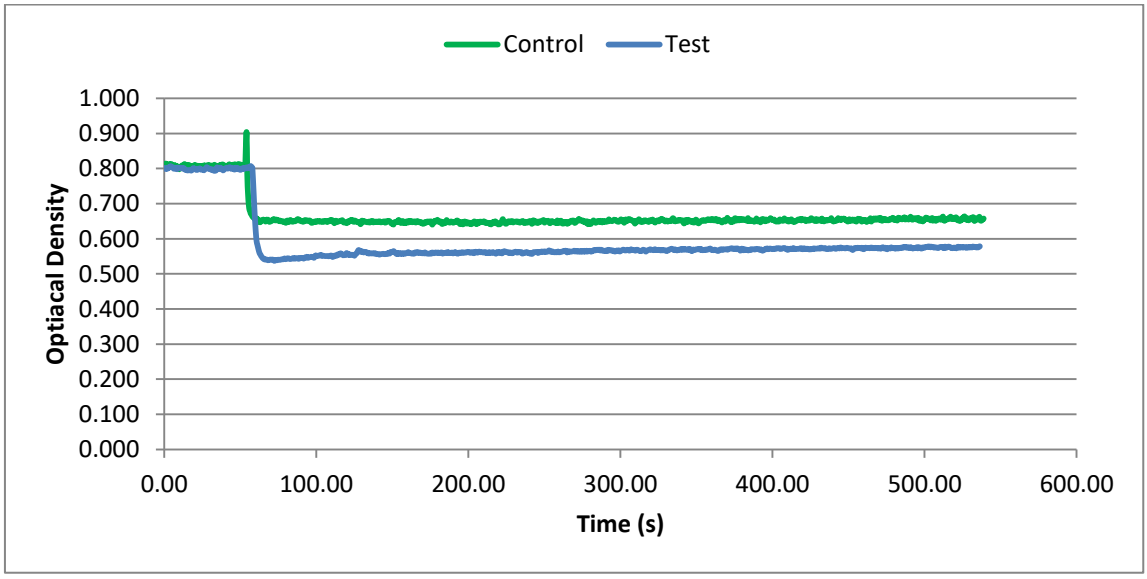

Figure 2.1: *Example HSR results. %HSR describes the degree by which the blue 'test' trace corrects to the level of the green 'control' trace.*

The ESC was performed using a similar method. 10µL of 0.1M EDTA (Sigma-Aldrich, Gillingham) was added to the diluted platelet sample to prevent platelet aggregation. 10µL of 1mM ADP (Sigma-Aldrich, Gillingham) was added to the test samples and 10µL of OBS to the control samples. The ADP triggered shape change in intact, functional platelets and this lead to an increase of OD. See Figure

2.2 for an example. The degree of increase was expressed as a percentage of the difference in OD between platelet rich plasma and platelet poor plasma.

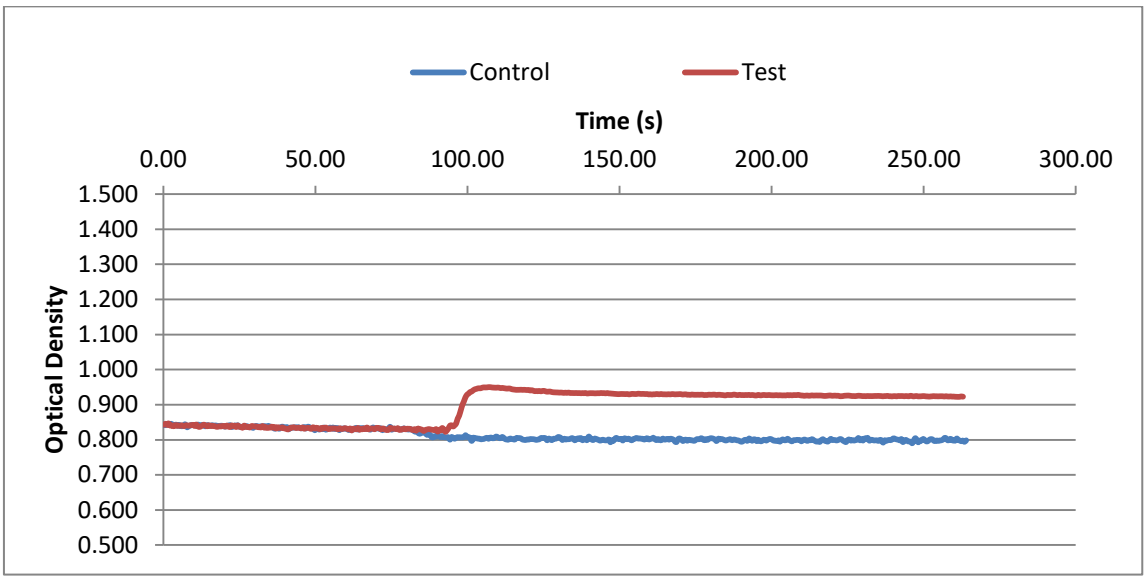

Figure 2.2: *Example ESC results. %ESC describes the change in the red 'test' trace as a proportion of the difference between PPP (blank) and PRP prior to activation.*

In order to calculate the ESC percentage, the raw data was downloaded from the aggregometer into a Microsoft Excel spreadsheet and converted to OD using the following formula as advised by Helena Biosciences:

Point Value x 0.000000001694600612 - 0.500302473804904

The OD of water was used to define 100% transmission and the OD obtained from a blocked channel was used to define 0% transmission. The PPP and PRP % transmission values (X and Y respectively) were determined against this scale and used to calculate extinction values (E) as follows:

$E = -log(T\%/100)$

Thus:

$E_{PPP} = -log(X/100)$

And:

$E_{PRP} = -log(Y/100)$

The degree of shape change observed during the test, expressed as a percentage of X – Y, is referred to as A. The extinction of shape change was calculated as follows:

$E_{Shape\ Change} = -log[(Y-A(X-Y)/100]$

The three calculated extinction values were then used to generate a final ESC percentage result using the formula below:

$ESC = (E_{Shape\ Change}-E_{PRP})100\%/(E_{PRP}-E_{PPP})$

Neither of these methods required calibration and routine quality control samples are not commercially available for platelet aggregometry tests. However, normal PRP specimens were processed on this aggregometer on a regular basis to ensure the equipment and reagents were functioning as expected.

2.4 Experimental Test Methods

Four new *in vitro* tests were identified as being user friendly, inexpensive and having the potential to measure platelet concentrate quality. It the beginning of the study, the ThromboLUX and iZON analysers were loaned to the haemophilia laboratory at OUH Trust free of charge for the purposes of evaluation in this study. The initial project plan was to undertake a large clinical study comparing ThromboLUX score with transfusion outcome in partnership with the NHSBT. The iZON assessment was intended to make an interesting aside that could be carried out whilst the project planning and approval aspects of the ThromboLUX study were in process.

The ROTEM instrument was available for clinical testing in the OUH haemophilia laboratory. It was decided to include it in the study when it became clear that the ThromboLUX did not perform as anticipated and adaptation of the project plan was required. The HSR and ESC tests were also set up at this time and it subsequently became clear that the HSR test showed more pronounced changes

with presumed platelet concentrate quality decline than most of the other test methods. For this reason, the AWE test was developed in house in an attempt to replicate the HSR results in a manner that was easier to perform and interpret and less prone to operator error or technical inconsistencies (e.g. inaccurate pippetting or timing). AWE tests were carried out using Sysmex CS series analysers that were used for routine coagulation testing in the OUH haemophilia laboratory.

2.4.1 iZON impedance analyser

The iZON system (iZON Science Ltd, Oxford) was specifically designed for measurement of MV and had not previously been used to test platelets. The platelet testing protocol used in this study was developed in partnership between the study team and iZON Science Ltd. The iZON qNano system assessed in the project is shown in Figure 2.3 below. The instrument is controlled and the data is presented by the iZON control Suite software on a linked computer. The measurement chamber is the small silver cylinder on top of the gold instrument. Pressure is provided by the black compressor unit and the tubing that runs from it to plug into the top of the measurement chamber. Nanopore stretch is controlled by the black dial on the right hand side of the instrument and changes are recorded in the software.

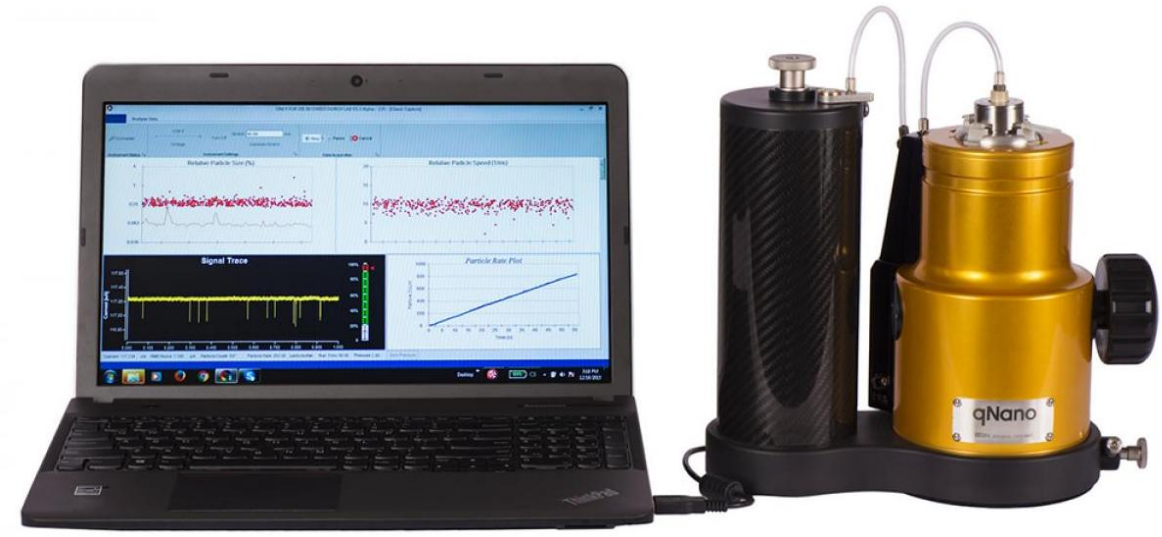

Figure 2.3: *iZON qNano analyser and software. Image from iZON Science Ltd company website: www.izon.com*

780nm beads were supplied by iZON Science Ltd and used to calibrate each run. Fixed, lyophilised platelets (Helena Biosciences Europe, Gateshead, UK) were prepared in 5mL tris buffered saline (Helena Biosciences Europe, Gateshead, UK) per vial. 40µL aliquots of this preparation were frozen at -40^{o}C and one was thawed to run as a biological internal quality control sample alongside each test run. Assessment of quality control acceptability was qualitative. Platelet samples for analysis were diluted in isotonic saline (Sigma-Aldrich, Gillingham) to give a platelet count of about 10 x 10^{9}/L.

Testing was carried out as follows:

<u>Instrument Setup:</u>

1. Turned on the iZON computer and loaded the iZON Control Suite software
2. Turned on the qNano instrument and connected it to the computer via the USB hub
3. Selected and fit the appropriate Nanopore – size 7P for platelet analysis (iZON Science Ltd)
4. Pipetted 80µL of electrolyte solution (0.9% NaCl – Baxter, UK) into the lower chamber
5. Fitted the upper chamber and added 80µL of electrolyte solution (0.9% NaCl – Baxter, UK) to it
6. Measured the stretch applied to the Nanopore using electronic callipers (provided by iZON Science Ltd) and entered this value into the 'Stretch' box on the 'Instrument Setup' screen
7. Clicked the 'Turn On' button on the 'Instrument Setup' screen and ensured a current was displayed
8. Ensured that normal responsiveness to altered stretch and voltage was observed.
9. Set the stretch and applied a voltage (45.5mm / 0.06v for platelets). Checked the current was 100nA ±10nA

<u>Data Acquisition:</u>

10. Ensured the system was clean by acquiring data for 1 minute to check no events were recorded

11. Removed the electrolyte solution (NaCl) from the upper chamber and replaced it with 50 – 80µL of sample that had been inverted 8 times to mix
12. Ensured the pore was open and that particles were detected
13. Entered the 'Data Acquisition' page and pressed 'Start' to begin collecting events
14. Collected at least 1000 events, ensuring that the trace remained stable during this time. Acquisition was paused while any instabilities were resolved
15. Pressed stop and filled in appropriate sample information in the pop up box. The 780nm beads were analysed with each run and saved as a calibrator. Fixed platelet samples were analysed with each run as biological controls.
16. When the sample analysis was completed, the sample was removed and the upper chamber was washed three times with electrolyte solution (0.9% NaCl – Baxter, UK)
17. Steps 10 to 16 were repeated for each of the subsequent samples
18. When data acquisition was completed, the Nanopore was removed, rinsed thoroughly with water, dried with a tissue and stored in a plastic cover

<u>Data Analysis:</u>

19. In the 'Analysis' screen, 'Open Signal Traces' was clicked to add previously acquired data to the List, which contained the data acquired during the current run
20. The 'Analyse the List' button was clicked
21. Individual signal traces were included in the data if the 'Include trace in XML file' option in the 'Data Output Settings' box was checked. Inclusion of traces greatly increased the data file size and slowed analysis but was sometimes desirable to ensure the validity of individual events. 'Data Output Settings' was accessed from the 'Data Acquisition' menu (click the lower right hand corner) under the 'Acquire Data' tab

22. ‘Open Particle Data Sets’ was clicked to open previously analysed data sets. These only included individual signal traces if they were generated during the original analysis.

23. Individual events were selected by clicking on the relevant data point in a scatter-plot under the ‘Analysis’ tab. This highlighted the event data in the ‘Translocation Data Points’ table at the bottom of the screen and enabled exclusion by unchecking the ‘Include’ box next to that event.

24. Calibration was required for display of absolute (rather than relative) size. This was achieved by clicking on the specimens ‘Calibrated’ box in the ‘Particle Data Sets’ list. Any calibration sample that was open in the list can be selected and applied.

25. Histogram BINs were individually calculated for each data set and needed to be altered to provide consistency when comparing multiple data sets. The desired BIN width was entered into the ‘View Settings’ box (accessed from the ‘View’ menu under the ‘Acquire’ tab).

26. If additional data processing was required the event data could be accessed in Excel by right clicking on a specific sample in the ‘Particle Data Sets’ list and selecting ‘Export to CSV’. This generated a CSV document which was saved in the iZON file for the sample in question.

2.4.2 ThromboLUX platelet quality analyser

The ThromboLUX analyser (LightIntegra Technology, Vancouver) had been developed specifically for measuring platelet concentrate quality and trialled extensively by the developers, who had published the results of their work with the analyser in several articles (Maurer-Spurej *et al*, 2006; Maurer-Spurej *et al*, 2007; Maurer-Spurej *et al*, 2009). Because of this, it was anticipated that the instrument would work as stated and that, after a brief technical verification that the ThromboLUX was performing correctly, this study would rapidly move on to a straightforward beta evaluation. Plans were made to work with the NHSBT and an ethical approval application was prepared to recruit patients participating in the NHSBT TOPPS and ATHENA studies to a sub-study undertaken to assess the ability of the ThromboLUX to predict transfusion efficacy.

When the ThromboLUX system was first delivered to OUH Trust in 2010, it had been redesigned from the prototype version that had been used during the initial evaluations. The new marketed version was a closed box system that was straightforward to use and could be applied either as a routine screening tool in the transfusion laboratory or on a point of care basis. See Figure 2.4. In addition, it had been adapted to use a disposable sample presentation system that drew a platelet concentrate specimen into a capillary tube when the analyser began the test.

Figure 2.4: *ThromboLUX analyser. Samples are presented in the holder at the front of the analyser, test details are input using the touch screen and results are displayed on the screen when the test is finished. Image from LightIntegra Technology company website: www.lightintegra.com*

Calibration of the optical aspects of the system was carried out by LightIntegra during installation and periodic maintenance. Routine calibrations during operating were not required. Vials of 'calibration beads' were supplied by LightIntegra for use with the ThromboLUX instrument.

However, these were used as an internal quality control measure, rather than a calibration material. Test specimens were undiluted platelet concentrate samples. Early attempts to analyse PRP generated from whole blood samples resulted in analytical errors and did not produce results so ThromboLUX analysis of PRP was not included in the study.

The ThromboLUX analytical method underwent several changes during the course of the study. The initial test method is shown below and subsequent changes are discussed afterwards.

ThromboLUX Analysis:

1. Switched on The ThromboLUX analyser and allowed to warm up for 30 minutes
2. Touched the screen and entered username and password to log in
3. Entered the settings menu to ensure the correct settings were applied for the sample in question. In particular, the correlation time and analysis viscosities needed to be altered between running calibration beads (water) and biological samples (plasma). The required values are shown in Table 2.1 below:

Table 3.1: *ThromboLUX viscosity settings*

Specimen (Medium)	Correlation Time	Calibration Viscosity	Viscosity at 37°C	Viscosity at 20°C
Calibration Beads (Water)	120000	6.93e-04	6.93e-04	1.002e-03
Biological (Plasma)	240000	6.93e-04	1.06e-03	1.56e-03

To alter any of the settings, the box in which the current setting was displayed was tapped, followed by the spanner at the bottom of the screen. The value was changed as required then the tick was tapped to return to the overview.

4. Selected the appropriate sampling method. Spiked disposables were inserted directly into platelet / calibration bead packs but carried a risk of introducing contaminants so were only used with platelet concentrates directly before disposal. Tubed disposables (designed to be sterile docked to platelet / calibration bead bags) were used in this study to withdraw samples from open containers. About 3mL of sample was withdrawn.

5. The 'Run Test' (platelet concentrate samples) or 'Maintenance Test' (calibration bead samples) mode was selected as appropriate and sample identifiers were entered using a barcode scanner or keyboard

6. The disposable was prepared and inserted into the test chamber. The arrow was tapped to initiate the test, which took about 20 minutes.

7. Upon completion, the test results (including the ThromboLUX score) were displayed on the screen. They could be retrieved at later dates by entering the access log mode, selecting test log or maintenance log as required and inserting a memory stick before clicking the download icon (down arrow).

Data processing was all performed by the test software but a brief overview is given here for reference. Each sample was measured at 37^{o}C, then at 20^{o}C, then a second time at 37^{o}C. The analyser measured the relative intensity of light (I) deflected by each particle category (MV, platelets and aggregates) and determined the mean radius (R) using dynamic light scattering principals. These measurements were used, along with the standard deviation (SD) of the measured particle radius, to calculate morphology values (M) for each particle categories as follows:

$(R - SD) \times I = M$

The platelet M (PM) and MV M (MVM) readings were used to calculate the overall ThromboLUX score as follows:

$(PM^{37a} - MVM^{37a}) + (PM^{20} - MVM^{20}) + (PM^{37b} - MVM^{37b}) = ThromboLUX\ score$

Measurements for aggregates were not directly included in the calculation but influence the ThromboLUX score by reducing the relative light intensity of the platelet and MV sized particle categories. See Figure 2.5 for an overview of the test principal.

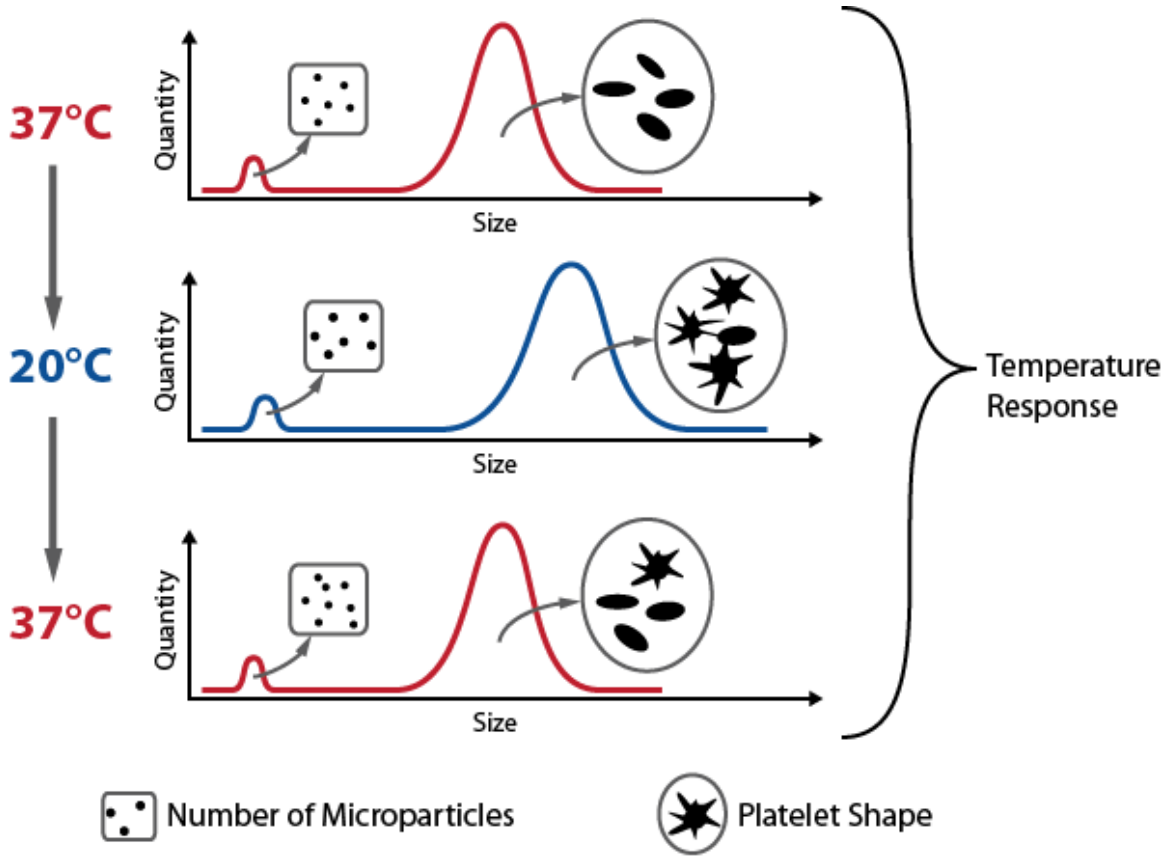

Figure 2.5: *Principal of the ThromboLUX test. Platelet and PMV size distributions are measured by DLS at 37°C, then 20°C, then 37°C again. A degree of activation is expected at 20°C but should be largely reversed in the second 37°C measurement in good quality platelet concentrates. PMV numbers should be low in good quality concentrates.*

When preliminary results were not as expected (see chapter 3), the first ThromboLUX analyser (TL 1) was exchanged for a second machine (TL 2). TL 2 had been re-engineered to accept samples presented in capillary tubes, rather than the disposable sampling system. Plain glass capillary tubes and a suitable syringe were provided by LightIntegra. At the time this change was made the software on the ThromboLUX analyser was upgraded to enable all tests (both maintenance and sample tests) to be run using a single setting, rather than selecting the 'Test' or 'Maintenance' mode separately as described in step 5. The TL 2 instrument software prompted the user to state

whether the specimen to be processed was a platelet concentrate sample of a calibration bead sample and automatically updated the viscosity settings appropriately.

Further testing was carried out with TL 2 and it became clear that analytical issues had not been resolved. TL 2 was exchanged for a third instrument (TL 3) at the beginning of the sample swapping stage of our study. Shortly after this, it was decided that the way fluctuations in the data and outlying data points were handled by the existing software was not robust enough and a different software programme, called Thrombosight, was developed. This software was not run on board the analyser but as a standalone programme on a PC. ThromboLUX users were advised to disregard the score generated on the instrument. The ThromboLUX data was instead downloaded to memory stick, transferred to the Thrombosight programme and analysed by this programme to generate the ThromboLUX score.

An additional technical overhaul was made between the end of the *in vitro* analysis of ageing platelet concentrates and the beginning of the clinical trial. The exact modifications made to the analyser at this point were not clear, excepting that it had been removed from OUH Trust without notice during the study team's absence, 'serviced and checked', returned upon request and subsequently reinstalled in the haemophilia laboratory. The laser was realigned at this point and it is not clear what difference this made from the previous setup. The ThromboSight software was also updated, allowing the study team to access the relative light intensity result for the MV fraction of each specimen tested during the final part of the project.

In addition to the software and hardware changes made to the analyser, there were a number of alterations in the result interpretation advice given by LightIntegra. Maurer-Spurej *et al* applied a cut-off value of 12 in their clinical study (Maurer-Spurej *et al*, 2009). We were told that scores above 14 were indicated satisfactory concentrate quality on receipt of TL 1 at the beginning of this study. The cut off value was changed to 16 when we began working with TL 2 and when TL 3 was installed LightIntegra advised that they were no longer able to define a global cut off value.

At this point, it was recommended that all centres performing ThromboLUX analysis determine their own, locally assigned quality cut off value.

2.4.3 Thromboelastometry

The ROTEM instrument (Tem International GmbH) detects resistance to oscillation of a pin in a cup caused by development of a clot. See Figure 2.6 for an overview. As clot amplitude (size) increases, resistance to pin rotation increases in a proportional manner. This reduces the extent to which the pin turns. The degree by which the pin turns is determined by optical analysis of light deflected from the pin to a detector (Tem International GmbH, 2016).

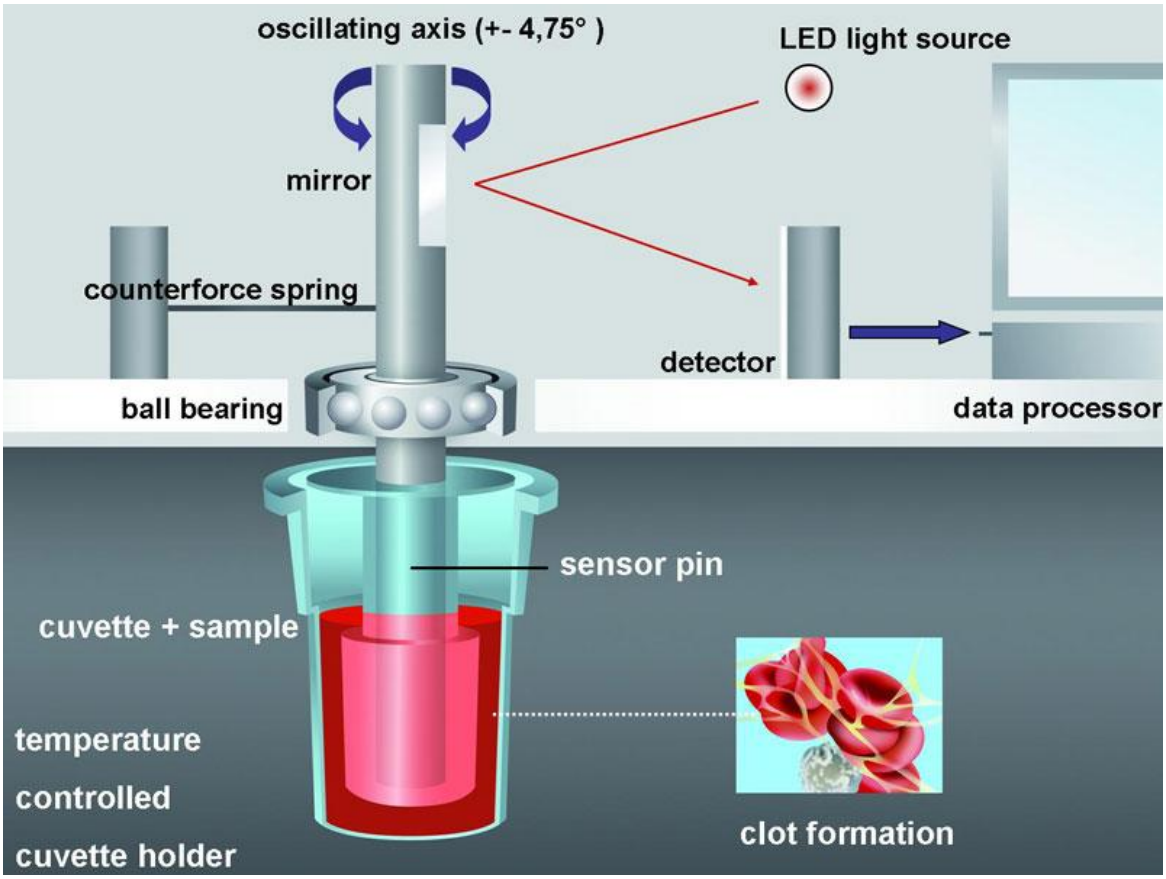

Figure 2.6: *Principal of ROTEM analysis. Image from LightIntegra Technology company website: https://www.rotem.de/en/methodology/thromboelastometry/*

Resistance over time is displayed in graphical representation by the analyser. The clot amplitude is shown on the Y axis and the time since the test began is shown on the x axis. See Figure 2.7.

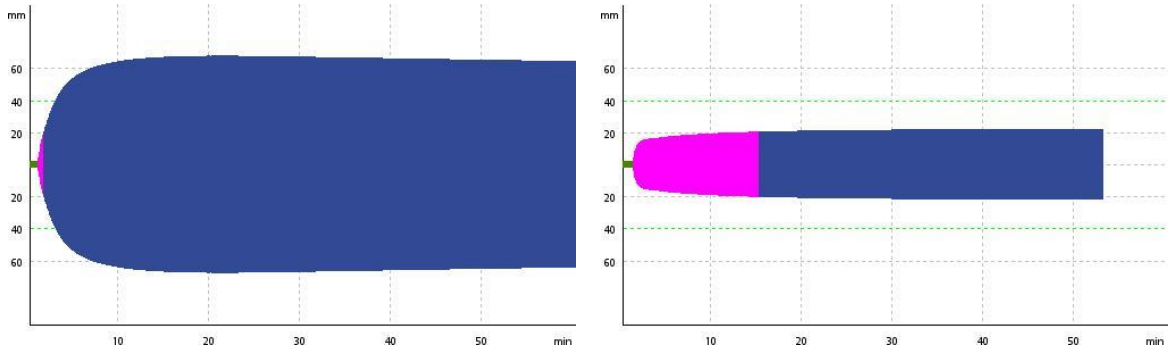

Figure 2.7: *Example ROTEM results. An EXTEM result is shown on the left and a FIBTEM result is shown on the right. The contribution of the samples platelet activity to clot formation is equal to the difference between the two results.*

The ROTEMs software uses the raw light data to generate an amplitude measurement. Clot amplitude (A) can then be used to calculate clot elasticity (CE), which is preferential for use measuring platelet function (Solomon *et al*, 2015). The relationship is as follows:

$CE = (100 \times A)/(100 - A)$

Clot elasticity may also be expressed in units of dyne/cm^2 (G). The G value is 50 times the CE value reported by the ROTEM.

There are a variety of different test protocols available from TEM for use on the ROTEM system, each with their own combination of reagents and pre-programmed pipetting procedure. For this study, the EXTEM and FIBTEM tests recommended for clinical analysis of platelet function were used. Both tests use a TF activator and the FIBTEM test also incorporates a platelet inhibitor (cytochalasin-D).

The first ROTEM platelet concentrate tests included in this study were carried out using neat samples. However, the sensitivity of the method at such high platelet counts was a matter of concern so subsequent tests were carried out on both neat and diluted samples. Sample dilutions were prepared in Cryocheck normal reference plasma (Precision BioLogic, Canada) to give a platelet count of 300 x 10^9 / L ± 10% as previously described.

The test method used to perform the ROTEM tests were as follows:

1. Switch on the ROTEM instrument, ensure all cup holders are placed against the back panel and leave for 30 minutes to warm up
2. Prepare sample dilutions as appropriate
3. Remove EXTEM, FIBTEM and SarTEM (CaCl) reagents from the fridge and leave for 5 minutes to come to room temperature
4. Log in to the ROTEM software and enter the 'Analysis' module
5. Add appropriate sample identification information to each one of the four channels to be run
6. Select the appropriate test for each of the four channels to be run. A typical run would consist of neat EXTEM in channel 1, neat FIBTEM in channel 2, diluted EXTEM in channel 3, diluted FIBTEM in channel 4.
7. Place disposable cup and pin covers into cup holders for each of the channels to be used
8. Select the first channel to be run and tap 'Start'
9. Follow the pipetting instructions on screen. For EXTEM these are:
    - Select tip
    - Place in SarTEM reagent
    - Press button to draw up reagent
    - Press button to draw up air space
    - Place in EXTEM reagent
    - Hold over cup and press button to expel reagents into cup
    - Discard and replace tip
    - Place in mixed sample
    - Press button to draw up sample
    - Hold over sample cup and press button to expel sample into cup
    - Place tip in bottom corner of cup and press button to draw up test mixture
    - Hold tip over cup and press button to expel tip mixture into cup (for mixing)
    - Place cup holder onto measurement pin
    - Press button on screen to confirm beginning of test
10. For FIBTEM the pipetting steps are:

- Select tip
- Place in SarTEM reagent
- Press button to draw up reagent
- Hold over cup and press button to expel reagent into cup
- Discard and replace tip
- Place in FIBTEM reagent
- Hold over cup and press button to expel reagent into cup
- Discard and replace tip
- Place in mixed sample
- Press button to draw up sample
- Hold over sample cup and press button to expel sample into cup
- Place tip in bottom corner of cup and press button to draw up test mixture
- Hold tip over cup and press button to expel tip mixture into cup (for mixing)
- Place cup holder onto measurement pin
- Press button on screen to confirm beginning of test

11. Allow all channels to run for at least one hour then stop any channels that are still running and transfer the data to the archive by tapping the 'Clear Channel' button
12. Data was retrieved from the Database module after analysis and transferred to a USB stick
13. The data files were then opened in Microsoft Excel to access a spreadsheet containing all parameters, including G

The ROTEM did not require any calibration but internal quality control samples were purchased from TEM Internationa GmbH and processed monthly as recommended by the company.

To evaluate platelet function the G values were used to calculate platelet elasticity as follows:

EXTEM G – FIBTEM G = Platelet G

In some cases, the results were also standardised for platelet count as follows:

(EXTEM G – FIBTEM G) / Platelet Count = G per Platelet

2.4.4 AWE test

The AWE test is a simplified and automated version of the HSR performed on a Sysmex CS-5100 or CS-2100 analyser that was developed as part of this study. It is potentially transferable to other automated analysers. As with the HSR, the AWE test measures the ability of platelets to pump out water as a marker of their viability and ability to function. Platelet concentrate (or PRP) samples were placed into 2mL sample tubes, loaded into an analyser rack and placed on board the analyser. Up to ten samples could be placed into each rack and the analyser then automatically pipetted 120 µL of each sample to its own test cuvette. After 10 seconds, 60 µL of water was added to each test cuvette and absorbance at 575nm was recorded for the next 180 seconds. Initially the absorbance of the test samples decreased as the platelets took up the reagent water. After reaching a minimum level at around 20 seconds, the absorbance of test samples containing viable platelets began to increase as the platelets pumped out the water.

The AWE test uses the Sysmex CS series immunoassay detection method to measure changes in OD that are reported in units of Absorbance per minute. This combination of settings is also used for measurement of prothrombin time in a routine clotting screen. The test reports the change in OD between the minimum absorbance recorded after addition of the water reagent and the absorbance level recorded after a period of recovery. There was, unfortunately, no option to blank each sample against its equivalent PPP sample within the current CS series software.

A variety of different test settings were evaluated. The test was trialled using both neat platelet concentrate samples and samples diluted in Cryocheck normal reference plasma (Precision BioLogic, Canada) to give a platelet count of 300 x 10^9/L (± 10%). Two different measurements were taken from each reaction trace: the variable result (VAR) refers to the degree of slope measured over the 15 second period during which the increase in OD is greatest and the fixed (FIX) result measured the slope over the 80 second period from 20 to 100 seconds. See Figure 2.8. Finally, all assays were performed in duplicate using both test cuvettes without stir-bars and test

cuvettes with stir-bars wherever possible in order to investigate the possible effect of samples settling during analysis. The stir bar tests are referred to as VSB (variable stir-bar) and FSB (fixed stir-bar).

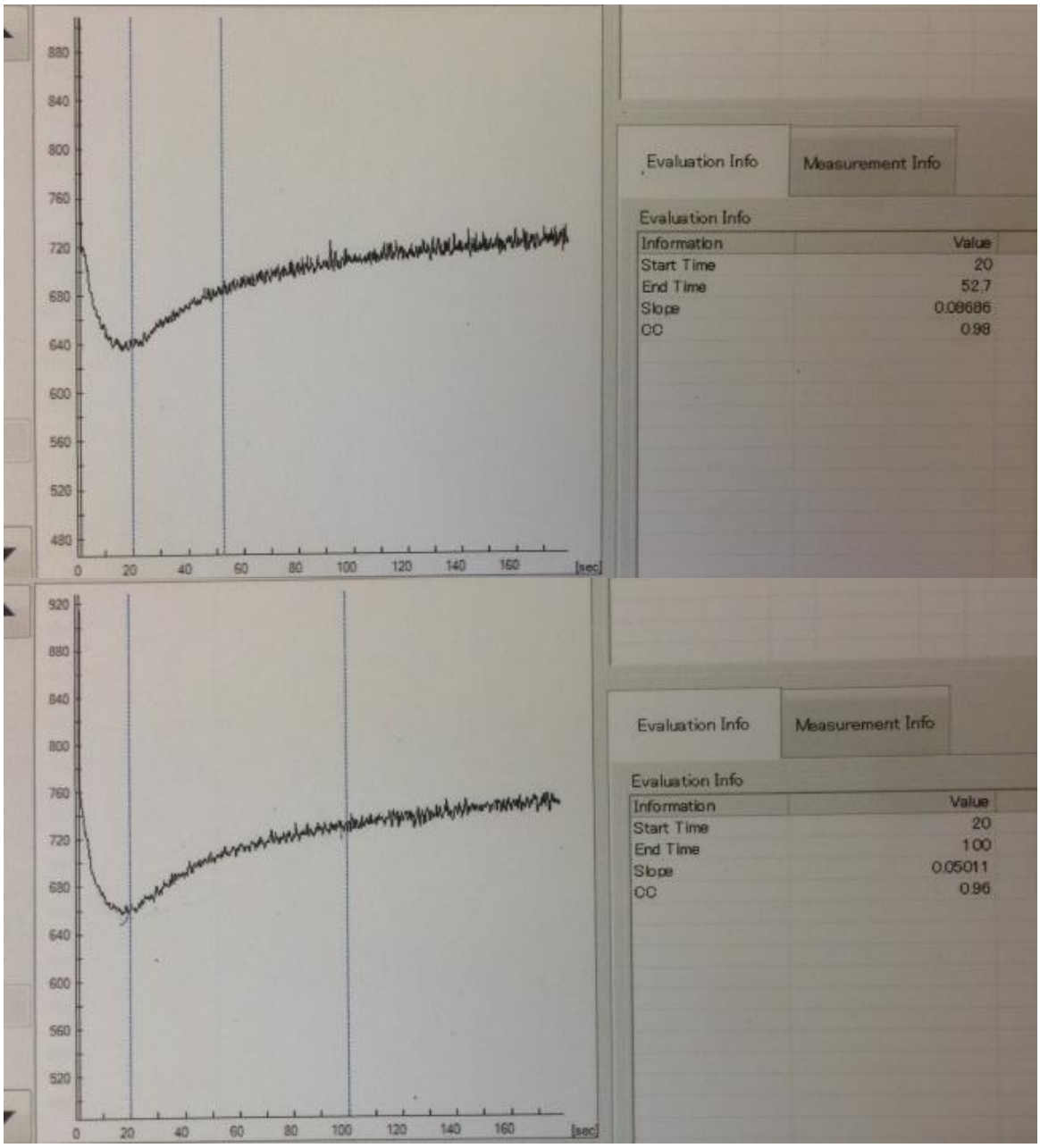

Figure 2.8: *Example AWE results. A test performed using the VAR measurement settings is shown in the top picture and a test performed using the FIX measurement settings is shown in the bottom picture. The graphs show absorbance on the y axis and time in seconds on the x axis. The 'slope' recorded in the table on the right hand side is the result that is reported. This measurement reflects the change in OD between the two vertical blue lines on each graph.*

3 IN VITRO RESULTS

3.1 *In-Vitro* Results Introduction

The first part of this study focused mainly upon establishing a panel of laboratory techniques to be taken forward into the clinical phase. All technical development and assessment was carried out using commercial quality control specimens where available, PRP samples taken from healthy volunteers and out of date platelet concentrates discarded by the OUH Transfusion laboratory when they expired at five or seven days old.

A large proportion of this work focussed on determining the cause of the uniformly high ThromboLUX scores. It was initially unclear whether the problem was technical in nature or specimen related. Specimen related problems could be related to the age, transport or storage of the platelet concentrates being tested or could reflect some small but significant difference between UK platelet concentrates and those produced in Vancouver, where the ThromboLUX analyser was developed. Technical problems could be specific to a particular instrument or linked to the methodology in general. To aid distinction between these factors, a series of sample exchange experiments were undertaken with other sites using the ThromboLUX analyser.

Following the initial development stage of the study, 10 pooled platelet concentrates were purchased from the NHSBT to gain further data with the ROTEM®, to try and establish a local cut-off value for the ThromboLUX and apply all available methods to specimens that are more closely comparable with the platelet concentrates used in clinical practice. All concentrates were tested at set time-points as they aged.

3.2 *In-Vitro* Results Methods

One of the challenges encountered during this project is the lack of reliable reference methods. HSR, ESC, pH testing and swirl assessment were performed as reference methods. Swirl and pH were not assessed during the very earliest stages of the study as the initial aims were to detect subtle quality deteriorations that were thought unlikely to be measurable by these relatively insensitive techniques. However, both methods were added later to flag very poor quality concentrates that we suspected were not being detected by the ThromboLUX. The novel techniques used during this phase were the iZON nanoparticle analyser, the ThromboLUX platelet quality analyser, the ROTEM® thromboelastometry system and the AWE test.

For the multiple site platelet concentrate exchange phase of the study, specimens were prepared and shipped to Oxford for testing from the NHSBT processing sites at Colindale and Brentwood, the Canadian National Blood Service and Sanquin in the Netherlands. Shipping methods varied from the controlled conditions used to transport clinical platelet concentrates to transport of capped containers at potentially inappropriate temperatures for platelet concentrate storage that should have induced poor quality. In addition to the Haematology Department at OUH Trust, testing was undertaken at the NHSBT Laboratory in Cambridge, the LightIntegra Laboratory in Vancouver, the Sanquin Research Laboratory in Leiden and the Haematology Department at the HAGA Hospital in The Hague.

The fresh platelet concentrate phase of the study provided an opportunity to further test the ThromboLUX analyser since the upgrades and modifications made during the previous study stages and determine whether the sensitivity of the instrument to age related platelet quality deterioration had improved. Additional testing with the ROTEM instrument was also performed and the initial stages of development of the AWE test were carried out.

3.3 *In-Vitro* Results of Reference Tests

The results obtained during the development, characterisation and assessment of a panel of platelet quality reference tests in the laboratory are shown below.

3.3.1 Automated blood cell analysis

Automated platelet counting and sizing was carried out using automated Sysmex FBC analysers. The Sysmex analysers use an impedance technique to determine the number of platelets in a sample and the overall proportion of the sample that is made up of platelets. MPV is then calculated from the impedance distribution. The concentrate volumes recorded by the NHSBT were used to calculate the platelet doses contained within the concentrates. In the initial validation stages of the study, results obtained from expired concentrates on the first day of testing (day 6, day 7 or day 9 after collection from the donor) were compared to results obtained on the final day of testing (day 11, day 12 or day 13 after collection) to investigate the effects of age related deterioration on automated blood cell indices. The platelet count, MPV and platelet dose results for 25 expired concentrates are shown in Table 3.1.

Table 3.1 also includes the results of T-tests carried out to compare the automated cell counting features of pooled and apheresis concentrates. These are shown at the bottom of the table. Unpaired, bi-directional T-tests were used to compare the two separate populations as the direction of change was unpredictable. It was found that the platelet count and dose differed significantly ($P = \leq 0.01$) between apheresis and pooled platelets, both at initial testing and final testing. However, the MPV showed no statistically significant change between the two collection types ($P = 0.14$ at initial testing and 0.23 at final testing).

In addition, the automated blood count test results obtained on the first and final days of testing were compared. The results of both paired and unpaired T-tests are shown in the right hand column.

Uni-directional T-tests were used as it was anticipated that the platelet count would decrease due to aggregation and disintegration and that the MPV would increase due to aggregation.

Table 3.1: *Automated blood count indices for all 25 expired platelet concentrates, for pooled platelet concentrates and for apheresis platelet concentrates. The results of an unpaired, bi-directional t- test comparing the platelet count, platelet dose and MPV of apheresis and pooled platelet concentrates are shown in the bottom row. The results of paired and unpaired, uni-directional T-tests comparing the platelet count and MPV of platelet concentrates measured in initial and final tests are shown in the right hand column.*

		Initial Test (Day 6, 7 or 9)			**Final Test (Day 11, 12 or 13)**			**P-value of uni-directional t-tests comparing initial and final results**	
	Volume (mL)	**Count (10^9/L)**	**MPV (fL)**	**Dose (10^9 platelets)**	**Count (10^9/L)**	**MPV (fL)**	**Dose (10^9 platelets)**	**Count (10^9/L)**	**MPV (fL)**
All (n=25)								**Paired:**	
Mean	212	1442	8.5	315	1349	8.7	301	0.00	0.03
Maximum	300	1767	9.6	427	1749	10.7	376	**Unpaired:**	
Minimum	172	1028	7.4	215	1004	7.3	217	0.137	0.212
Pooled (n=12)								**Paired:**	
Mean	273	1259	8.7	371	1191	8.9	349	0.01	0.02
Maximum	300	1628	9.1	427	1435	9.7	376	**Unpaired:**	
Minimum	257	1028	8.2	337	1004	8.4	326	0.36	0.22
Apheresis (n=13)								**Paired:**	
Mean	190	1573	8.4	294	1462	8.6	283	0.01	0.16
Maximum	201	1767	9.6	348	1749	10.7	339	**Unpaired:**	
Minimum	172	1226	7.4	215	1161	7.3	217	0.36	0.22
P-value of unpaired, bi-directional t- test comparing Pooled and Apheresis values		<0.01	0.14	0.01	<0.01	0.23	<0.01		

Platelet count and MPV differed significantly within the pooled concentrate group and the test sample as a whole when paired T-tests were used, indicating deterioration of specific concentrates. However, unpaired T-tests did not reveal significant changes, suggesting there was no clear separation between the newly expired and extremely aged concentrate groups as a whole. The apheresis concentrates also showed statistically significant reductions in platelet count using paired T-tests but the MPV did not change significantly. This may reflect decreased aggregation in apheresis concentrates, potentially indicating reduced quality deterioration, or it may be due to increased micro- vesiculation and thus possibly related to increased quality deterioration. Overall, the differences observed over time for individual concentrates were not great enough to overcome the high inter-concentrate variability that is shown in Table 3.2.

Table 3.2: *Co-efficient of variation (CV) for initial and final automated platelet count and volume results for all concentrates and for the pooled and apheresis concentrate sub-groups.*

	Platelet count		MPV	
	Initial CV (%)	**Final CV (%)**	**Initial CV (%)**	**Final CV (%)**
All concentrates	16	15	6	9
Pooled concentrates	14	13	4	5
Apheresis concentrates	10	11	7	12

The platelet count and calculated unit dose differed significantly ($p = \leq 0.01$) between apheresis and pooled platelets but the MPV showed no difference. Platelet count deteriorated within individual units (apheresis $p = 0.007$, pooled $p = 0.006$) and MPV increased in pooled units ($p = 0.019$). These differences were not detectable between the two product groups using unpaired tests.

3.3.2 pH measurement

pH measurement was carried out during the ageing platelet concentrate phase of the study. For each concentrate the pH was initially between 7 and 7.5. It generally increased a little for the day two and five measurements, presumably as the platelets recovered from the sub-optimal conditions experienced during processing and transport and then began to drop again. One concentrate dropped below pH 7 on day 9, 8 concentrates had dropped below pH 7 on day 12 and all concentrates had dropped below pH 7 by day 14. All concentrates recorded pH levels of less than 6.5 by the end of testing at day 16. See Figure 3.1.

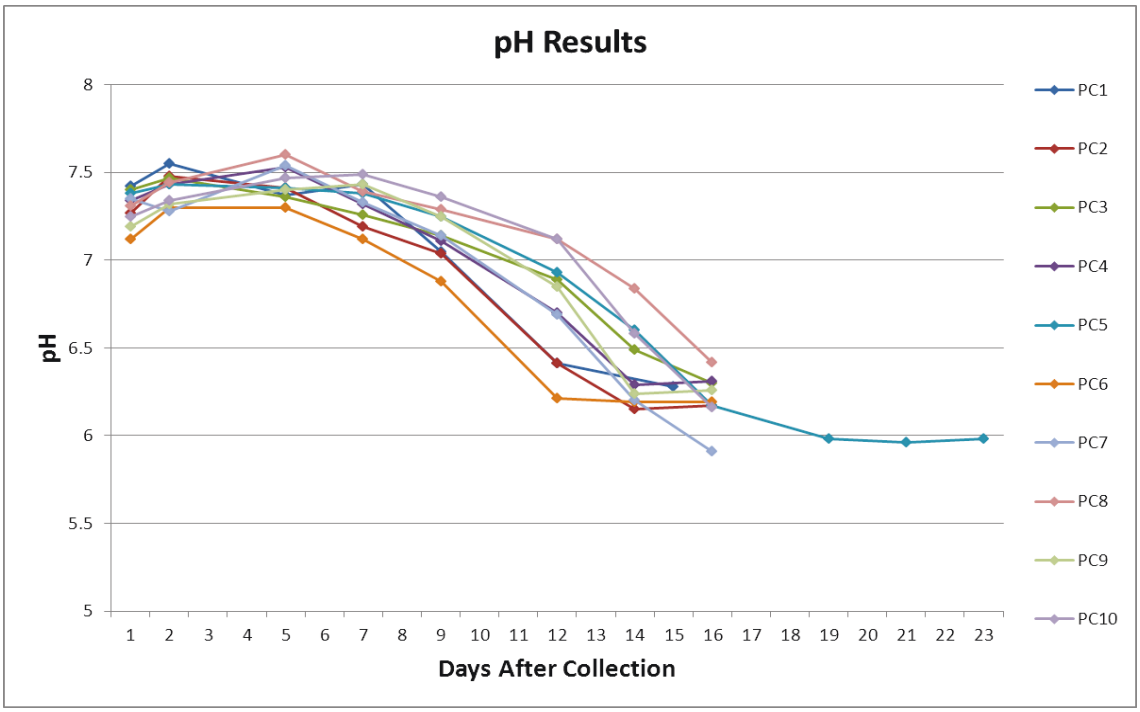

Figure 3.1: *pH results for fresh platelet concentrates PC1-PC10.*

3.3.3 Swirl assessment

As with pH measurements, swirl assessment was undertaken at OUH Trust from the ageing platelet concentrate phase of the study onwards. All purchased concentrates were initially swirl positive and each became swirl negative during the study period. However, the amount of time

for which they were swirl positive varied considerably between concentrates, with the first recorded swirl negative day being anywhere between day 5 and day 16. See Table 3.3.

Table 3.3: *First swirl negative day for each concentrate.*

Concentrate Number	First day swirl negative
PC1	12
PC2	7
PC3	5
PC4	12
PC5	14
PC6	7
PC7	14
PC8	16
PC9	12
PC10	12

3.3.4 ESC and HSR assays

HSR and ESC assays were carried out on four expired platelet concentrates. All were tested on at least two separate occasions, at least three days apart. The results obtained are shown in Table 3.4.

Table 3.4: *HSR and ESC results for expired concentrates stored under appropriate conditions.*

Concentrate ID number	Day post collection	HSR (%)	ESC (%)
104	D6	158	18.0
	D9	70	11.2
	D12	53	11.0
	D15	27	5.2
105	D6	69	28.0
	D9	75	16.8
	D12	57	19.5
	D15	13	10.1
106	D7	77	13.4
	D10	55	8.8
	D13	74	15.6
107	D9	65	22.4
	D12	24	8.8

Although there is no set normal range for these tests, Holme *et al* (1998) reported HSR results of 58-81% and ESC results of 20-28% for fresh citrate phosphate dextrose (CPD) preserved PRP in a multi-laboratory study. Most of the concentrates initially gave results within this range, which reduced over time as the platelets aged.

To ensure that these techniques were able to detect extremely poor platelet quality, they were used to test platelet concentrate samples that had been kept at 37°C and -40°C for six days prior to undergoing testing on day 12 after collection. Storage at 37°C induces increased levels of platelet apoptosis and reduced platelet viability (Bertino *et al*, 2003). Freezing reduces both the quantity and viability of platelets in a sample (Taylor, 1981). The results of this experiment are shown in Table 3.5.

Table 3.5: *HSR and ESC results for expired concentrates measured on Day 12 after collection, following storage from Day 6 at standard, high and low temperatures.*

Concentrate	Storage Condition	HSR (%)	ESC (%)
104	22°C (standard)	53	11.0
	37°C	4675	-7.3
	-40°C	89	-0.9
105	22°C (standard)	57	19.5
	37°C	257	5.6
	-40°C	113	1.6

The HSR results were unexpectedly raised. This may have been due to increased osmotic fragility of the platelets, leading to rupture and associated increase in optical density within the test sample. The ESC was low in all samples as expected. These results suggested that the HSR test is unreliable in extremely poor quality platelet concentrates and that the ESC test is preferable.

For the most part, the HSR and ESC tests both gave results within the expected range for the purchased ageing concentrates and dropped as the concentrates aged. There were occasional outliers and it was unclear whether these were caused by technical or concentrate related

factors. All tests were performed in duplicate and individual results that were obviously affected by error were discarded and repeated wherever possible. See Figures 3.2 and 3.3.

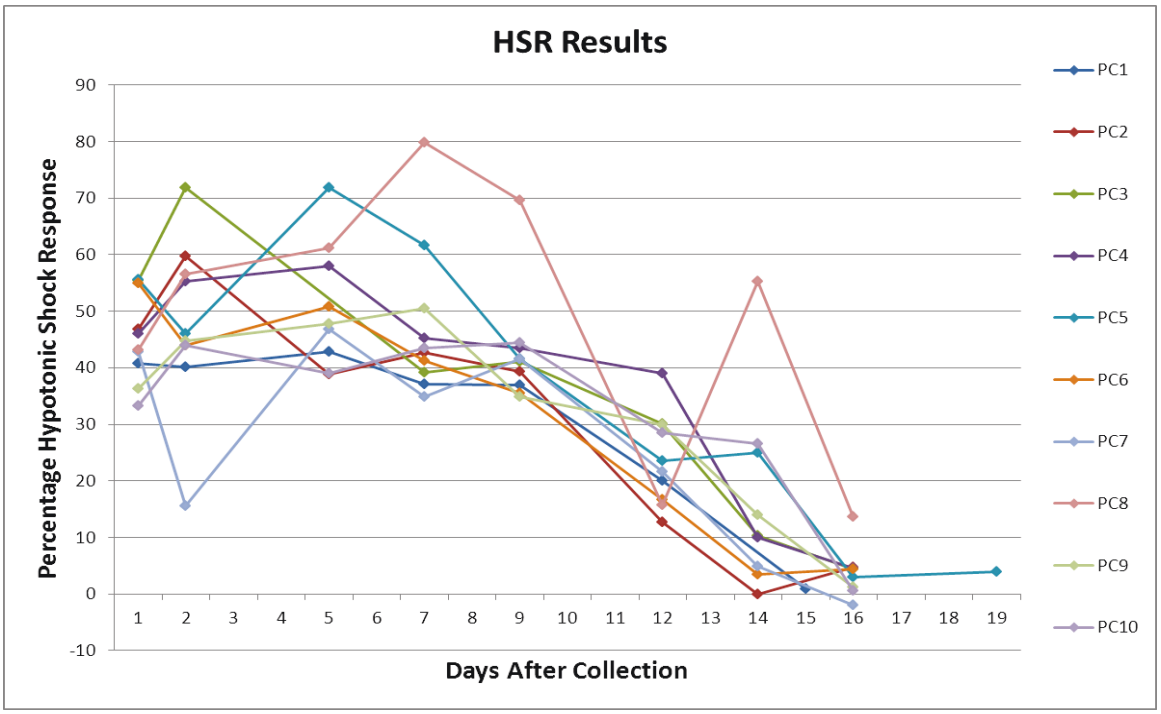

Figure 3.2: *HSR results for fresh platelet concentrates PC1-PC10.*

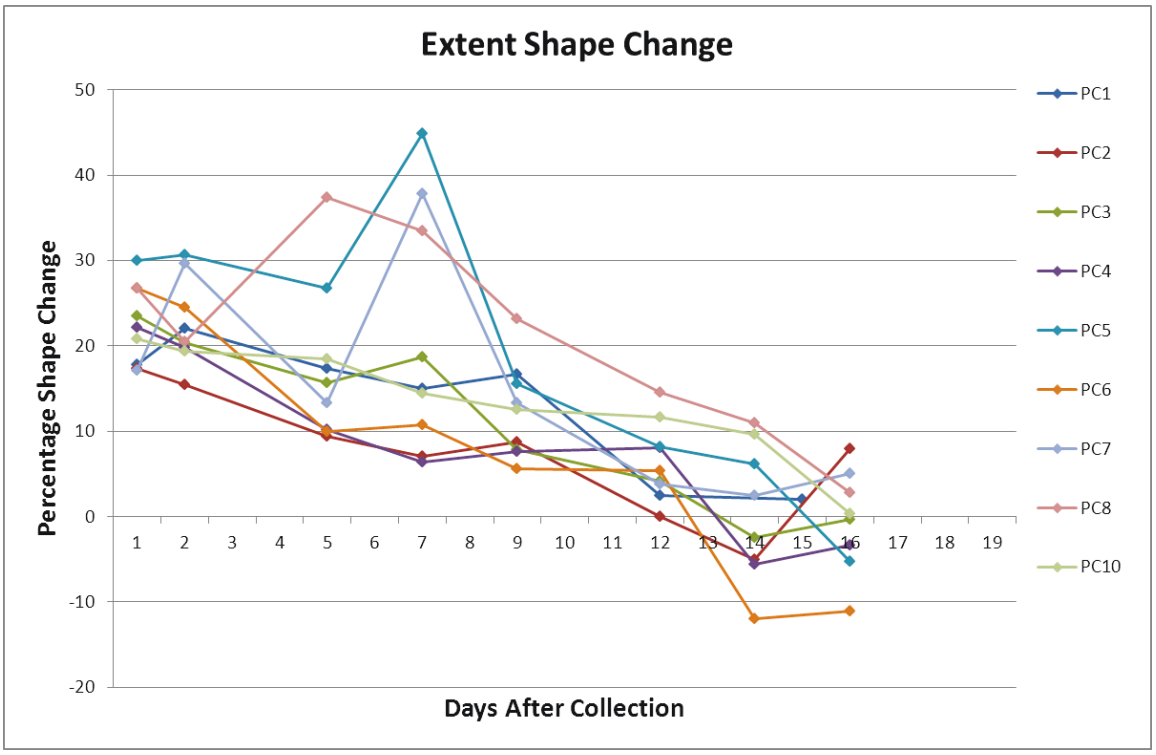

Figure 3.3: *ESC results for fresh platelet concentrates PC1-PC10.*

3.4 iZON Impedance Analyser *In-Vitro* Results

The iZON impedance analyser was designed to measure small particles. Blood platelets are at the upper end of its detection limit. Within the early stages of this study, its ability to produce an accurate platelet distribution curve and determine platelet surface charge was investigated.

The numbers of PMV (defined here as <2000nm particles) in some of the expired platelet concentrates was observed to increase as the concentrates aged. Figure 3.4 shows iZON platelet distribution peaks measured at three different time points from a single platelet concentrate that demonstrates appearance of a smaller MV peak separate from the main platelet population. As the presence of large MV in platelet concentrates is a potential marker of platelet activation during platelet storage (Harrison *et al*, 2010) the ability to detect this population may have utility.

However, it was not possible to generate reliable charge measurements within this study. The instrument was difficult to use and setting up reliable measurements was very time consuming. Using the software available at the time, measurements at multiple analysis pressures were required. Recurrent pore blockages occurred, particularly at lower analysis pressures and with samples from older platelet concentrates, perhaps due to some degree of activation and / or aggregation of the sample. This made it impossible in some cases to obtain enough events for a reliable reading. There was also concern that some of the results eventually recorded after multiple measurement attempts were not representative of the true state of the sample. Restarting measurements and stopping and starting data acquisition multiple times within a single measurement may have led to inadvertent selection of less activated platelets within the measurement. It was decided at this point that the ability of the iZON instrument to measure platelet sized particles was not yet understood well enough to be a realistic prospect for use in future stages of the study.

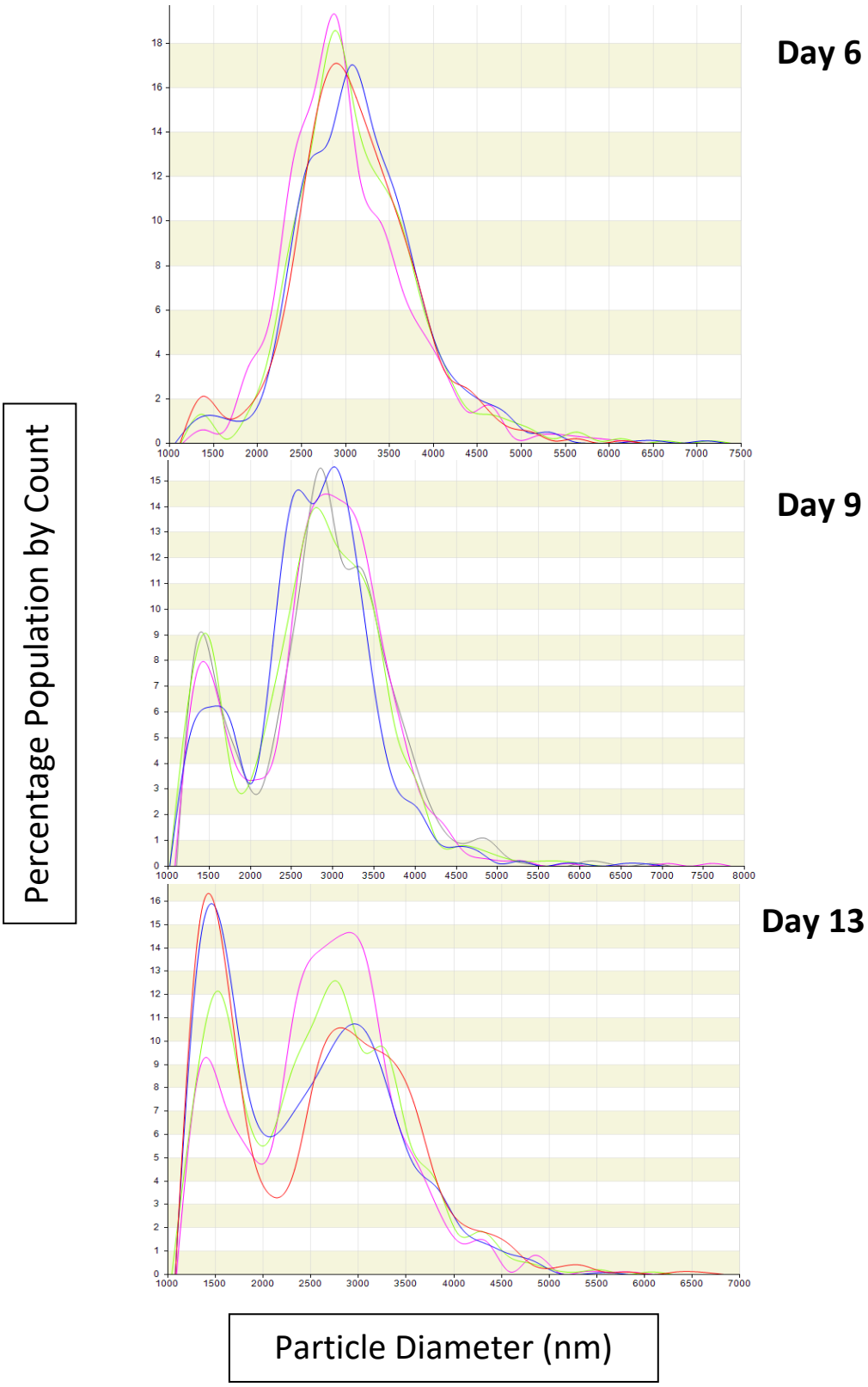

Figure 3.4: *iZON platelet distribution curves obtained at four different analysis pressures (red, pink, blue and green lines) for platelet concentrate 11 on days 6, 9 and 13 after collection*

3.5 ThromboLUX Analyser *In-Vitro* Results

The laboratory assessment of the ThromboLUX analyser is presented below.

3.5.1 Initial laboratory assessment of the ThromboLUX

The between batch precision of the first ThromboLUX analyser delivered to our laboratory (TL 1) was assessed using calibration beads supplied by LightIntegra Ltd. The company advised that scores of between 19 and 22 should be obtained when testing these beads. They were initially tested on eleven separate occasions and gave a CV of 11.3% as shown in Table 3.6. The average score was within the expected range.

Table 3.6: *Results of a between batch precision study using ThromboLUX scores for a commercial calibration bead preparation supplied by LightIntegra Ltd*

Test Date	TL 1 Beads Score (Expected Range 19 – 22)
24/11/2010	20.3
25/11/2010	18.7
26/11/2010	18.3
29/11/2010	20.0
30/11/2010	19.8
01/12/2010	18.5
02/12/2010	18.0
03/12/2010	23.5
06/12/2010	17.1
09/12/2010	24.2
10/12/2010	21.3

Average	20.0
SD	2.3
CV	11.3
Max	24.2
Min	17.1

To give an indication of the within batch precision and assess the variability of results obtained from biological samples, an expired platelet concentrate was tested five times on day six after

collection, five times on day nine after collection and five times on day twelve after collection. The results are shown in Table 3.7. The co-efficient of variation (CV) observed within this experiment varied from 8.2% on day 6 after the platelets were collected from the donor to 18.8% on day 9 after collection.

Table 3.7: *Results of a within batch precision study using ThromboLUX scores for an expired platelet concentrate.*

Day	Score
6	19.9
	23.9
	23.2
	22.4
	24.8
9	24.2
	21.4
	20.9
	16.7
	28.0
12	24.8
	23.4
	31.6
	25.7
	29.3

TL 1 Results	
Average	22.8
SD	1.9
CV	8.2
Average	22.2
SD	4.2
CV	18.8
Average	27.0
SD	3.4
CV	12.6

All platelet concentrates gave scores above the acceptable quality cut-off value advised at that time (>14) up to the end of testing, even when reference methods indicated a complete lack of viability within the concentrate. Some concentrates showed increasing quality scores with increasing age. This was in disagreement with the data of Maurer-Spurej *et al* (2009), who found that roughly one in six platelet concentrates gave a poor (<14) score prior to expiry at five days after collection from the donor. The study performed by Maurer-Spurej *et al* (2009) used the prototype version of the

ThromboLUX analyser. This data was the first indication that the two analyser models were not entirely equivalent.

Due to these inconsistencies, a technical fault with the TL 1 ThromboLUX analyser was suspected and it was exchanged for a second device of the same specifications (TL 2) in the hope that this would resolve the issues observed. In addition, the analyser was moved to a room on its own, where interferences from other equipment could be ruled out, and powered through an uninterrupted power supply (UPS) to guard against irregularity of the power supply in case this was an interfering factor. However, the between batch precision, as measured using calibration bead preparations, remained unchanged with the new instrument as is shown in Table 3.8.

Table 3.8: *Repeated between batch precision study using ThromboLUX scores for calibration bead preparations using instrument 2 in conjunction with a UPS system*

Date	TL 2 Beads Score (Expected Range 19 – 22)
09/05/2011	23.4
11/05/2011	23.1
13/05/2011	18.7
16/05/2011	24.3
20/05/2011	19.7
23/05/2011	21.5
26/05/2011	19.9
01/06/2011	19.2

Average	21.2
SD	2.1
CV	10.1
Maximum	24.3
Minimum	18.7

The change of instrument unfortunately made no difference to the unexpectedly high scores obtained for expired platelet concentrate samples. The specimens stored at 37°C and -40°C (discussed above in relation to the HSR and ESC tests) were also tested on the TL 2 analyser and the scores obtained are shown in Table 3.9. Although the sample stored at -40°C gave scores below the

cut-off of 14 for both samples, the 37°C samples still appeared to have acceptable quality and, in the case of concentrate 104, had even improved.

Table 3.9: *ThromboLUX scores for four expired platelet concentrates, including samples stored at inappropriate temperatures obtained using instrument 2.*

Days post collection / storage temperature	ThromboLUX Score			
	Concentrate 104	**Concentrate 105**	**Concentrate 106**	**Concentrate 107**
Day 6	31.2	36.5	21.8 (day 7)	
Day 9	30.9	33.1	36.6 (day 10)	21.5
Day 12	28.2	23.4	33.5 (day 13)	19.9
Day 15	25.3	31.3		
37°C	31.1	30.5		
-40°C	8.1	13.6		

3.5.2 Comparison of ThromboLUX at multiple sites

Because of the apparent problems with sensitivity to poor platelet concentrate quality, the study went on to arrange and participate in a series of sample swapping exercises with other laboratories using the ThromboLUX device. At the beginning of this phase of the study, the TL 2 was exchanged for a third ThromboLUX analyser: TL 3. The initial sample swapping experiment was conducted using both TL 2 and TL 3 and served as a baseline comparison of the two machines. The details and results of five separate sample swapping experiments are documented below. See table 3.10 for an overview.

Table 3.10: *Overview of testing carried out during the five sample exchange experiments*

Experiment	Samples	Oxford (TL 2)	Oxford (TL 3)	Vancouver	Leiden	Cambridge	The Hague
Leiden and Vancouver Concentrates	Leiden 1	Tested on arrival	Tested on arrival and after one week		Tested on day of arrival in Oxford		
	Leiden 2	Tested on arrival	Tested on arrival and after one week		Tested on day of arrival in Oxford		
	Vancouver 1	Tested on arrival and following day	Tested on arrival and following day				
	Vancouver 2	Tested on arrival and following day	Tested on arrival and following day				
	Vancouver 3	Tested on arrival and following day	Tested on arrival and following day				
	Vancouver 4	Tested on arrival and following day	Tested on arrival and following day				
	Vancouver 1 (frozen)	Tested on day after arrival	Tested on day after arrival				
	Vancouver 4 (frozen)	Tested on day after arrival	Tested on day after arrival				
Colindale Concentrates	Colindale 1		Tested on days 1, 2, 3, 7 and 9 after arrival. HSR, ESC and ROTEM tests also performed	Tested on days 1, 2, 3, 6, 8, 9 and 15 after arrival			
	Colindale 2		Tested on days 1, 2, 3, 7 and 9 after arrival. HSR, ESC and ROTEM tests also performed	Tested on days 1, 2, 3, 6, 8, 9 and 15 after arrival			
Vancouver Concentrates	Vancouver sample 1		Tested on day 11 after donation collection	Tested on days 2, 11 and 14 after donation collection. Kunicki score on days 10, 11 and 14			
	Vancouver sample 2		Tested on day 11 after donation collection	Tested on days 2, 11 and 14 after donation collection. Kunicki score on days 10, 11 and 14			

Brentwood Concentrates	Brentwood tube 1	Tested in duplicate on day of arrival		Tested on day of arrival in Oxford	
	Brentwood tube 2	Tested in duplicate on day of arrival		Tested on day of arrival in Oxford	
	Brentwood concentrate	Tested in duplicate on day of arrival		Tested on day of arrival in Oxford	
Second Colindale Concentrates	A) Concentrate 1, 37°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	B) Concentrate 1, 4°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	C) Concentrate 1, -40°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	D) Concentrate 1, 22°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	E) Concentrate 2, 37°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	F) Concentrate 2, 4°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	G) Concentrate 2, -40°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	H) Concentrate 2, 22°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	I) Concentrate 3, 37°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	J) Concentrate 3, 4°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	K) Concentrate 3, -40°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	L) Concentrate 3, 22°C	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	M) 20 day old concentrate 1	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival
	N) 20 day old concentrate 2	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival	Tested on day of arrival

Leiden and Vancouver Concentrates - June 2011

Capped specimens from two Dutch platelet concentrates that had been tested using a different ThromboLUX analyser at the Sanquin Research Laboratory in Leiden were sent to the Haematology Department in Oxford. Four additional platelet concentrates from Vancouver were also transported to Oxford at this time. All of these concentrates were tested on two separate ThromboLUX analysers in Oxford: TL 2 and TL 3, which was subsequently left at OUH in place of TL 2. The Vancouver concentrates were tested again on the following day. The Leiden concentrates were also tested using a different ThromboLUX analyser located in Leiden and after a further week stored without gas exchange. Samples from two of the Vancouver concentrates were frozen and thawed prior to testing. All of these results are shown in Table 3.11.

Table 3.11: *ThromboLUX scores for four platelet concentrates produced in Vancouver, capped samples from two platelet concentrates produced in Leiden and samples taken from two of the Vancouver concentrates that were stored at inappropriate temperatures.*

Test Date	Sample	TL 2	TL 3	Leiden Result
23/06/2011	2000nm Beads	23.8	24.1	
	Vancouver PC 1	16.6	21.4	
	Vancouver PC 2	4.4	9.6	
	Vancouver PC 3	20.4	31.1	
	Vancouver PC 4	31.2	28.3	
	Leiden PC 1 (exp 23/06)	18.3	25.5	22.4
	Leiden PC 2 (exp 28/06)	5.5	3.2	11.2
24/06/2011	2000nm Beads	22.5		
	Vancouver PC 1	23.6	17.9	
	Vancouver PC 2	5.5	7.7	
	Vancouver PC 3	26.1	28.8	
	Vancouver PC 4	19.9	24.3	
	Vancouver PC 1 (Frozen)	19.1	22.2	
	Vancouver PC 4 (Frozen)	23.9	28.0	
30/06/2011	Leiden PC 1 (exp 23/06)		19.4	
	Leiden PC 2 (exp 28/06)		7.4	

Although there was notable inter-analyser variability, Leiden concentrate 1 consistently gave high scores and Leiden concentrate 2 gave low scores. However, Leiden concentrate 1 still gave a high score (19.4) when the concentrate reached 15 days old and Leiden concentrate 2 appeared to improve following storage without gas exchange for a week.

The results produced by the two instruments were compared using a bi-directional paired T-test. This analysis identified the two sets of results as being significantly different (P = 0.036). Rather than showing that the initial ThromboLUX analyser demonstrated a positive bias, this experiment proved instrument 2 to produce significantly lower results than instrument 3. The correlation between results recorded by the two instruments was 0.66 as shown in Figure 3.5.

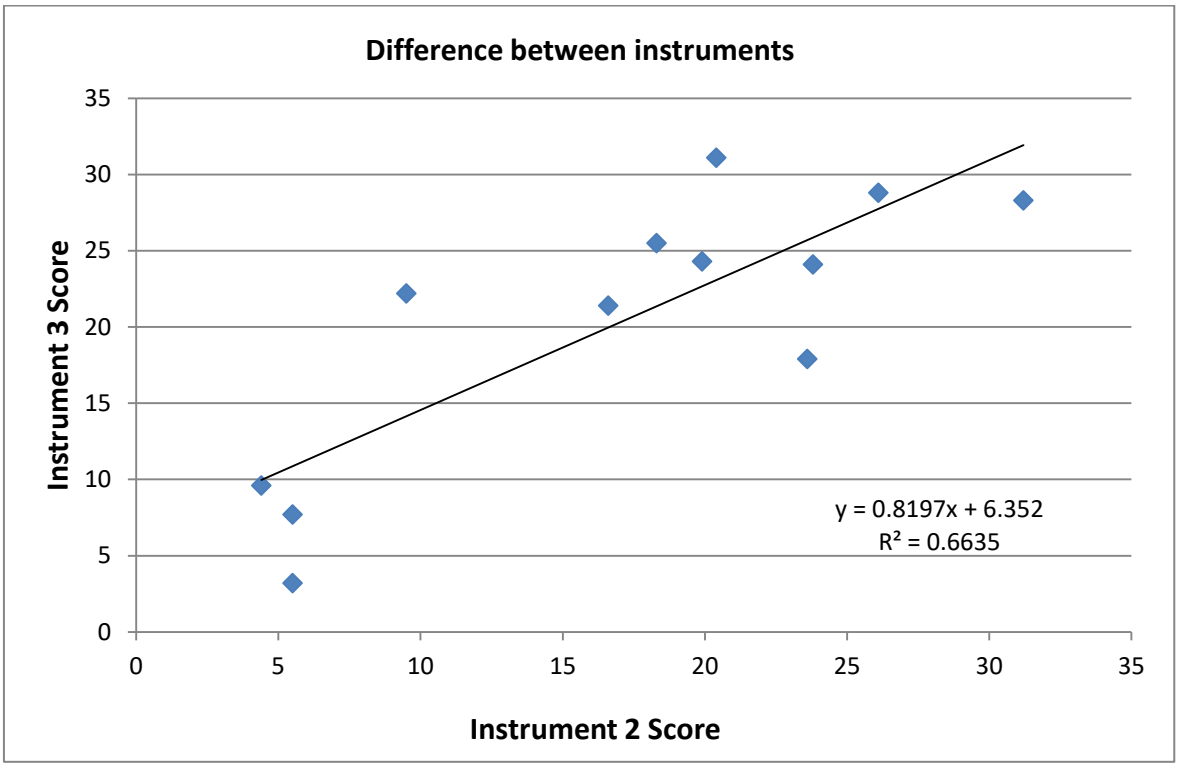

Figure 3.5: *Comparison of ThromboLUX scores obtained for the Vancouver and Leiden platelet concentrates using instrument 2 and instrument 3.*

Despite some variability between the two instruments, both were able to identify two platelet concentrates as having scores below the cut-off value of 14. Vancouver concentrate 2 gave low scores on both days of testing and Leiden concentrate 2 produced a low score in the UK and in Leiden. ThromboLUX scores of these levels had not been seen at all in UK concentrates. The results for the frozen platelet concentrates unexpectedly indicated acceptable quality. These results suggested that a malfunction which only affected the analysers used locally for this study was unlikely because the problem was apparent with multiple instruments.

Colindale Concentrates - September 2011

Two platelet concentrates were collected at the NHSBT processing centre in Colindale, split into paediatric platelet bags and dispatched under controlled conditions (in an insulated blood box with a temperature logger) to both Oxford and Vancouver. ThromboLUX Scores obtained at both locations were mostly comparable and remained consistently above the cut-off value used to identify poor quality platelets until at least day 15 (tested in Vancouver only). A clearer deterioration was observed in the platelet only G value (EXTEM – FIBTEM) obtained using the ROTEM® system, the HSR and the ESC. These results are shown in Figures 3.6 and 3.7.

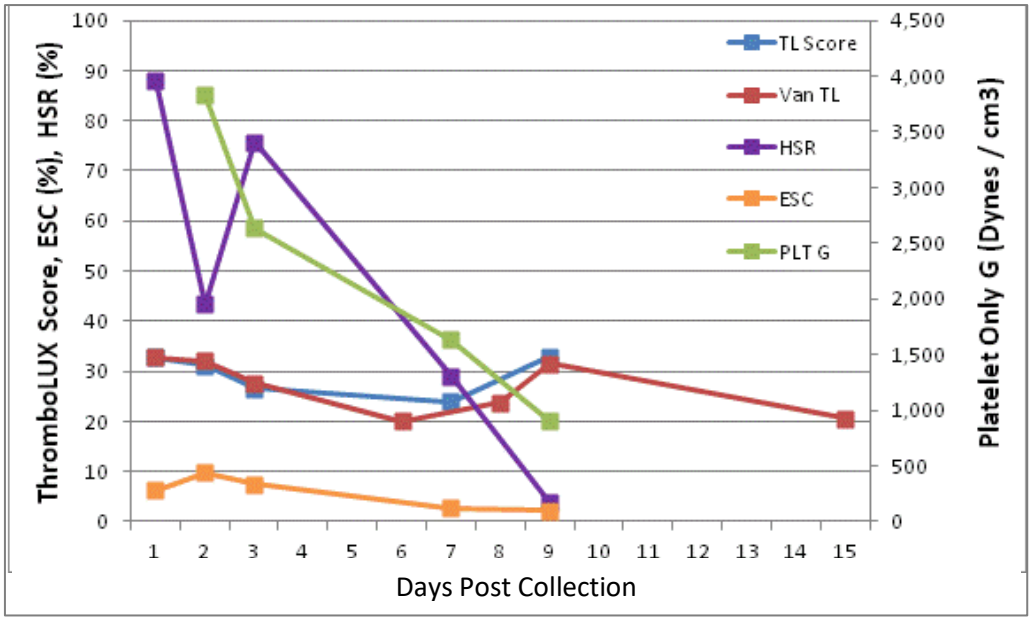

Figure 3.6: *Quality test results for Colindale platelet concentrate 1. ThromboLUX scores were obtained on Oxford (TL Score - blue) and Vancouver (Van TL – red). HSR (purple), ESC (light blue) and ROTEM® (green) tests were also carried out in Oxford.* ROTEM® *results are expressed as platelet only G (EXTEM G – FIBTEM G).*

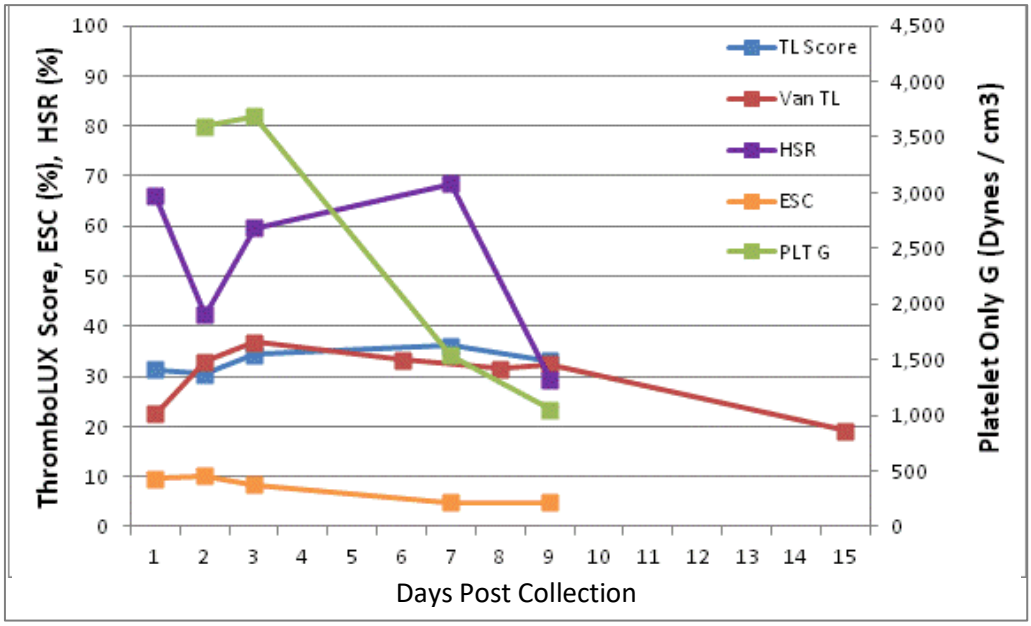

Figure 3.7: *Quality test results for Colindale Platelet Concentrate 2. ThromboLUX scores were obtained on Oxford (TL Score - blue) and Vancouver (Van TL – red). HSR (purple), ESC (light blue) and ROTEM® (green) tests were also carried out in Oxford.* ROTEM® *results are expressed as platelet only G (EXTEM G – FIBTEM G).*

In addition, swirl assessments and microscopy were carried out in Vancouver. Swirl persisted for both concentrates until day nine and microscopy showed that a high percentage of discoid

platelets were present on day 2, indicating that the concentrates were of good quality at that point. See Figure 3.8.

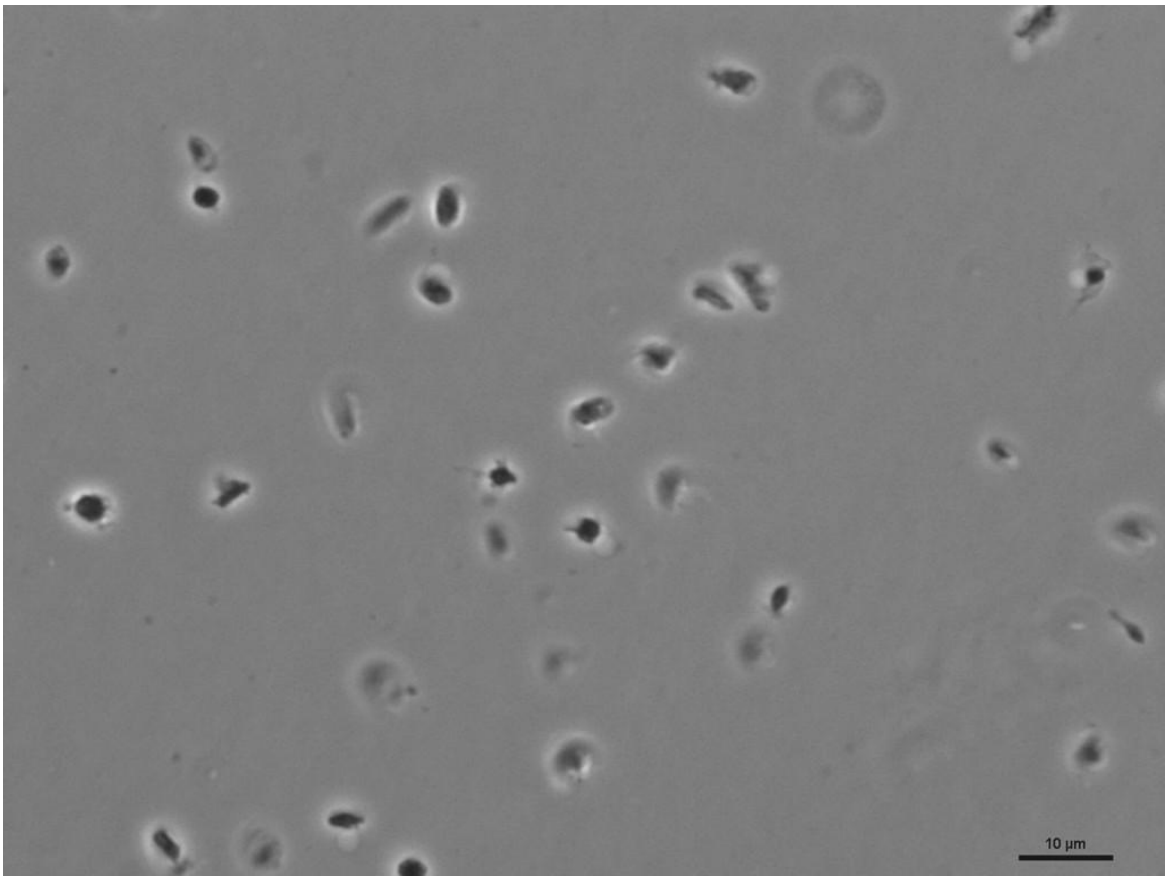

Figure 3.8: *Phase contrast microscopy image of Colindale platelet concentrate 1 showing a high percentage of discoid platelets. Courtesy of the LightIntegra Laboratory, Vancouver.*

Vancouver Concentrates – October 2011

Samples from two platelet concentrates were sent from the LightIntegra Laboratory in Vancouver to Oxford and tested in both locations. Additionally, Kunicki scores were generated in Vancouver. The results are shown in Figures 3.9 and 3.10. They showed concentrate 1 to be consistently poor quality from day 10 onwards and concentrate 2 to progressively deteriorate in quality. ThromboLUX scores obtained in Vancouver showed deterioration between days two and 11 but then increased on day 14. Neither concentrate gave scores below the cut-off of 14. The day 11 Oxford scores were considerably higher than equivalent Vancouver measurements.

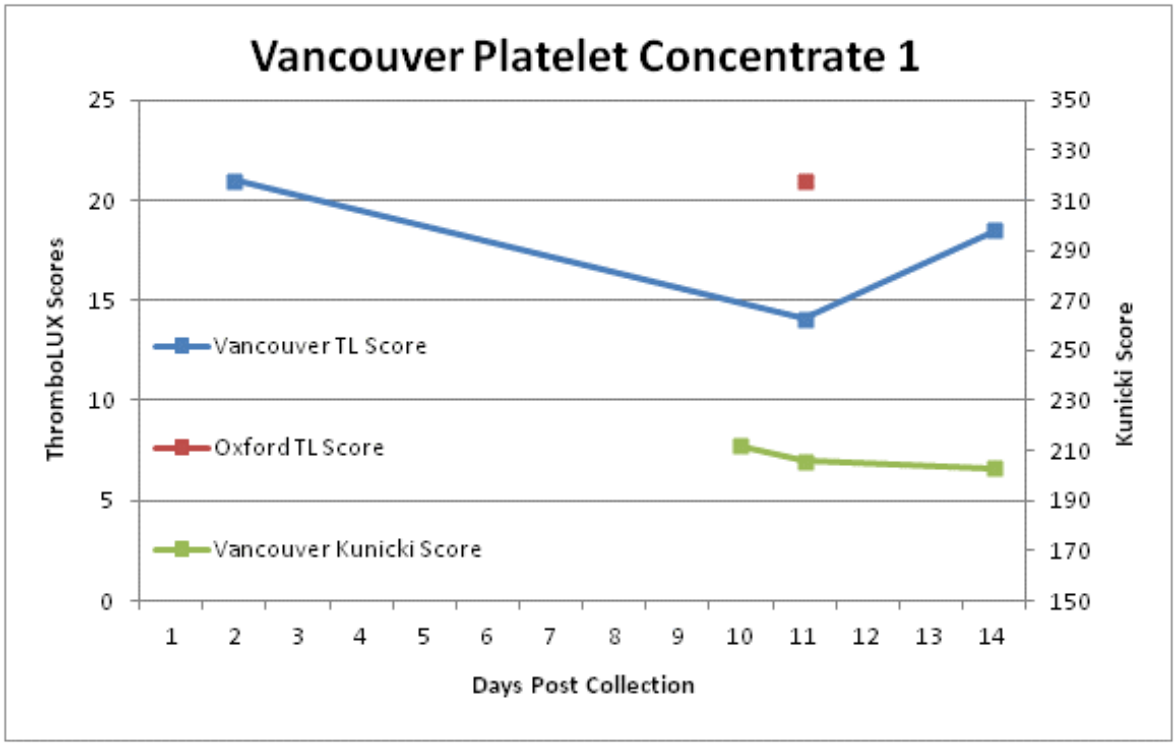

Figure 3.9: *ThromboLUX scores from Vancouver (blue) and Oxford (red) and Kunicki scores from Vancouver (green) for Vancouver sample 1*

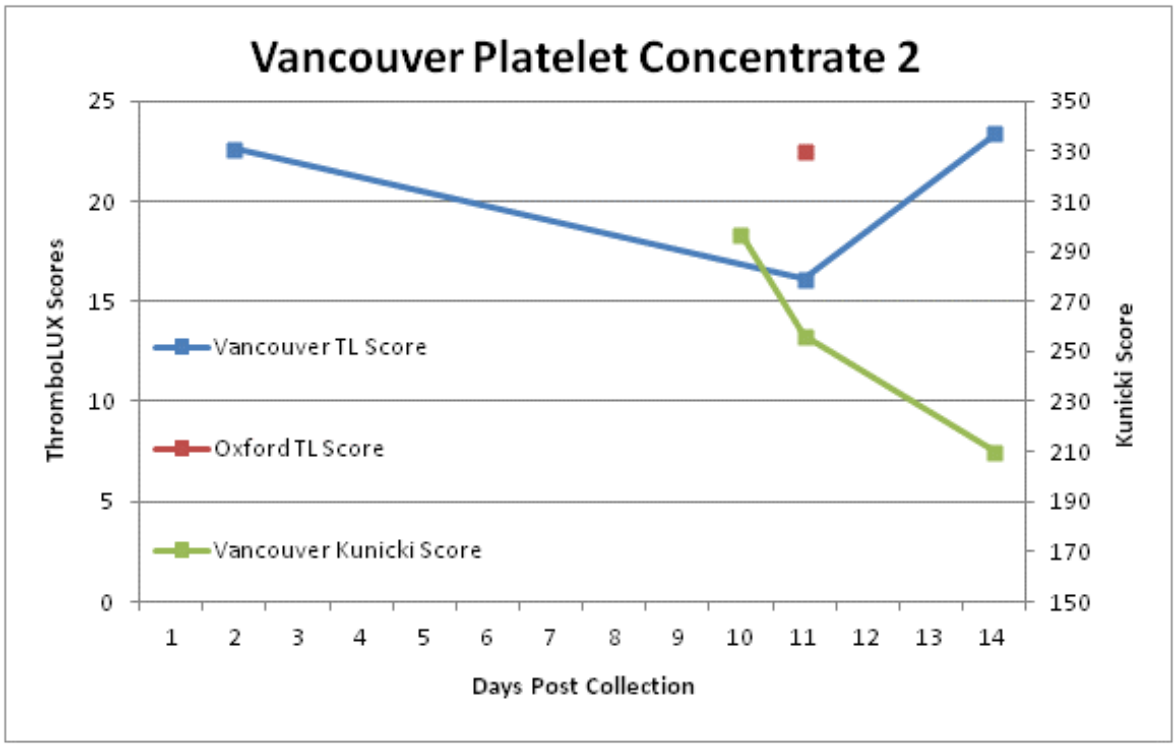

Figure 3.10: *ThromboLUX scores from Vancouver (blue) and Oxford (red) and Kunicki scores from Vancouver (green) for Vancouver sample 2*

Brentwood Concentrates – April 2012

The Oxford analyser underwent maintenance and upgrades, including a switch to the external ThromboSight software, that were hoped to improve its sensitivity to poor quality concentrates prior to testing two capped platelet concentrate specimens and one bagged platelet concentrate from Brentwood. These samples had been tested in Cambridge the previous day. All three specimens were collected 11 days before testing in Cambridge and the capped specimens had been stored without gaseous exchange for four days. The specimens were transported under controlled temperature conditions and it was anticipated that they would give comparable scores between the two sites. However, as can be seen in Figure 3.11, the Cambridge scores were much higher than those obtained in Oxford, raising the possibility that the maintenance performed on the Oxford machine had, indeed, increased its sensitivity to poor platelet quality, at least in comparison to the Cambridge analyser. Both capped specimens gave markedly different results during repeat analysis (Oxford 2) compared to the initial scores (Oxford 1) but both Oxford results for the concentrate were similar.

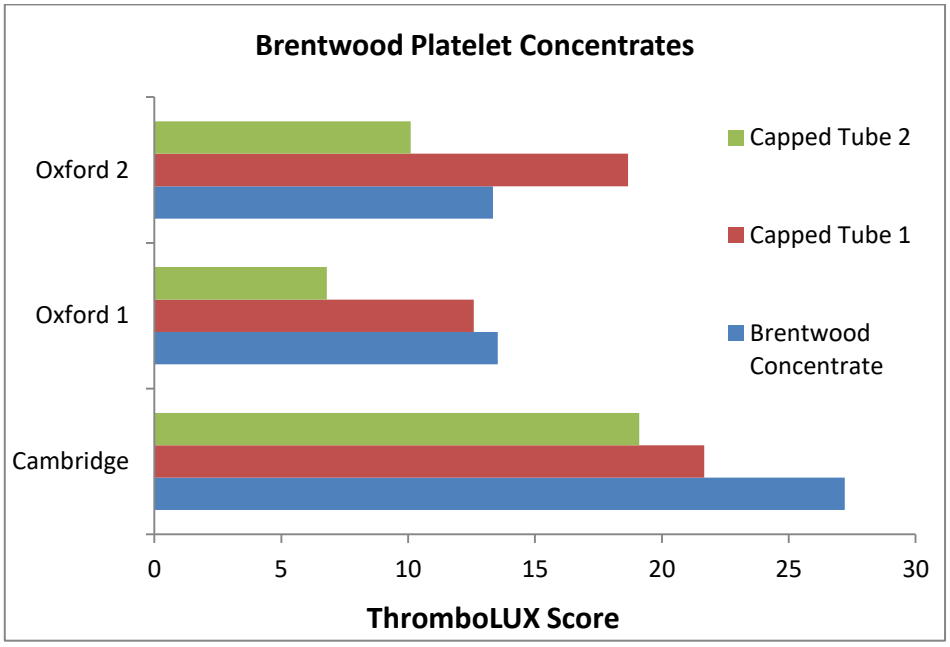

Figure 3.11: *ThromboLUX scores from Oxford and Cambridge for one platelet concentrate (blue) and two capped specimens (red and green) taken from other concentrates. Tests were performed in duplicate at Oxford (Oxford 1 and Oxford 2)*

Second Colindale Concentrates – July 2012

A final comparative testing experiment was performed in July 2012. Three double dose apheresis platelet donations were collected in Colindale and split into 4 x 80mL breathable paediatric platelet bags and 12 x 10mL breathable sample pouches. The 10mL pouches were stored at either 37°C, 4°C or -40°C for 24 hours prior to dispatch. In addition, two 20 day old platelet concentrates were each split into four 80mL packs. One 80mL bag, one 37°C pouch, one 4°C pouch and one -40°C pouch from each of the three fresh donations and one 80mL bag from each of the out of date concentrates was sent to each of the following locations: Oxford, Cambridge, Leiden and The Hague. Each site received a total of 14 specimens for analysis three days after collection. The results are shown in Figure 3.12 and summarised in Table 3.12.

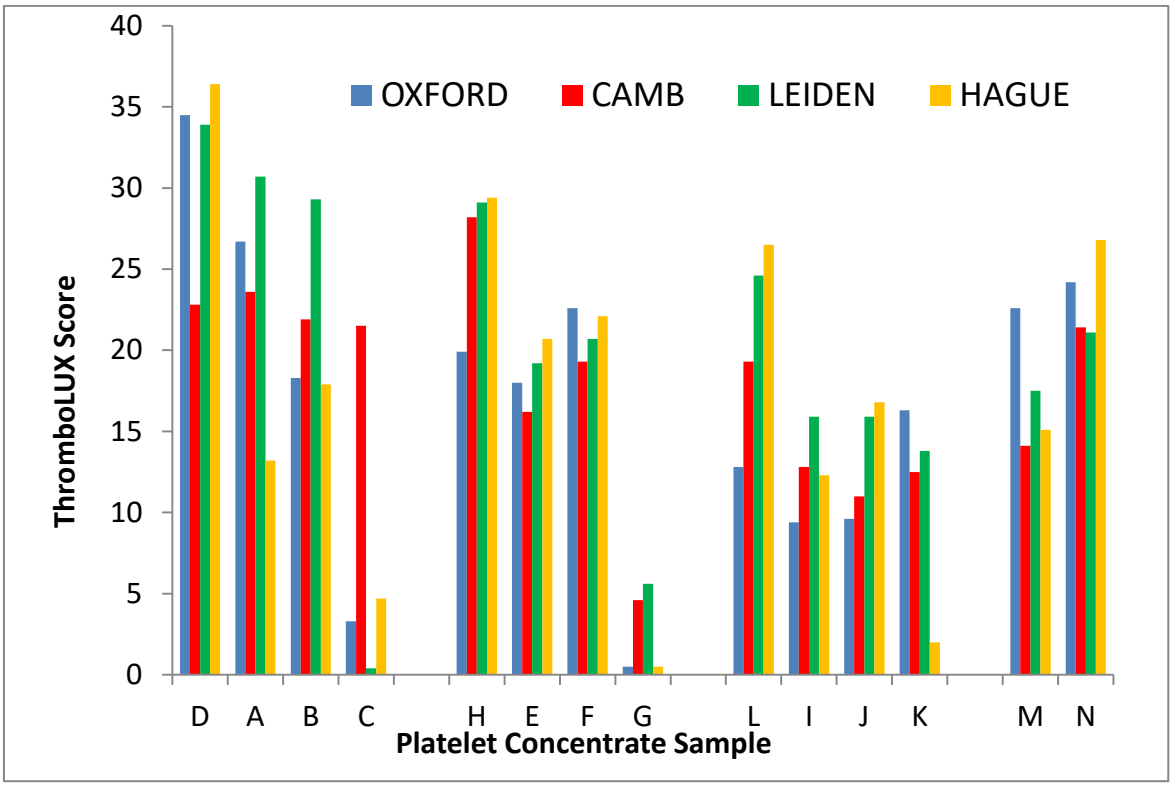

Figure 3.12: *ThromboLUX scores from Oxford (blue), Cambridge (red), Leiden (green) and The Hague (yellow) for three fresh platelet concentrates, stored at 22°C (D, H and L) and pouches derived from each of these stored at 37°C (A, E and I), 4°C (B, F and J) and -40°C (C, G and K) for 24 hours. Samples M and N were taken from 20 day old platelet concentrates.*

All platelet concentrates expected to be of good quality gave high scores at all four sites. However, the ThromboLUX was not sensitive to the age induced changes in platelet concentrate quality present in packs 4 and 5. In addition, the results for platelet concentrates stored at inappropriate temperatures, known to induce quality deterioration (Bertino *et al*, 2003; Taylor, 1981) were only intermittently reduced and a degree of variability was observed between the results obtained at the four different sites. See Table 3.12.

Table 3.12: *Qualitative summary of the results obtained for the 14 samples derived from five platelet concentrates tested in Oxford, at Cambridge, in Leiden and at The Hague.*

Pack 1	**Treatment**	**Qualitative Assessment of Results**
D	Platelets (70ml) 22ºC storage (day 3)	Good quality at all four sites
A	Pouch (8ml) 24h storage at 37ºC	Good quality at all sites except the Hague, which recorded borderline poor quality
B	Pouch (8ml) 24h storage at 4ºC	Good quality at all four sites
C	Pouch (8ml) 24h frozen / thawed	Very poor quality at all sites except Cambridge, which recorded good quality
Pack 2	**Treatment**	**Results**
H	Platelets (70ml) 22ºC storage (day 3)	Good quality at all four sites
E	Pouch (8ml) 24h storage at 37ºC	Good quality at all four sites
F	Pouch (8ml) 24h storage at 4ºC	Good quality at all four sites
G	Pouch (8ml) 24h frozen / thawed	Very poor quality at all four sites
Pack 3	**Treatment**	**Results**
L	Platelets (70ml) 22ºC storage (day 3)	Good quality at all sites except Oxford
I	Pouch (8ml) 24h storage at 37ºC	Borderline poor quality at all four sites
J	Pouch (8ml) 24h storage at 4ºC	Borderline poor quality at all four sites
K	Pouch (8ml) 24h frozen / thawed	Borderline poor quality at all sites except the Hague, which recorded very poor quality
Pack 4	**Treatment**	**Results**
M	Time expired (40ml) (day 20)	Borderline poor quality at Cambridge and the Hague, good quality at Oxford and Leiden
Pack 5	**Treatment**	**Results**
N	Time expired (40ml) (day 20)	Good quality at all four sites

A number of the samples tested gave consistent results at all four sites and some gave low results consistent with poor quality. However, a significant proportion of the results were still inconsistent or unexpectedly high, suggesting that the analytical issues were not completely resolved.

3.5.3 ThromboLUX ageing platelet concentrate results

Ten platelet concentrates were purchased from the NHSBT and tested on days 1, 2, 5, 7, 9, 12, 14 and 16 after collection using a variety of reference and experimental methods. Concentrate one was not tested on day 14 and concentrate five had additional testing carried out on days 19 and 23 to determine whether the ThromboLUX score dropped in extremely aged concentrates. The results of all tests carried out are shown side by side for each of the ten concentrates in Figures 3.13 to 3.22 below. The results of the reference tests were discussed in section 3.3 above. The results obtained using each of the novel tests will be discussed in the relevant section below.

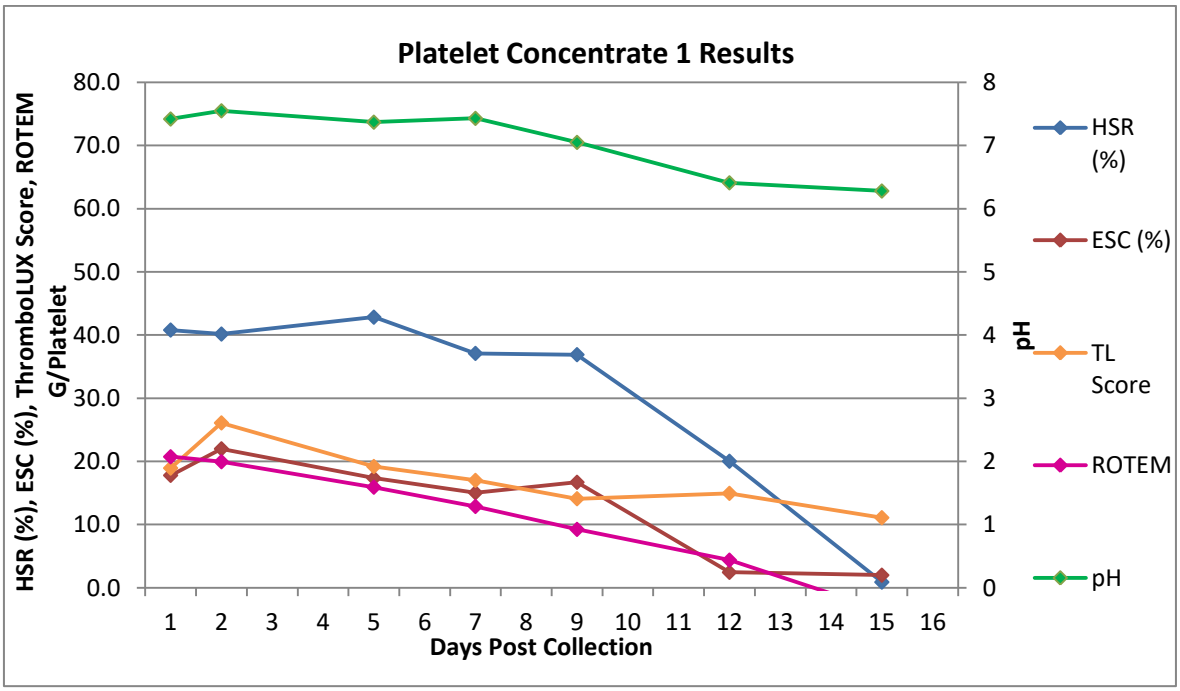

Figure 3.13: *Platelet concentrate 1 results. The HSR % (blue), ESC % (red), ThromboLUX score (orange) and ROTEM® G/platelet (pink) are plotted against the primary y axis. pH (green) is plotted against the secondary axis.*

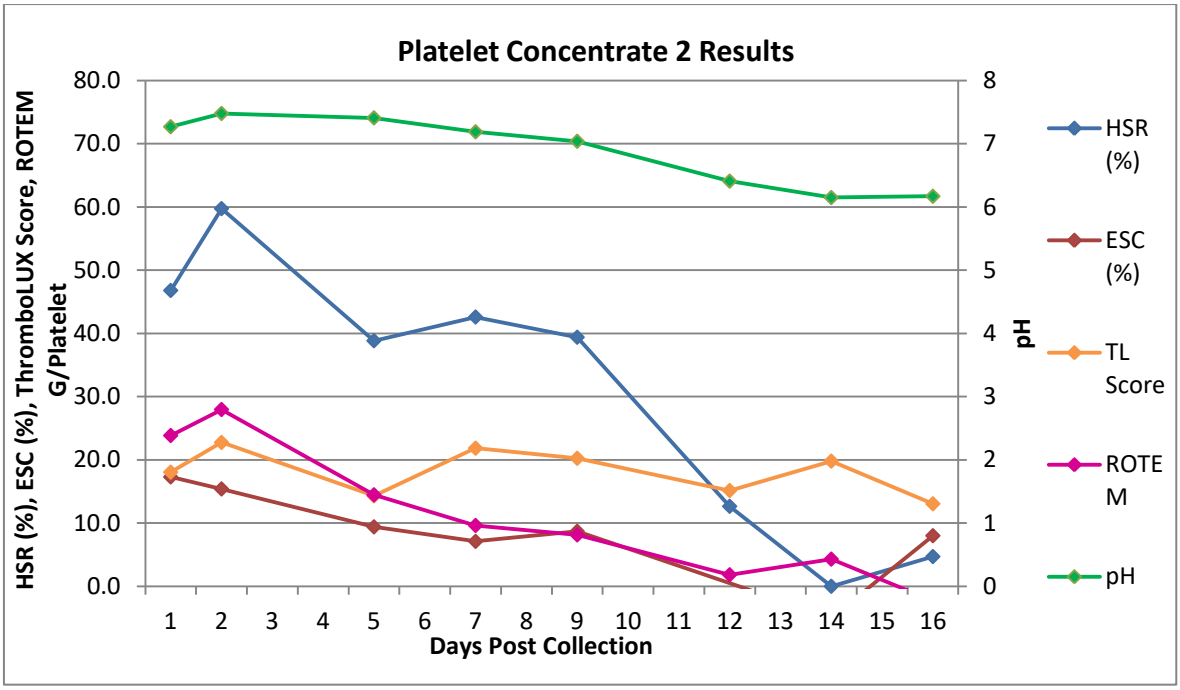

Figure 3.14: *Platelet concentrate 2 results. The HSR % (blue), ESC % (red), ThromboLUX score (orange) and ROTEM® G/platelet (pink) are plotted against the primary y axis. pH (green) is plotted against the secondary axis..*

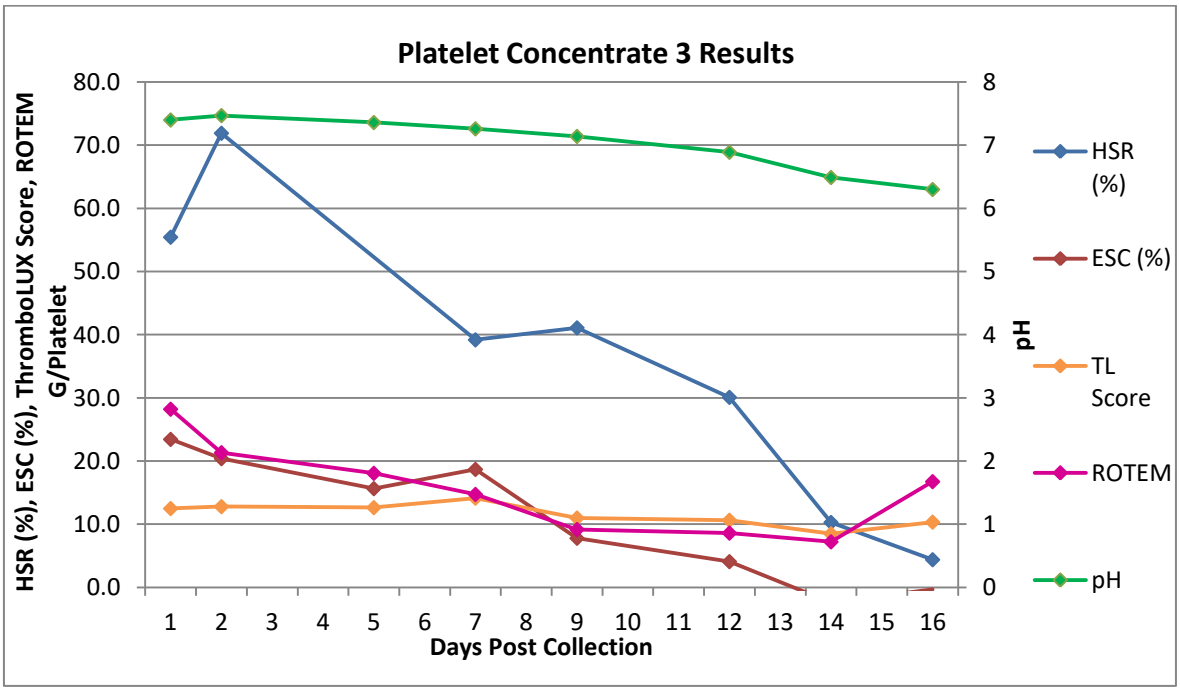

Figure 3.15: *Platelet concentrate 3 results. The HSR % (blue), ESC % (red), ThromboLUX score (orange) and ROTEM® G/platelet (pink) are plotted against the primary y axis. pH (green) is plotted against the secondary axis..*

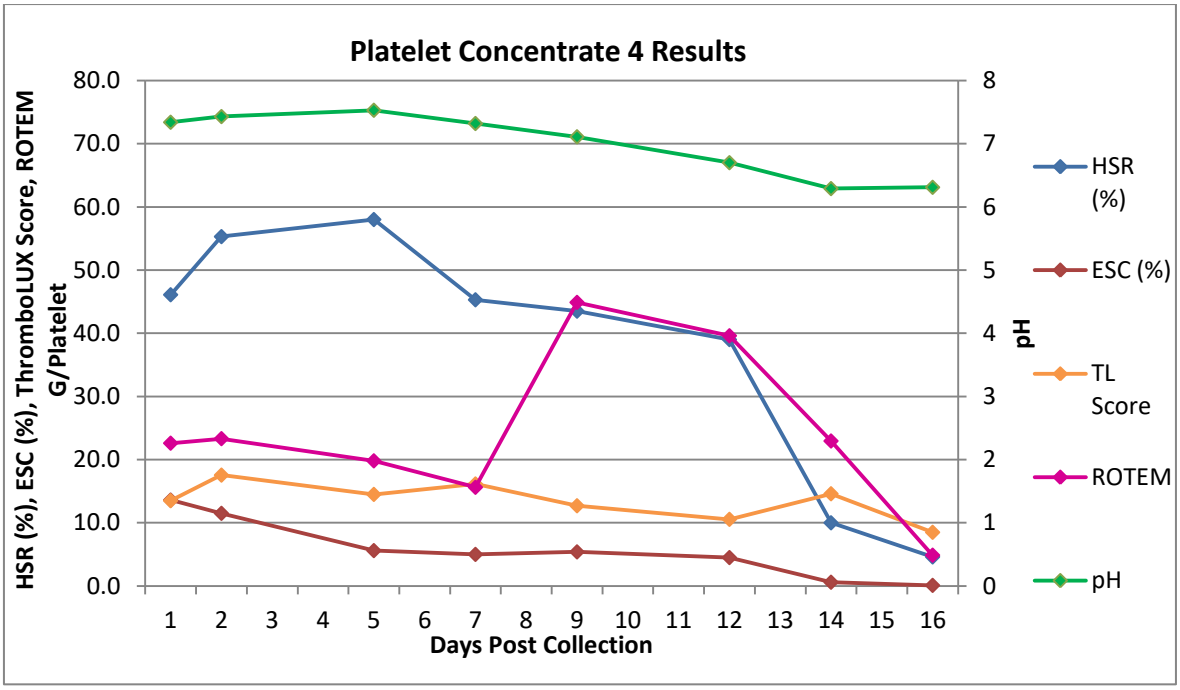

Figure 3.16: *Platelet concentrate 4 results. The HSR % (blue), ESC % (red), ThromboLUX score (orange) and ROTEM® G/platelet (pink) are plotted against the primary y axis. pH (green) is plotted against the secondary axis.*

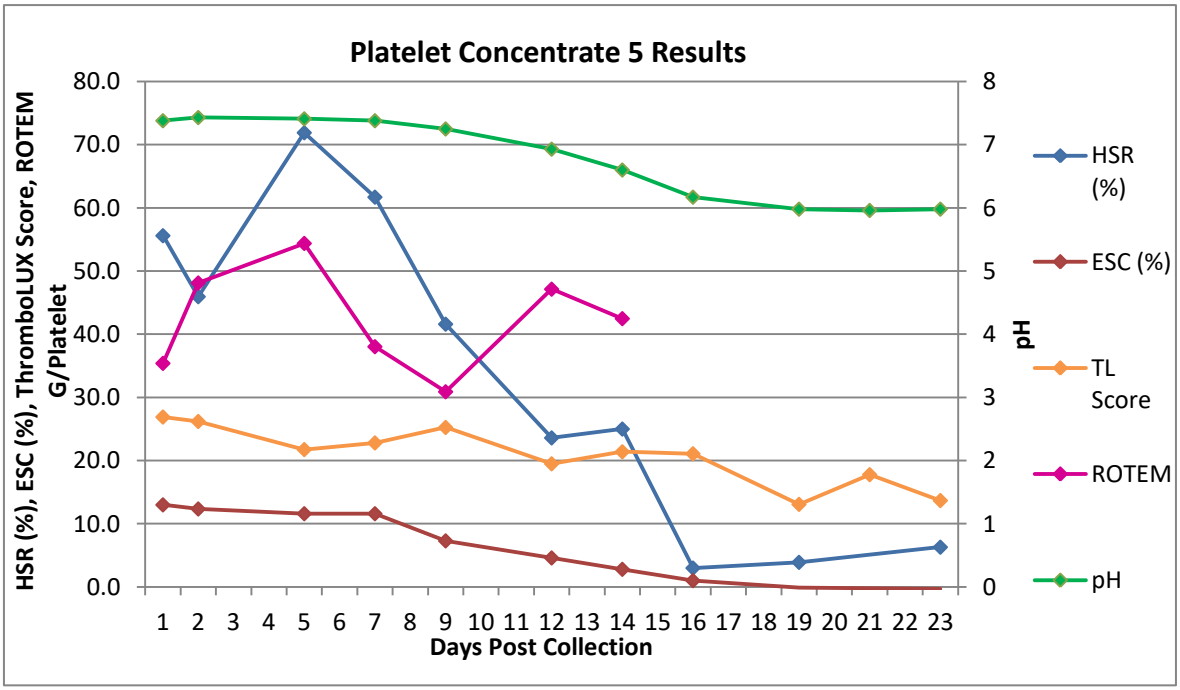

Figure 3.17: *Platelet concentrate 5 results. The HSR % (blue), ESC % (red), ThromboLUX score (orange) and ROTEM® G/platelet (pink) are plotted against the primary y axis. pH (green) is plotted against the secondary axis.*

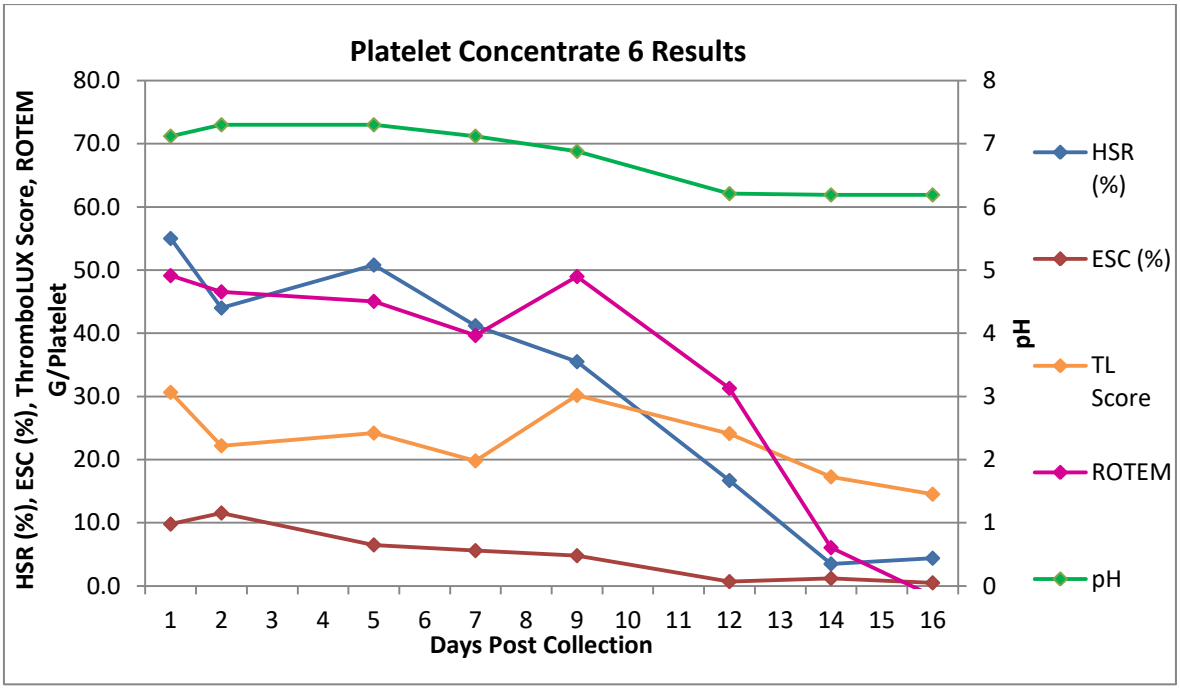

Figure 3.18: *Platelet concentrate 6 results. The HSR % (blue), ESC % (red), ThromboLUX score (orange) and ROTEM® G/platelet (pink) are plotted against the primary y axis. pH (green) is plotted against the secondary axis.*

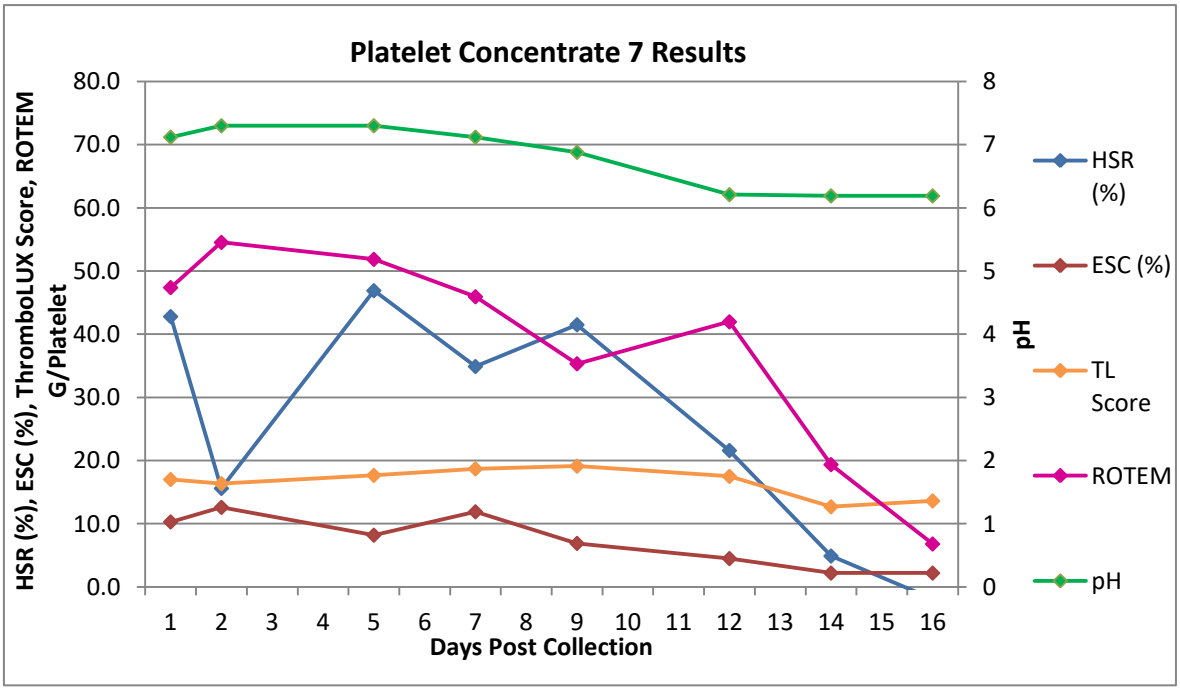

Figure 3.19: *Platelet concentrate 7 results. The HSR % (blue), ESC % (red), ThromboLUX score (orange) and ROTEM® G/platelet (pink) are plotted against the primary y axis. pH (green) is plotted against the secondary axis.*

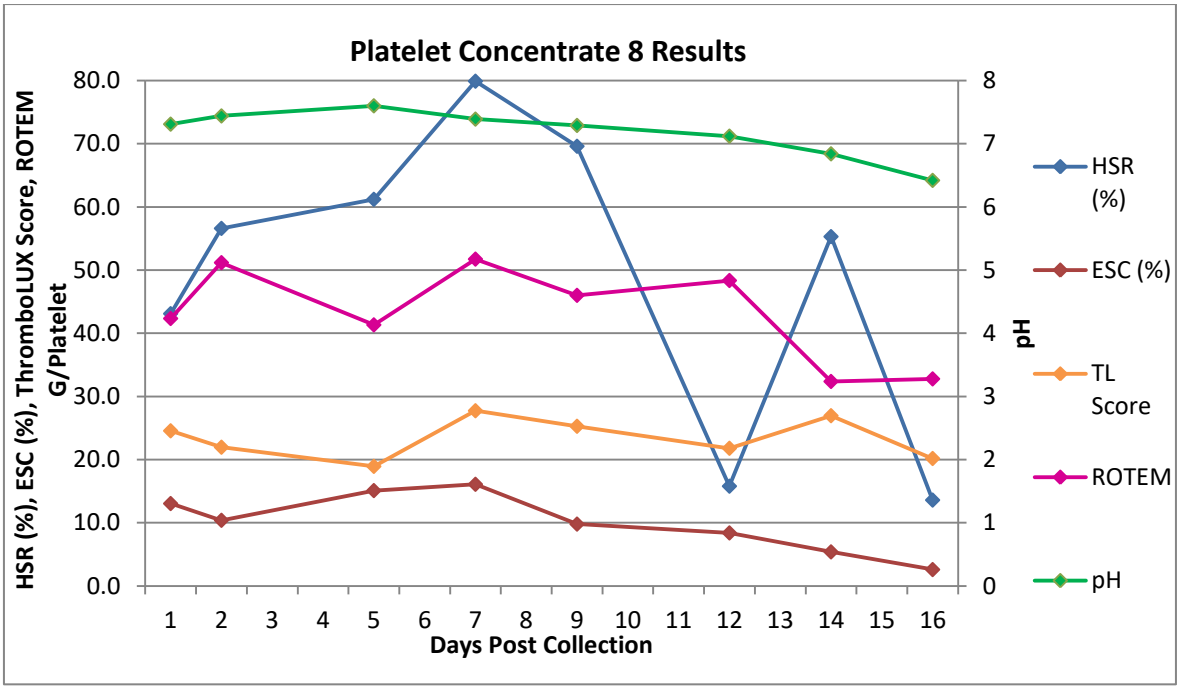

Figure 3.20: *Platelet concentrate 8 results. The HSR % (blue), ESC % (red), ThromboLUX score (orange) and ROTEM® G/platelet (pink) are plotted against the primary y axis. pH (green) is plotted against the secondary axis..*

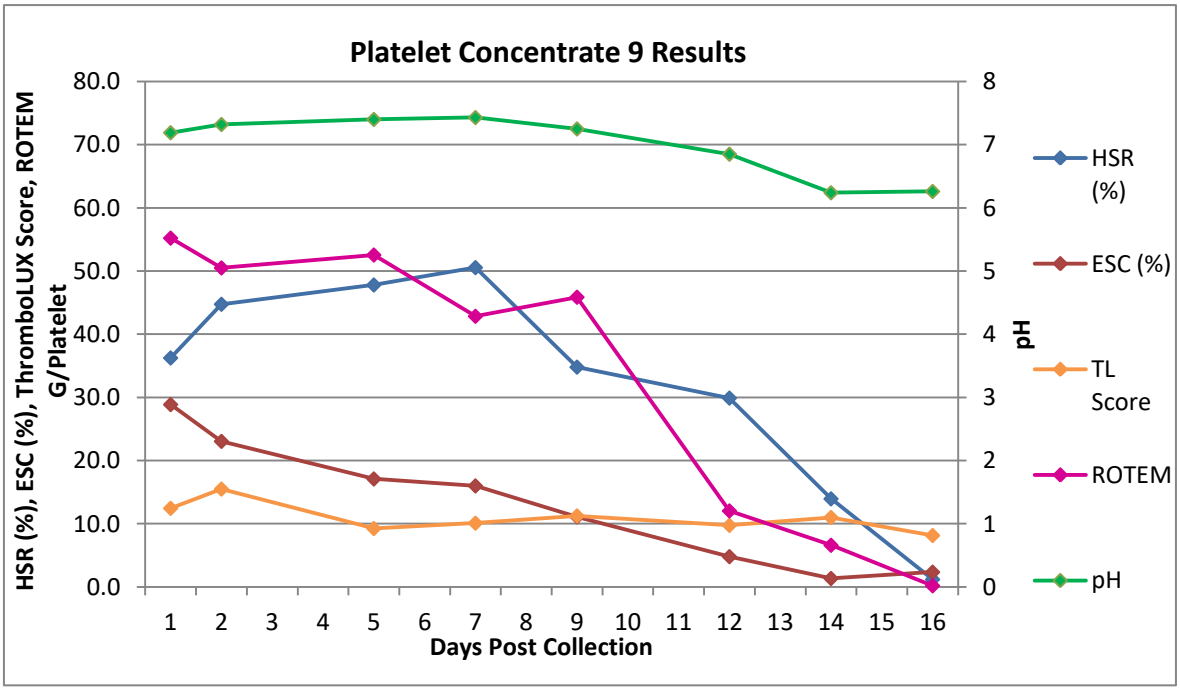

Figure 3.21: *Platelet concentrate 9 results. The HSR % (blue), ESC % (red), ThromboLUX score (orange) and ROTEM® G/platelet (pink) are plotted against the primary y axis. pH (green) is plotted against the secondary axis.*

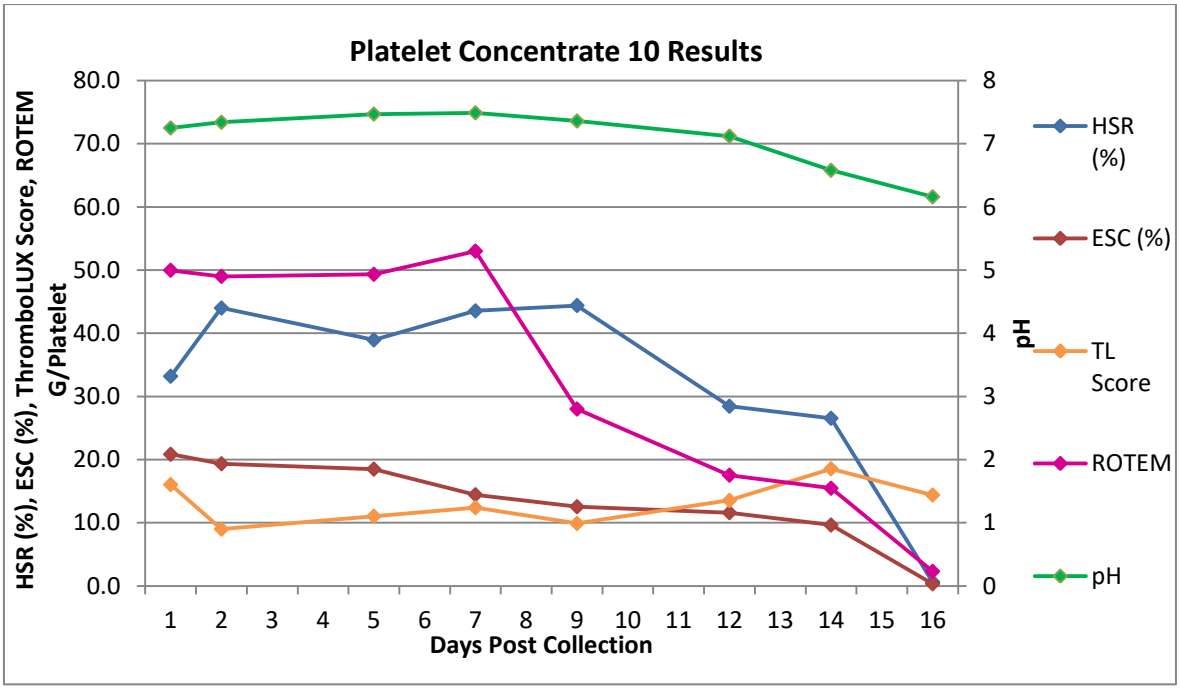

Figure 3.22: *Platelet concentrate 10 results. The HSR % (blue), ESC % (red), ThromboLUX score (orange) and ROTEM® G/platelet (pink) are plotted against the primary y axis. pH (green) is plotted against the secondary axis.*

The ThromboLUX results for all ten concentrates are shown together in Figure 3.23. Some concentrates showed a degree of deterioration over time. However, the differences were usually small, after 15 days and it was difficult to pick out a potential 'cut-off' value with any confidence. Concentrate 5 was tested at two additional time-points. Even at 23 after collection days it did not show clear and definite deterioration of quality upon comparison to the previously defined cut-off values suggested by LightIntegra at different stages of the study.

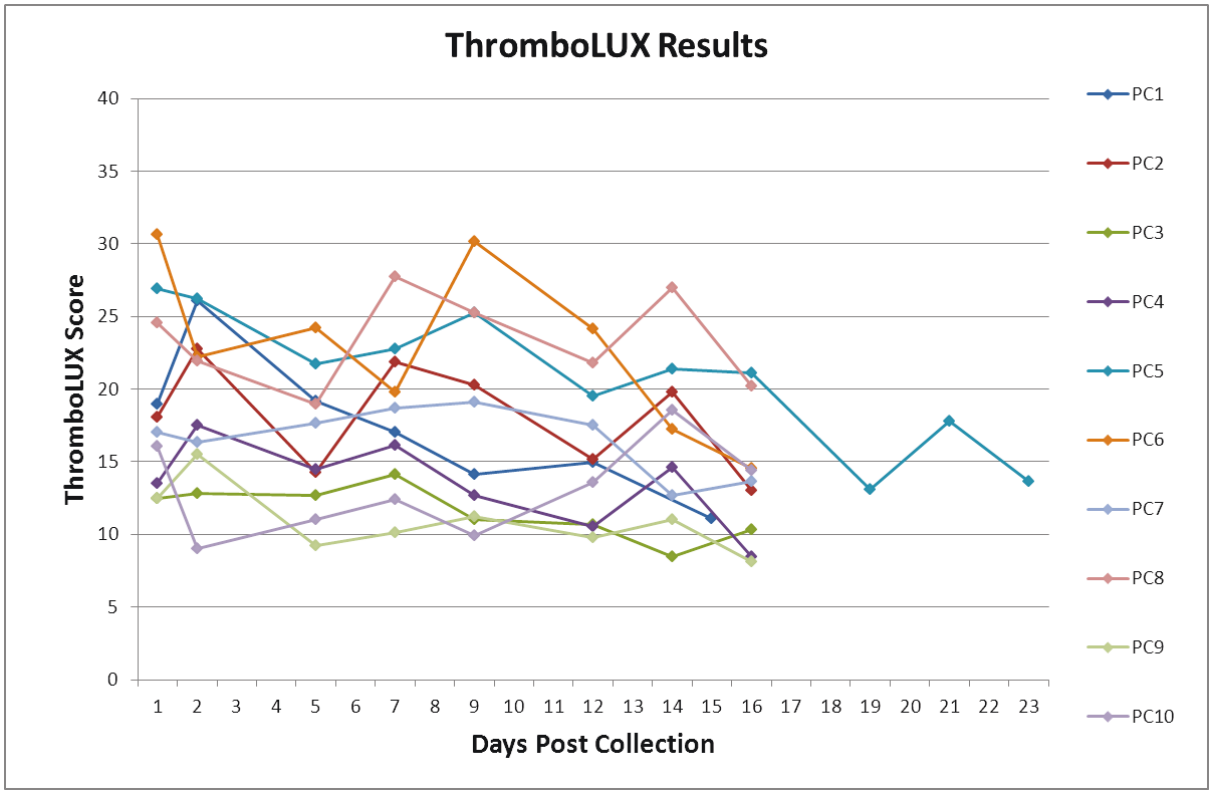

Figure 3.23: *ThromboLUX results for fresh platelet concentrates PC1-PC10.*

Samples from Platelet Concentrate 1 were stored at 22°C (appropriately stored control), 37°C, 50°C and -40°C (inappropriate storage temperatures known to damage platelet quality) prior to testing with the ThromboLUX analyser. Results were convincingly reduced for the sample stored at -40°C and slightly reduced for the sample stored at 50°C. No negative effect of storage at 37°C was observed. See Table 3.13.

Table 3.13: *ThromboLUX scores for platelet concentrate samples stored at various temperatures*

Storage Condition	Count	pH	TL Score	TL Score 2	TL Mean
22°C	1194	6.41	13.5	16.4	15.0
37°C	1053	6.12	20.5	11.6	16.1
50°C	418	6.24	12.4	13.4	12.9
- 40°C	1183	6.99	7.6	6.1	6.9

3.6 ROTEM Thromboelastometry *In-Vitro* Results

The laboratory assessment of the ROTEM analyser is presented below.

3.6.1 Initial laboratory assessment of the ROTEM

The EXTEM and FIBTEM tests available on the ROTEM® analyser underwent limited assessment within the expired concentrates stage of the study. Initial qualitative results for two of the expired platelet concentrates suggested a detectable difference with increasing age as shown in Figure 3.24.

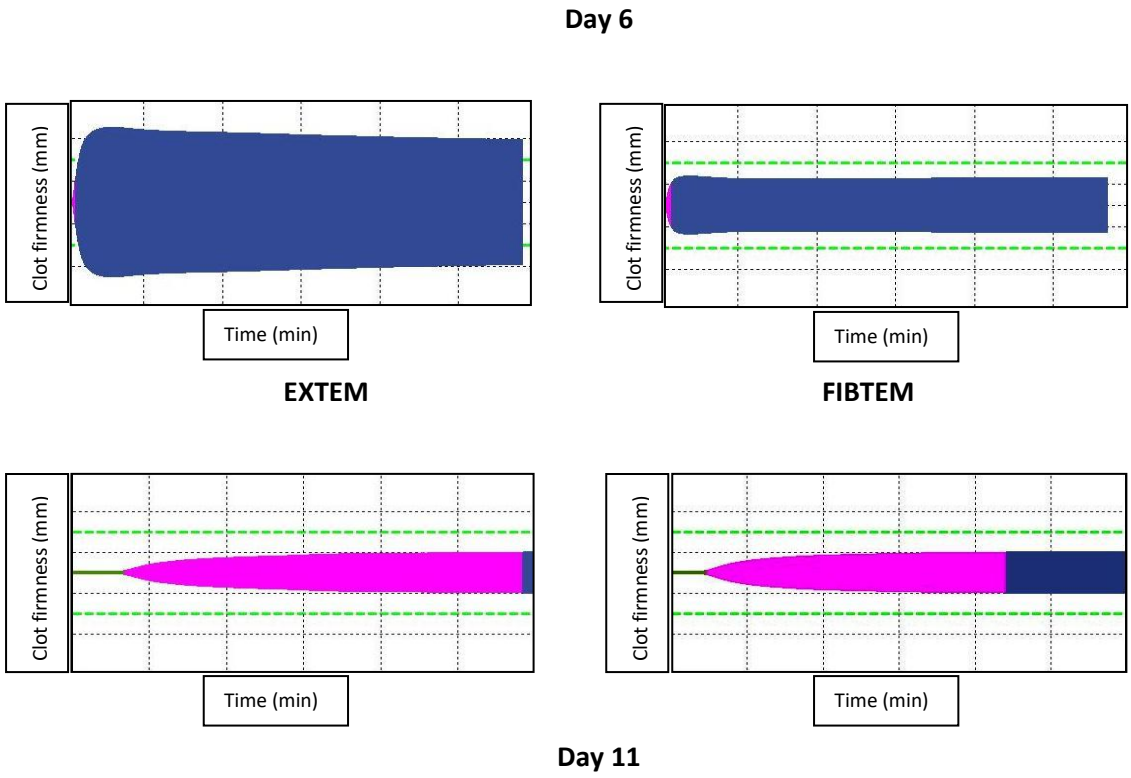

Figure 3.24: *ROTEM® results for expired platelet concentrate 103 on days 6 (top) and 11 (bottom). The images are graphic representations of the clot firmness over time. The EXTEM test results are shown on the left and the FIBTEM results are shown on the right.*

Following this initial investigation, it was decided that future testing should use platelet concentrate samples diluted in fresh frozen plasma (FFP) to avoid exceeding the ROTEM®s upper limit of sensitivity and compensate for the deterioration of plasma protein elements over time. A single

batch of FFP, prepared from a pool of four units, was separated into multiple aliquots that were defrosted and used to dilute platelet samples when required. The platelet concentrates were diluted to give a platelet count of 300 x 10^9 / L (± 10%) and gave qualitatively similar results. See Figure 3.25.

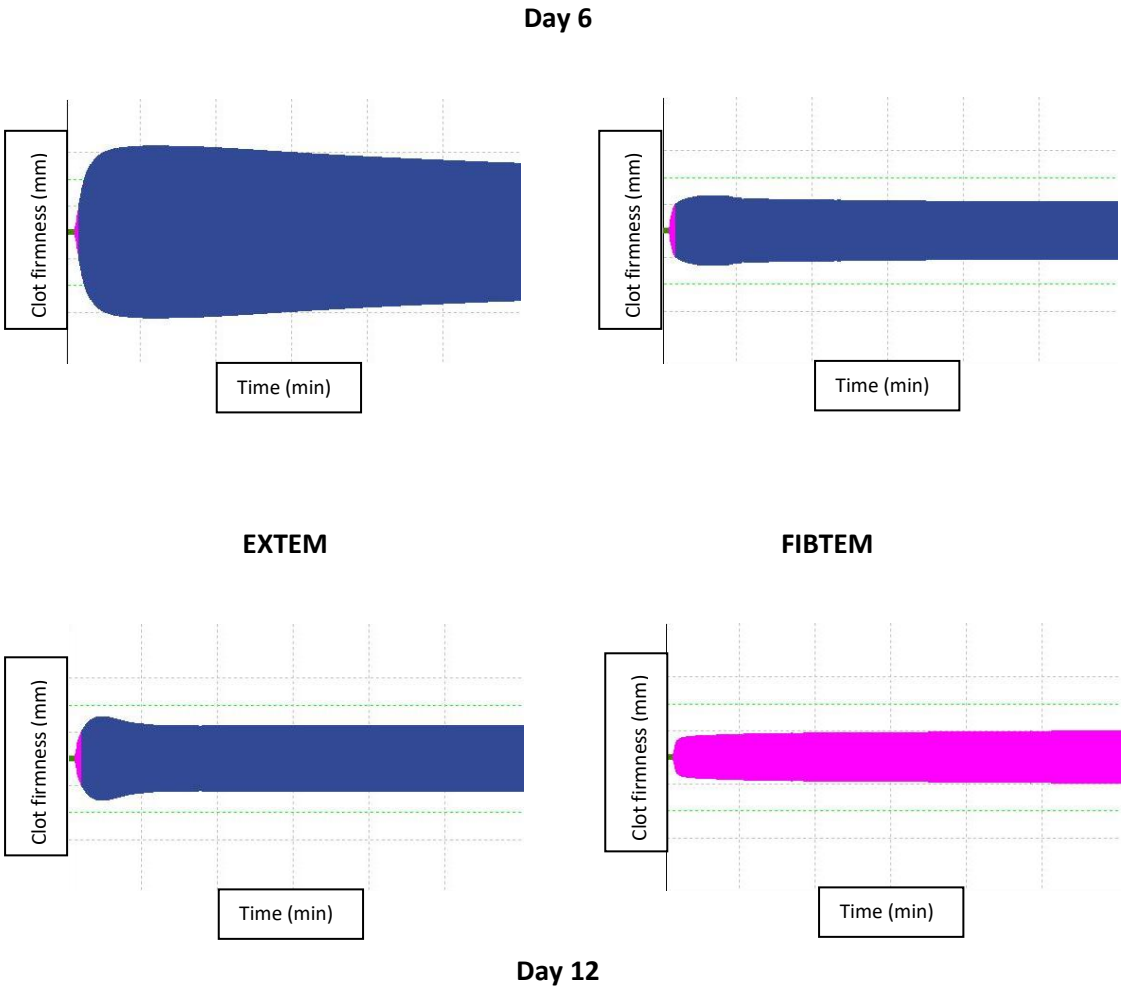

Figure 3.25: *ROTEM® results for expired platelet concentrate 104 on days 6 (top) and 11 (bottom). The images are graphic representations of the clot firmness over time. The EXTEM test results are shown on the left and the FIBTEM results are shown on the right.*

The five expired platelet concentrates tested on dilution during the method development stage of the project were found to show reduced clot strength with increasing age. The results are shown in Table 3.14. Of note, the platelet concentrate specimens stored at 37°C and -40°C (discussed above in relation to the HSR, ESC and ThromboLUX tests) showed markedly reduced clot firmness.

Table 3.14: *ROTEM® G value results obtained using the EXTEM and FIBTEM tests for five expired platelet concentrates including samples from two of them that were stored at inappropriate temperatures*

Concentrate Identification No.	Test Day / Condition	EXTEM G (Dyne/cm^2)	FIBTEM G (Dyne/cm^2)	EXTEM – FIBTEM G (Dyne/cm^2)
104	Day 6	9209	1746	7463
	Day 9	2746	1109	1637
	Day 12	2250	1085	1165
	Day 15	818	763	55
	37°C	657	705	-48
	-40°C	1323	1611	-288
105	Day 6	7324	1059	6265
	Day 9	1039	1093	-54
	Day 12	2336	1176	1160
	Day 15	1125	1153	-28
	37°C	809	704	105
	-40°C	1517	1270	247
106	Day 7	6009	1579	4430
	Day 10	3404	1757	1647
	Day 13	3987	1353	2634
107	Day 9	4619	1510	3109
	Day 12	1644	1541	103
108	Day 7	3328	1757	1571
	Day 10	4264	1430	2834
	Day 11	3336	1409	1927

These results suggested that the ROTEM® could detect deterioration of platelet function with age. A paired, uni-directional T-test was performed to see whether deterioration was evident between the first and last day that expired platelet concentrates were tested. Significant differences were found between six to nine day old concentrates and twelve to fifteen day old concentrates in terms of their EXTEM ($P = 0.02$) and EXTEM – FIBTEM ($P = 0.02$) results. To compensate for activity differences due to slight variations in dilution platelet counts, it was decided that future results could also be expressed as (EXTEM G – FIBTEM G) / platelet.

3.6.2 ROTEM ageing platelet concentrate results

The ROTEM results for all ten concentrates are shown alongside the pH, ESC, HSR and ThromboLUX results in Figures 3.13 – 3.22 that are presented above in section 3.5. They are also shown for all ten concentrates in Figure 3.26 below. For the most part, a general trend of deterioration over time is observed. However, there is a clear split in results: concentrates PC1 to PC3 gave much lower results at all time-points than concentrates PC5-PC10. Concentrate PC4 initially gave low results but increased dramatically to give high results from day 9 onwards. The batch of EXTEM reagent was changed on day 9 of testing PC4 and the new batch continued to be used for the rest of this phase of the study. It seems likely that the ROTEM results are batch dependant.

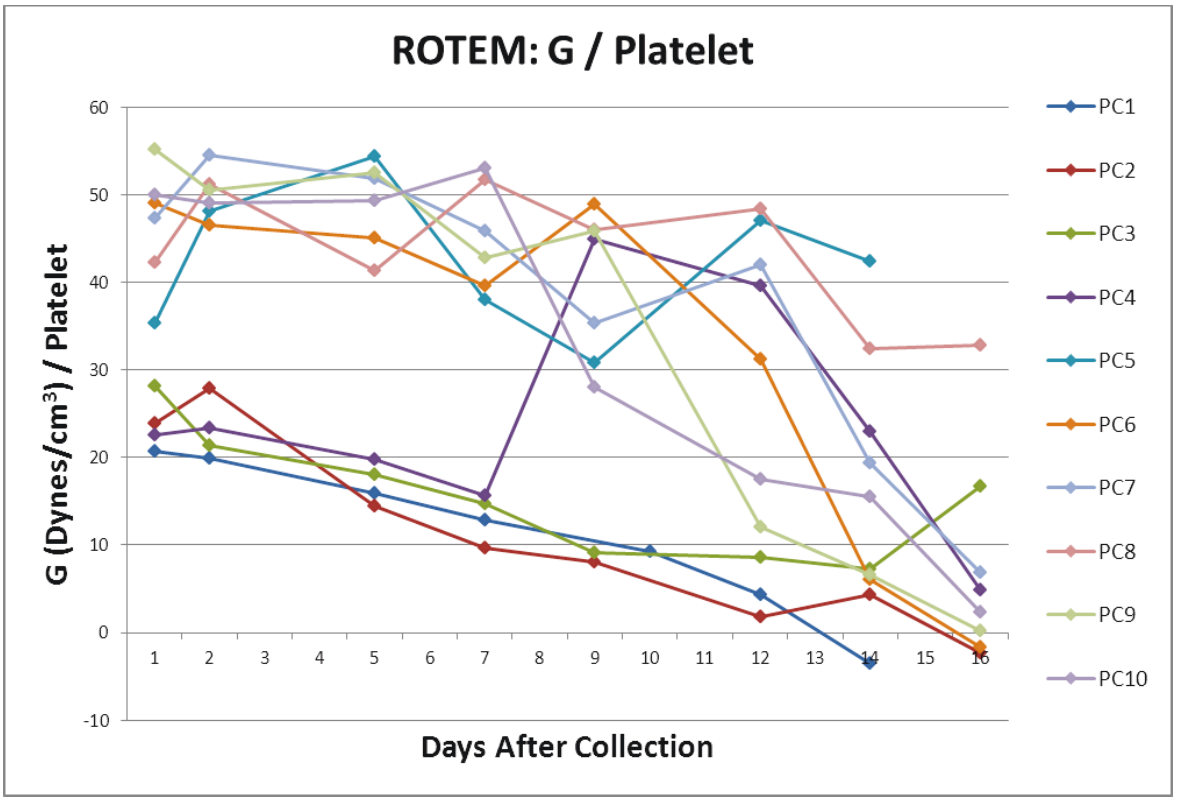

Figure 3.26: *ROTEM results for fresh platelet concentrates PC1-PC10.*

The ROTEM® was able to detect storage at extreme temperatures with a high degree of sensitivity compared to an appropriately stored control sample. Samples of Platelet Concentrate 1 were stored at 22°C (appropriately stored control), 37°C, 50°C and -40°C (inappropriate storage temperatures known to damage platelet quality). The results for all samples are shown in Table 3.15.

Table 3.15: *ROTEM® results for platelet concentrate samples stored at various temperatures. G/PLT is the EXTEM G – FIBTEM G per platelet in the test sample.*

Storage Temperature	EXTEM G	FIBTEM G	EXTEM-FIBTEM G	G/PLT
22°C	5,184	1,398	3,786	12.9
50°C	1,544	1,072	472	4.2
37°C	1,504	1,416	88	0.3
-40°C	1,837	2,043	-206	-0.8

3.7 AWE Test *In-Vitro* Results

The laboratory assessment of the AWE test is presented below.

3.7.1 Initial laboratory assessment of the AWE test

In vitro assessment of the new AWE test, developed on the Sysmex CA 5100 automated coagulometer as part of this project, was carried out using three PRP samples taken for the purpose of method development, four fresh platelet concentrates purchased as part of the ageing platelet concentrates section of this study and six platelet concentrate samples that were included in the clinical trial (see chapter 4). PC7, PC8, PC9 and PC10 from the Ageing Platelet Concentrate section of the study were tested using the AWE test over a period of 16 days. See Figures 3.27 and 3.28. The tests carried out during this phase of the study were performed using the VAR and FIX test settings without stir-bars.

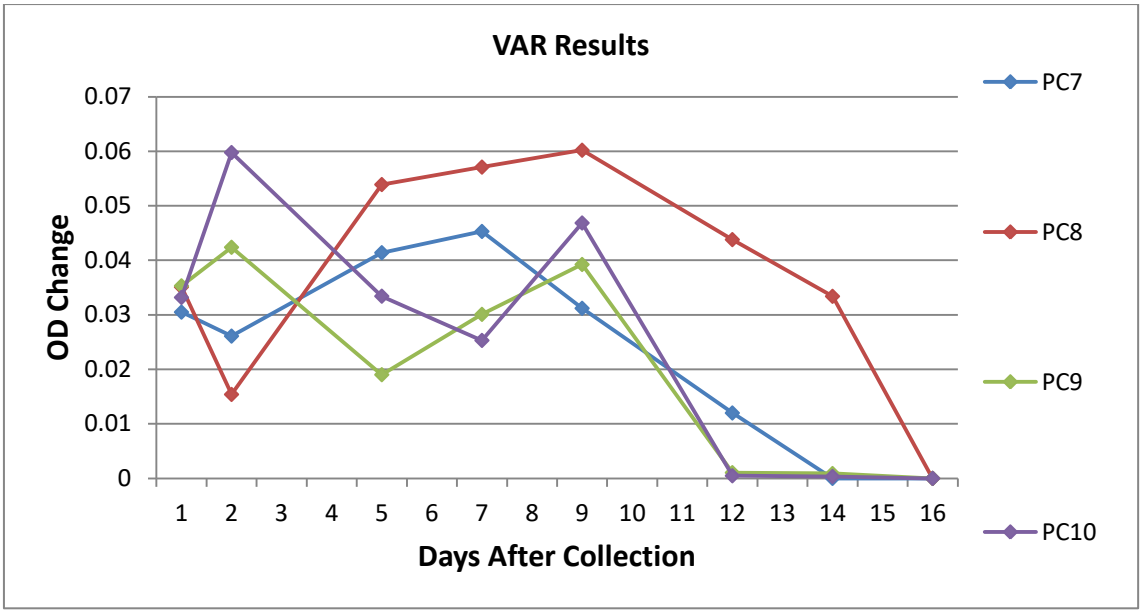

Figure 3.27: *VAR measurement setting AWE test results for platelet concentrates PC7, PC8, PC9 and PC10 from the in vitro stage of the study*

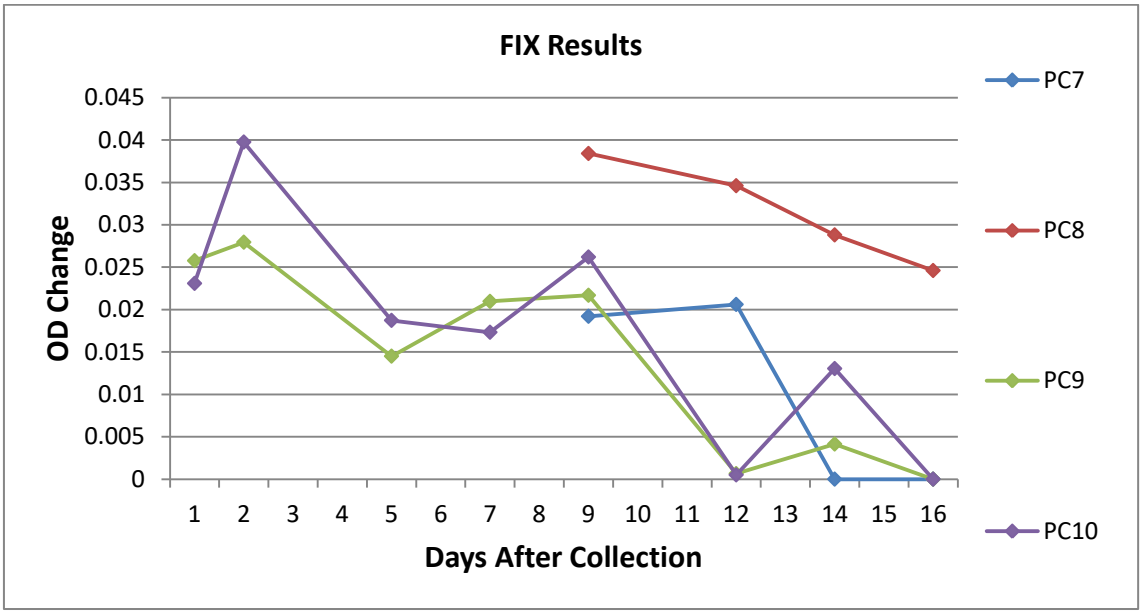

Figure 3.28: *FIX measurement setting AWE test results for platelet concentrates PC7, PC8, PC9 and PC10 from the in vitro stage of the study*

These initial results suggested that the AWE test results may fall with increasing platelet concentrate age and deteriorating platelet function. It was decided that further validation of the method was indicated.

The first platelet concentrate included in the clinical stage of the study was tested ten times for each of the four AWE test settings, using both neat and diluted samples, on the day of transfusion and again after storage in the laboratory for five days. The average results, standard deviation (SD) and CV for each parameter is shown in Table 3.16.

Table 3.16: *Average, SD and CV values for PC 001. All four measurement settings were applied to neat specimens and diluted specimens that were tested ten times each on two separate occasions.*

Sample	Day	Measure	VAR	FIX	VSB	FSB
PC 001 Neat	**11/09/2015**	Average	0.13964	0.08295	0.12367	0.07079
		SD	0.01490	0.00764	0.00549	0.00168
		CV (%)	10.671	9.211	4.436	2.372
	16/09/2015	Average	0.02306	0.02334	0.02437	0.02398
		SD	0.00163	0.00106	0.00113	0.00064
		CV (%)	7.051	4.540	4.648	2.680
PC 001 Diluted	**11/09/2015**	Average	0.03644	0.02346	0.02721	0.01677
		SD	0.00917	0.00253	0.00455	0.00149
		CV (%)	25.152	10.782	16.710	8.853
	16/09/2015	Average	0.00489	0.00697	0.00647	0.00750
		SD	0.00044	0.00090	0.00136	0.00097
		CV (%)	8.914	12.958	21.087	12.875

The CV obtained for each test set range from 0.024 (FSB measurement settings for the neat sample tested on the day of transfusion) to 0.252 (VAR measurement settings for the diluted sample tested on the day of transfusion). With only one exception (PC 001 diluted sample, VAR test settings), the tests that used stir-bars showed better precision than the tests that did not. The tests that used fixed settings were always more precise than the ones that used variable

settings. Although some of the CV measurements show poor precision by traditional laboratory standards, they are not unusual for platelet tests, which are notoriously difficult to standardise. The ranges of the results obtained are also shown in a visual format in Figures 3.29 to 3.33.

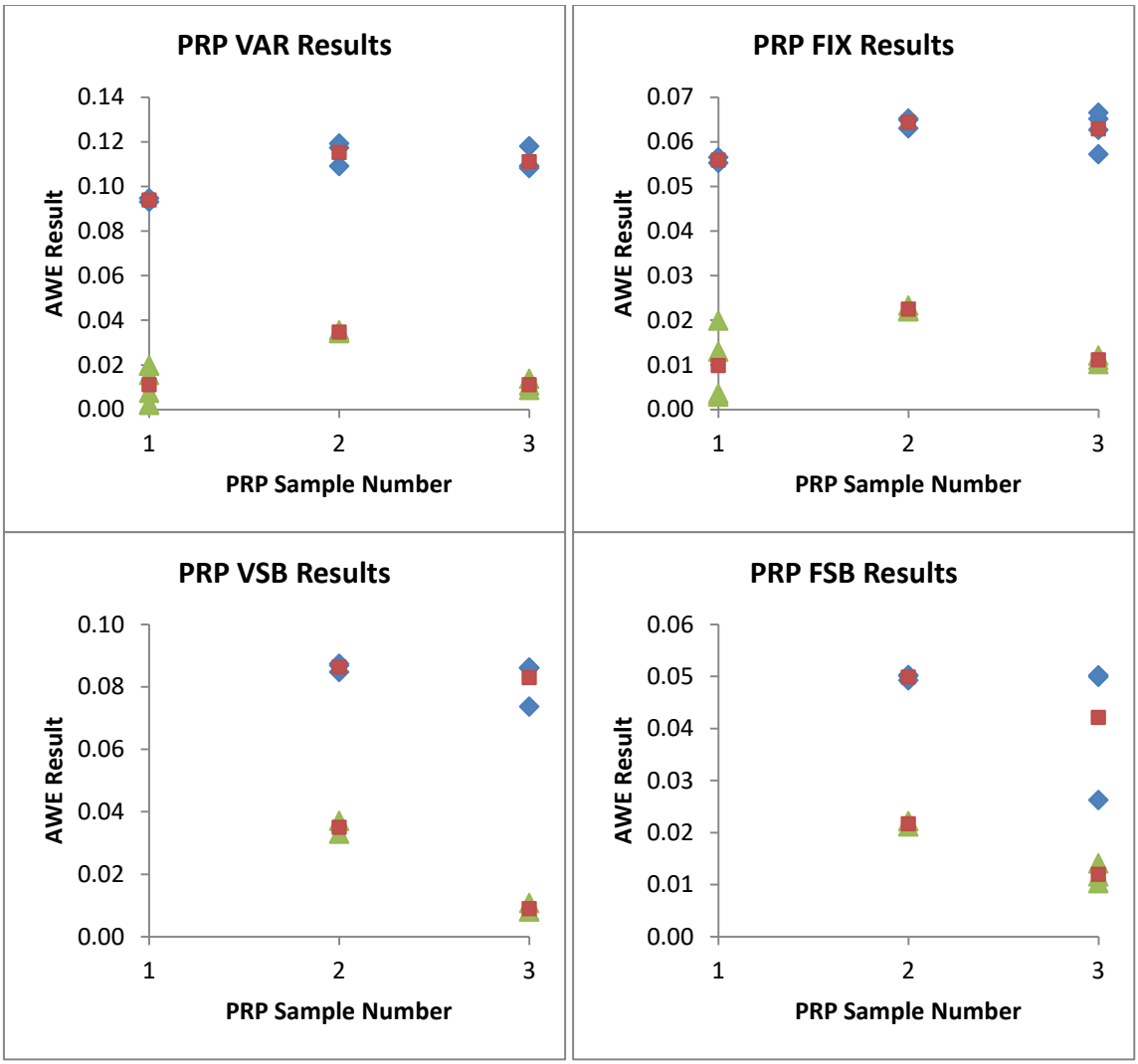

Figure 3.29: *Graphical representation of all AWE VAR settings results (top left), FIX settings results (top right), VSB results (bottom left) and FSB results (bottom right) obtained for the PRP samples tested. Blue diamonds show Fresh PRP results, green triangles show Aged PRP results and red squares show the averages of each group.*

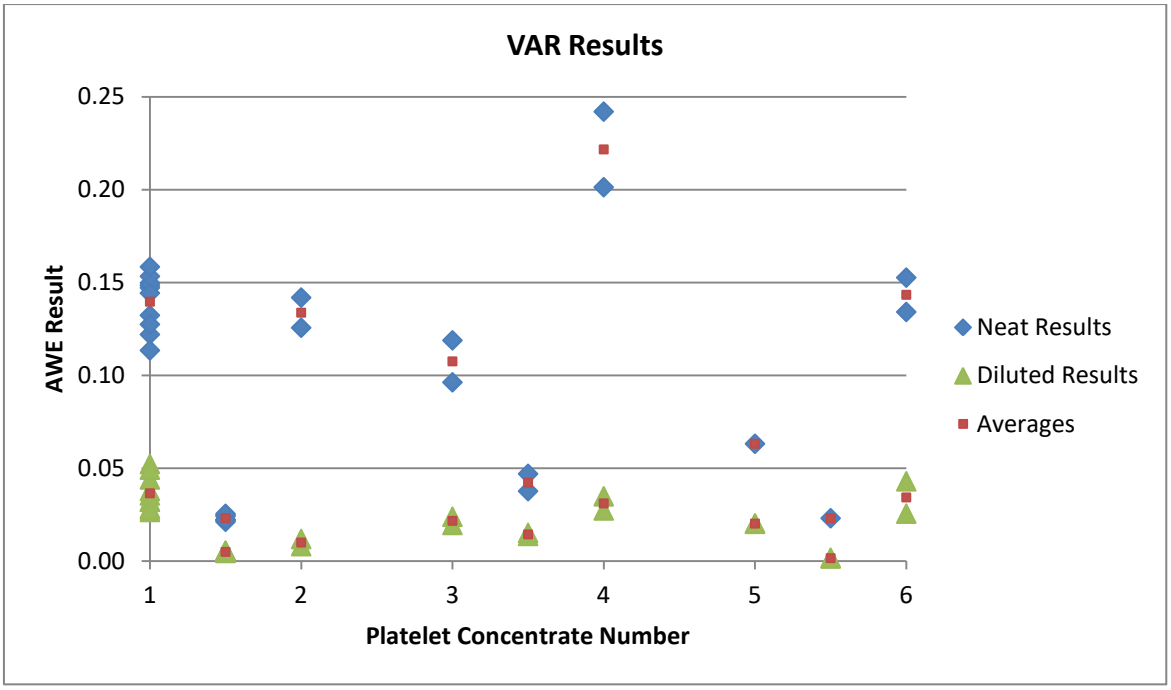

Figure 3.30: *VAR results obtained for all of the PC samples tested. Aged sample results are shown at positions of 1.5, 3.5 and 5.5 on the X axis for PC 001, 003 and 005.*

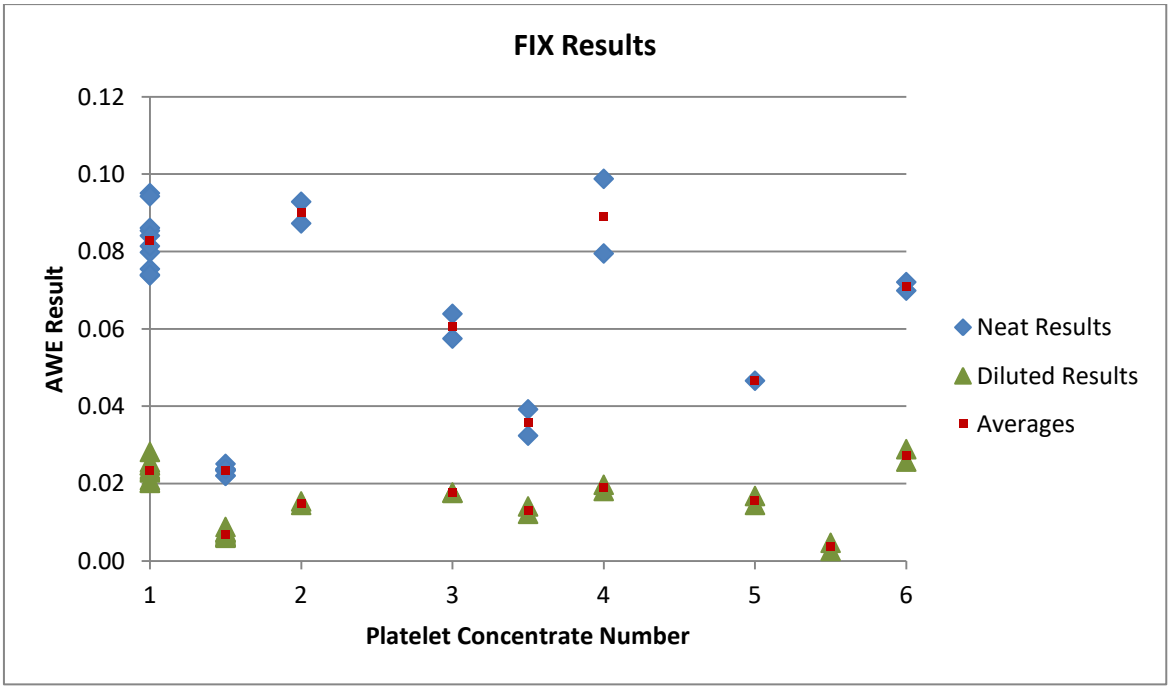

Figure 3.31: *FIX results obtained for all of the PC samples tested. Aged sample results are shown at positions of 1.5, 3.5 and 5.5 on the X axis for PC 001, 003 and 005.*

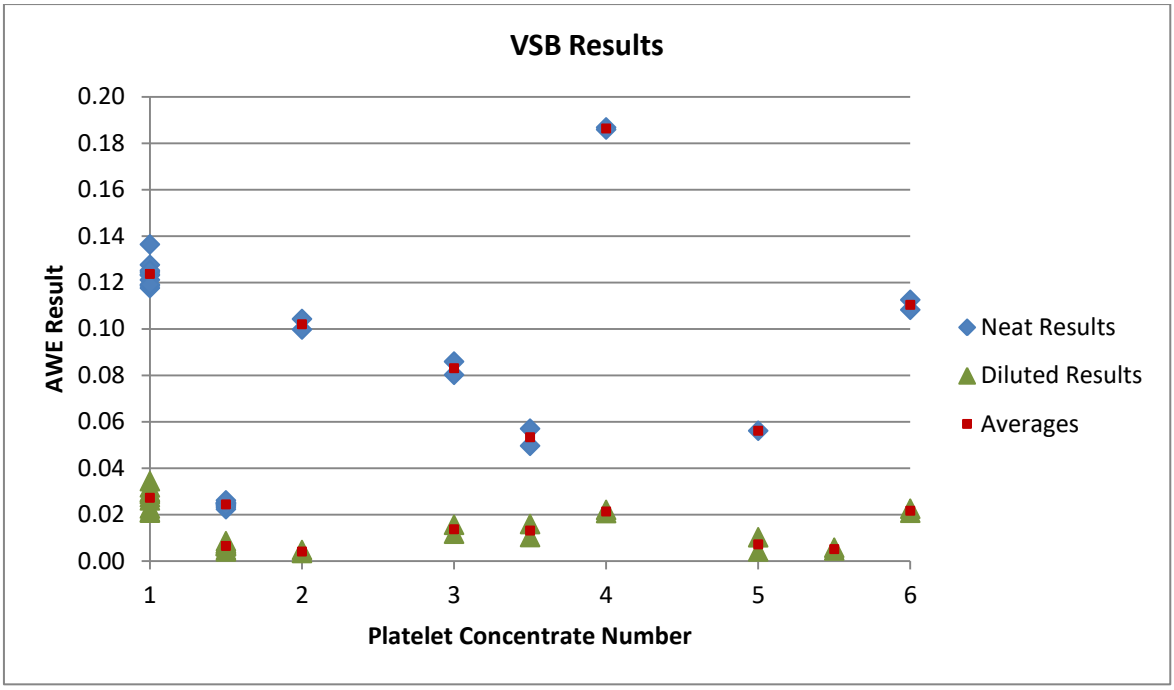

Figure 3.32: *VSB results obtained for all of the PC samples tested. Aged sample results are shown at positions of 1.5, 3.5 and 5.5 on the X axis for PC 001, 003 and 005.*

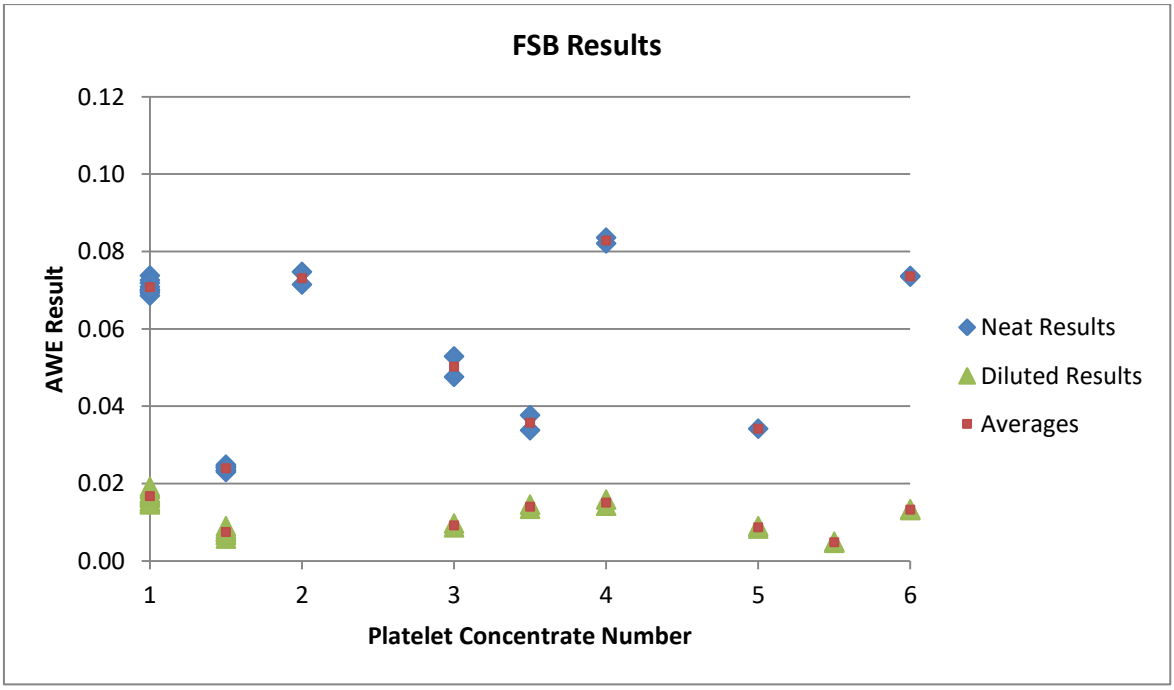

Figure 3.33: *FSB results obtained for all of the PC samples tested. Aged sample results are shown at positions of 1.5, 3.5 and 5.5 on the X axis for PC 001, 003 and 005.*

All three PRP samples and four of the PC samples were tested at more than one time point. Between testing, the specimens were stored in a capped container in the laboratory without any agitation – conditions that are not considered compatible with maintenance of platelet viability. The three PRP samples showed clear deterioration between the two tests. See Figures 3.34 to 3.36.

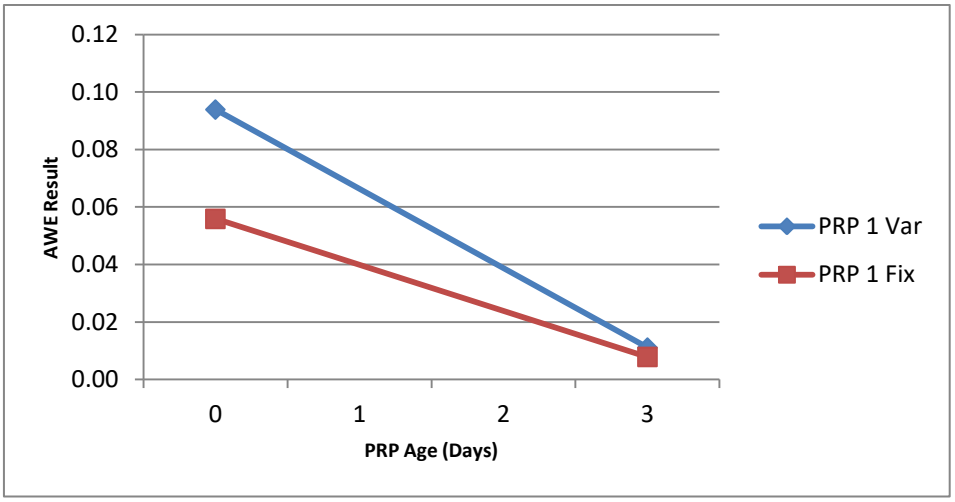

Figure 3.34: *Change in AWE results for PRP sample 1*

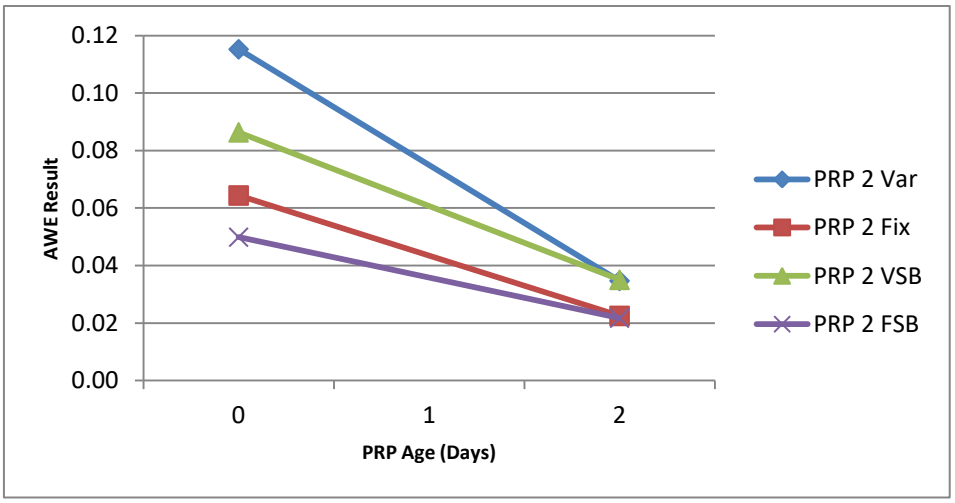

Figure 3.35: *Change in AWE results for PRP sample 2*

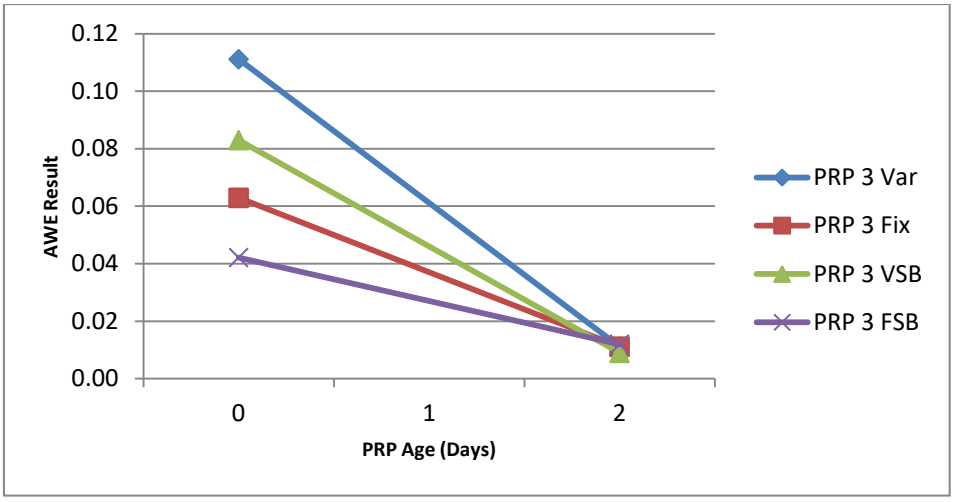

Figure 3.36: *Change in AWE results for PRP sample 3*

PC 001 also showed clear deterioration (see Figure 3.37).

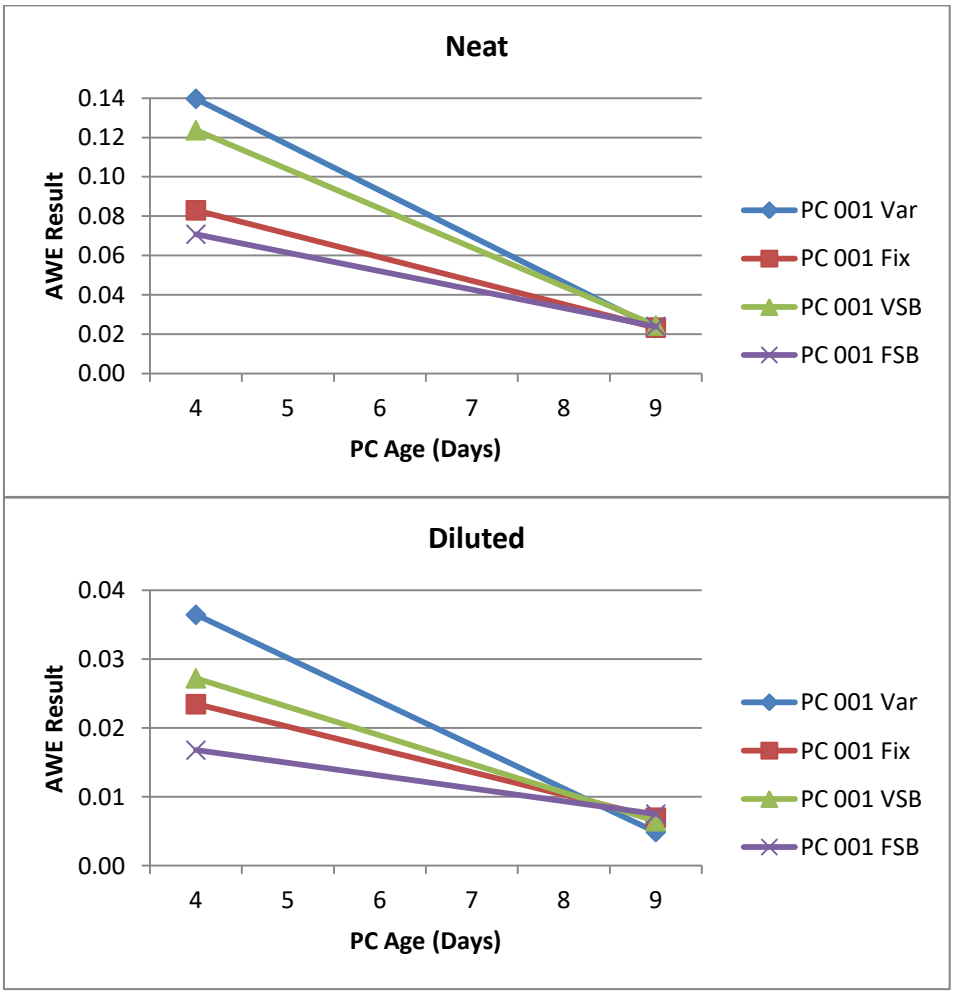

Figure 3.37: *Change in neat (top) and diluted (bottom) sample AWE results for PC 001*

Every test performed on PC 002 on the second test date gave a 'No Polynomial Adjustment' (NPA) error. This error is reported by the analyser when there is no detectable change in the OD during the measurement period. The NPA errors observed for platelet concentrate or PRP samples most likely occur because the platelets in the sample have deteriorated to the extent that they are no longer able to undergo measurable recovery from water uptake during the test.

PC 003 showed deterioration by all of the settings that did not use stir-bars (see Figure 3.38). The diluted stir-bar test results did not deteriorate but the second testing time point was only one day after the day of transfusion and this may not have been sufficient time for the platelet function in the sample to deteriorate sufficiently for detection.

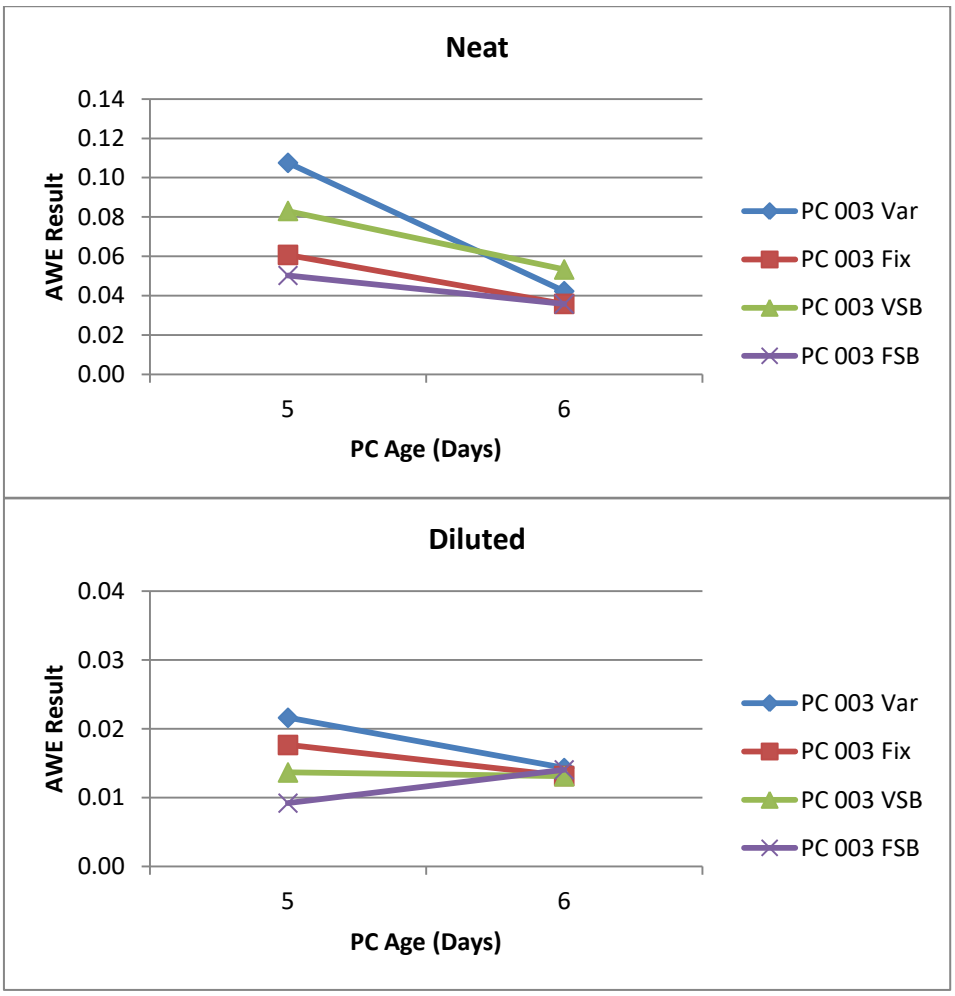

Figure 3.38: *Change in neat (top) and diluted (bottom) sample AWE results for PC 003*

Only diluted test results were available at a second time-point for concentrate 005. As for concentrate 003, deterioration was more clearly evident using the non-stir-bar settings. See Figure 3.39.

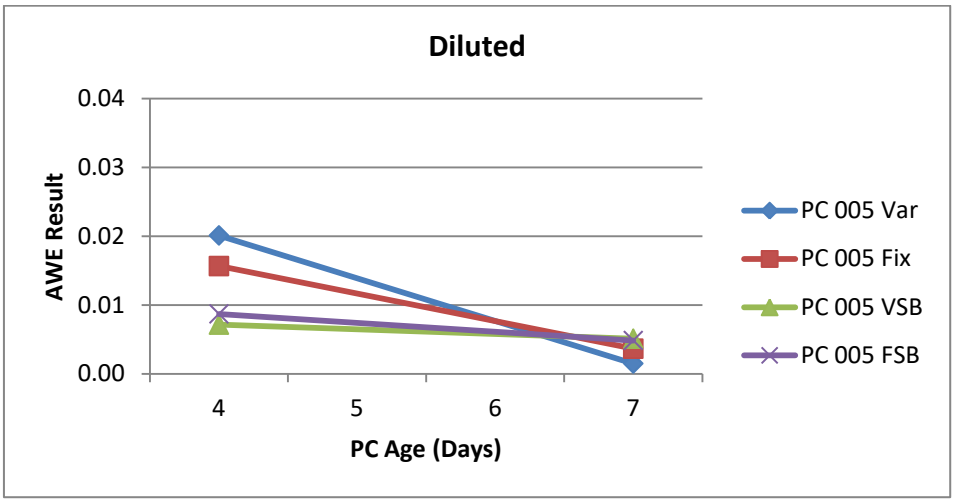

Figure 3.39: *Change in diluted sample AWE results for PC 005*

There was no obvious relationship between concentrate age and the level of AWE test results. See Figures 3.40 and 3.41.

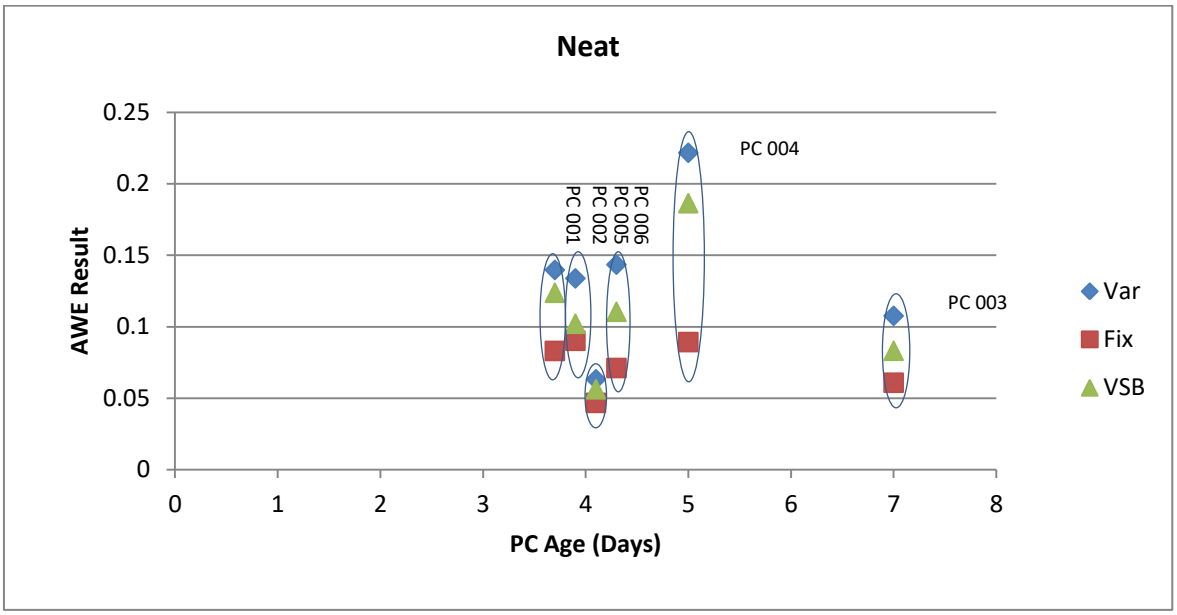

Figure 3.40: *Platelet concentrate age against neat sample test results*

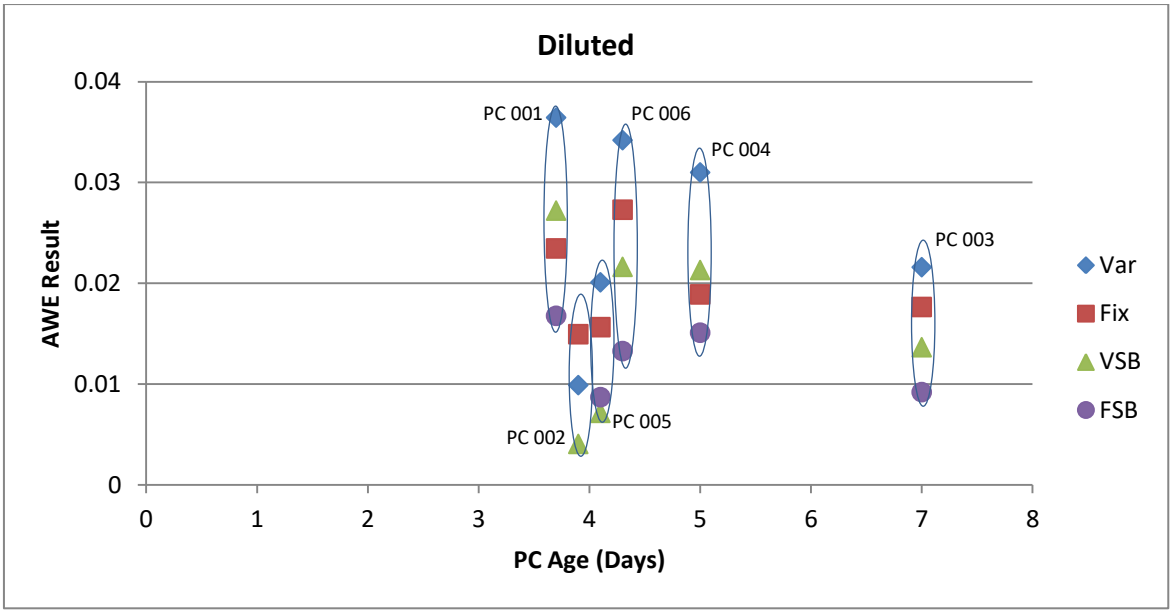

Figure 3.41: *Platelet concentrate age against diluted sample test results*

A series of tests were carried out on PC 003 to determine whether the platelets in the specimens were settling out in the sample tubes prior to or during analysis. Samples were mixed and aliquotted into tubes ready for testing. The first set of samples was loaded onto the machine for testing at time 0 but the other sets were retained. Subsequent sets were analysed (without further mixing) at 6, 12, 18, 24, 60 and 120 minutes. The results are shown in Figures 3.42 – 3.45. There does not appear to be any appreciable settling effect, with the possible exception of the diluted samples tested at 60 and 120 minutes. In practice, this means that short delays in testing while the specimens are waiting for sampling on the analyser are unlikely to have a significant effect on the test results.

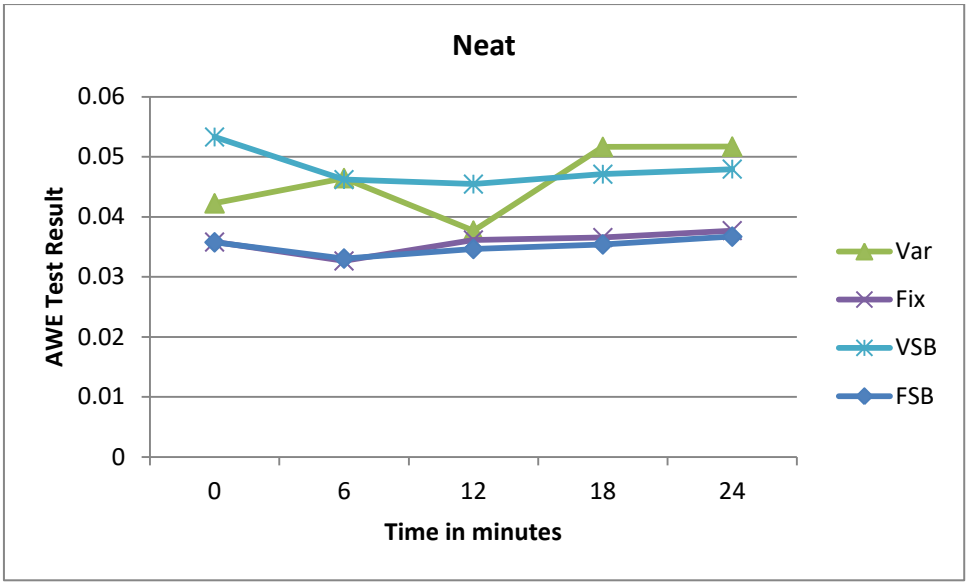

Figure 3.42: *Sample settling experiments for neat samples tested at 0, 6, 12, 18 and 24 minutes*

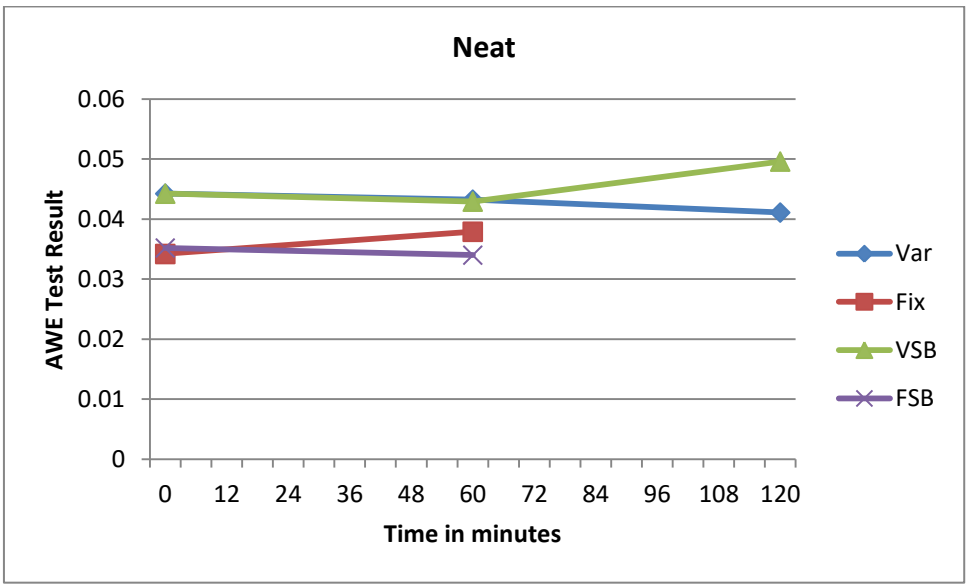

Figure 3.43: *Sample settling experiments for neat samples tested at 0, 60 and 120 minutes*

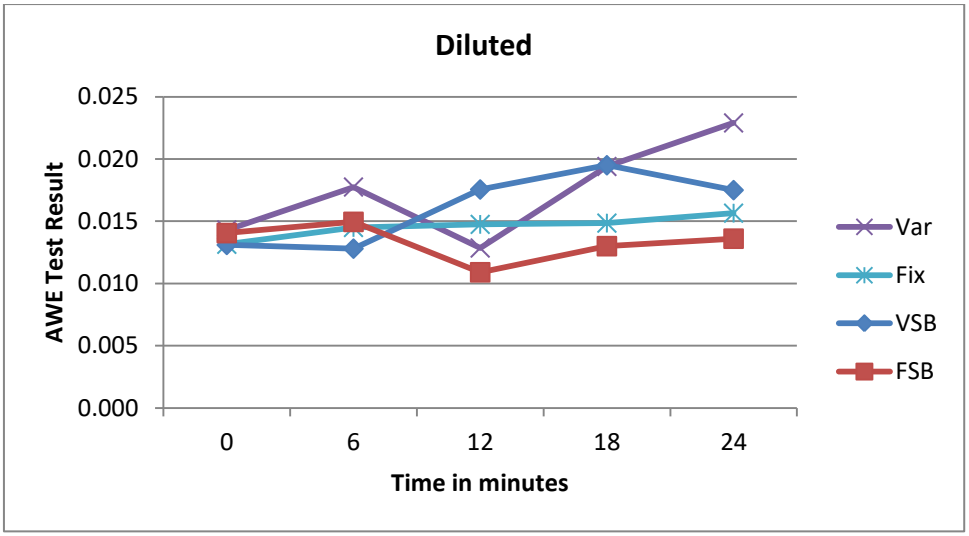

Figure 3.44: *Sample settling experiments for diluted samples tested at 0, 6, 12, 18 and 24 minutes*

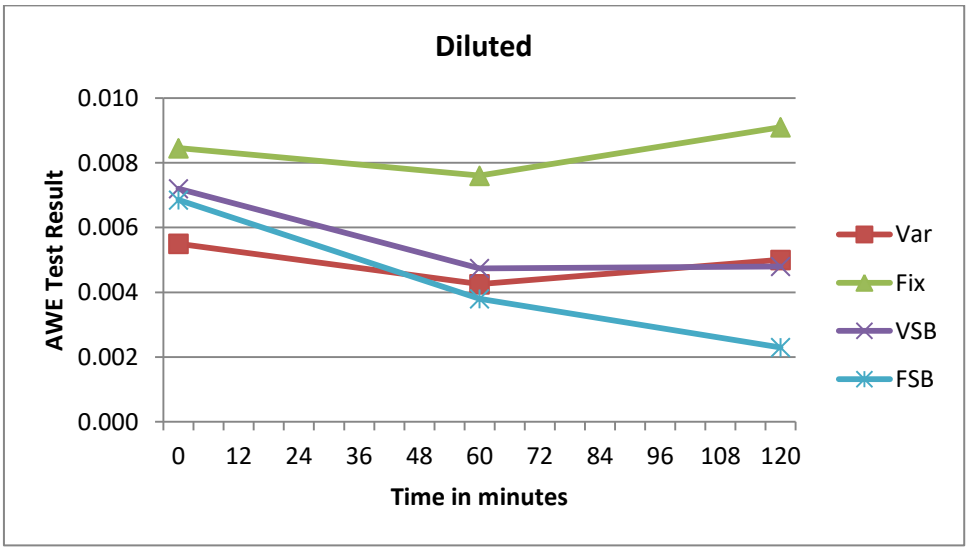

Figure 3.45: *Sample settling experiments for diluted samples tested at 0, 60 and 120 minutes*

In traditional HSR tests, the platelets response to water that is added to the test sample is blanked against a control test that uses isotonic OBS instead of water. This allows the results to be standardised to compensate for differences in the starting OD of the concentrates under investigation. In order to confirm that the OD change observed during the AWE test is due to a

water expulsion response and ensure that the observed OD change is not due purely to a dilutional effect, a buffer control test was run alongside the PC 002 water tests. See Table 3.17.

Table 3.17: *Owren's buffer experiment results for PC 002. The neat (N1, N2) and diluted (D1, D2) samples were tested using both water and buffer. The calculated results obtained by subtracting the buffer test results from the water test results are also shown.*

Test	VAR	FIX	VSB	FSB
N1	0.12560	0.09287	0.09972	0.07148
N2	0.14185	0.08728	0.10426	0.07475
Buffer N1	0.01856	0.01854	NPA	NPA
Buffer N2	NPA	0.02599	NPA	0.00121
N1 - Buffer	0.10704	0.07433	0.09972	0.07148
N2 - Buffer	0.14185	0.06129	0.10426	0.07354
D1	0.00800	0.01450	0.00470	0.00000
D2	0.01180	0.01540	0.00343	0.00000
Buffer D1	0.00260	NPA	NPA	NPA
Buffer D2	NPA	NPA	0.00670	NPA
D1 - Buffer	0.00540	0.01450	0.00470	0.00000
D2 - Buffer	0.01180	0.01540	-0.00327	0.00000

More than half of the tests gave NPA errors as the OD changes in the Owren's buffer tests were undetectable or negative. Those numerical results that were generated were so low that the effect of adjustment was negligible. The additional measurement did not increase the apparent precision of the results. It was suspected that the very slight OD changes that were detected in the AWE control tests represented the natural variation of OD in the sample, rather than any specific response or dilution effect. It was decided that the 'control' aspect of the test added no value and was a waste of resources so it was dropped from the study.

3.7.2 AWE Test ageing platelet concentrate results

The AWE test results for the final four patients were discussed in the test development section of the study (3.4.4) but are shown again here for convenience. See Figures 3.46 and 3.47. There are

only a limited number of results available but they did appear to show deterioration over time so the method was taken forward for further evaluation in the clinical phase of the study.

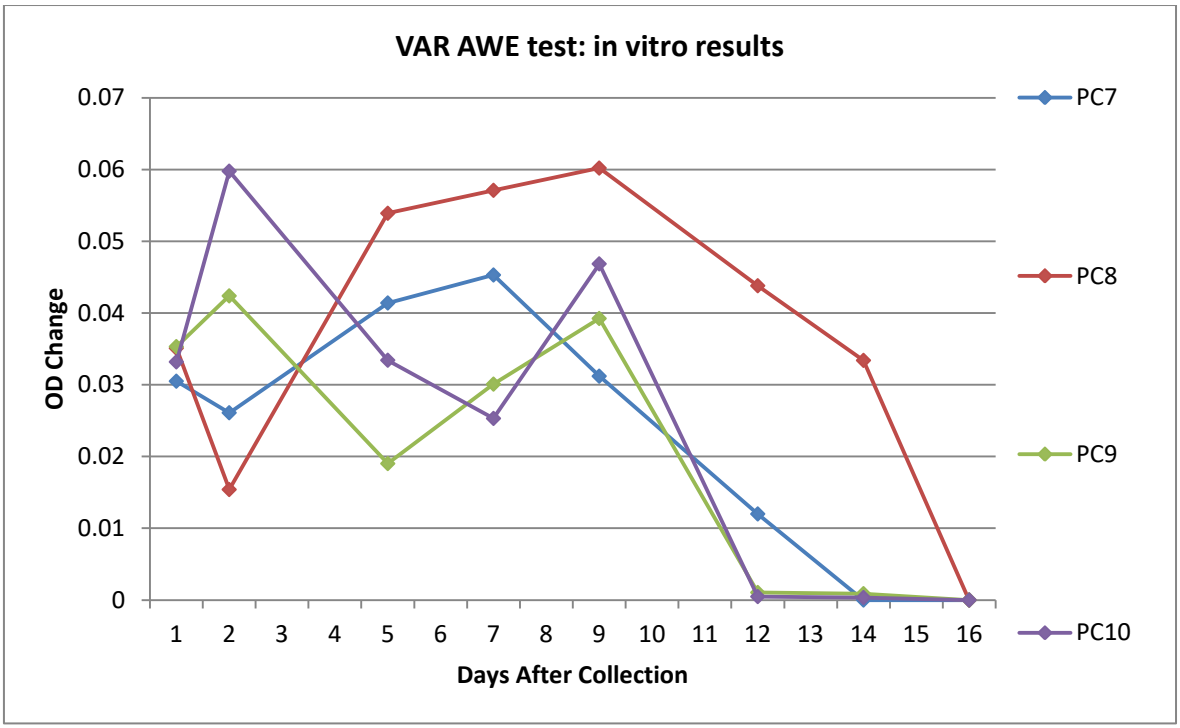

Figure 3.46: *VAR setting AWE Test results for PC7 to PC10*

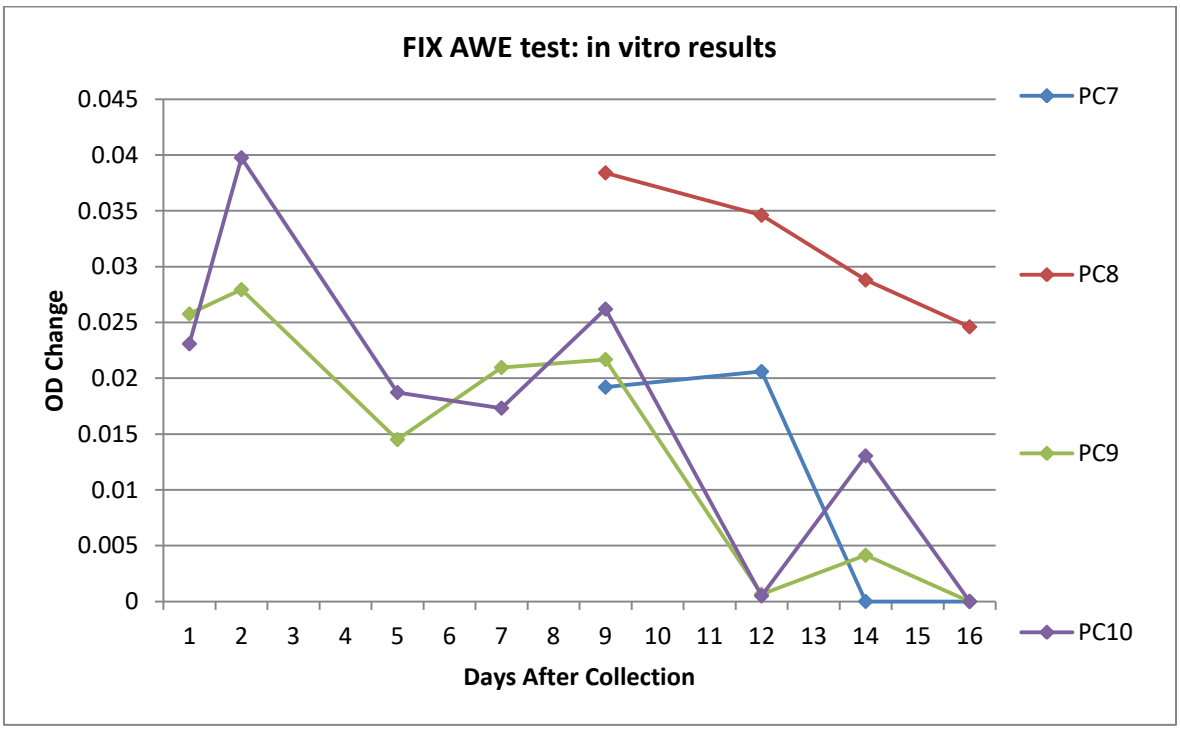

Figure 3.47: *FIX setting AWE Test results for PC7 to PC10*

3.8 *In Vitro* Results Discussion

In this initial stage of the study, significant differences were observed between pooled and apheresis platelet concentrates in terms of platelet count and total platelet dose. Some differences were also found between the way the two groups of concentrates deteriorated with age, which would be consistent with the observations of Paglia *et al* (2014, 2015) that apheresis and pooled platelet concentrates deteriorate in different ways.

The HSR, ESC, pH and swirl control tests were set up and observed to be working satisfactorily. All showed the expected deterioration of results over time for the ten fresh platelet concentrates tested. The ESC and HSR tests both gave abnormal results after the platelet concentrate quality was artificially reduced in the laboratory. Unfortunately, the expertise to use the technically challenging Kunicki scoring method with confidence was not available and attempts to source a suitable image capture system within the budget of the study were not successful. Although disappointing, this test was not felt to be critical to the success of the project due to availability of the alternative control methods listed above.

Three of the fresh platelet concentrates purchased from the NHSBT (PC2, PC3 and PC6) lost their capacity to 'swirl' before the end of day seven and were, presumably, of poor quality after this. The results given for these concentrates by the other reference tests were amongst the lowest observed but did not in any case stand out as obvious outliers. They were evenly spread across the range of results obtained using the ThromboLUX and ROTEM analysers.

The two experimental methods originally identified for investigation, the iZON and the ThromboLUX, both failed to perform as expected. The iZON had not previously been used to analyse platelets or platelet concentrate derived specimens. The results it generated were poorly reproducible and difficult to process. As this method was in the relatively early stages of

development and solutions to these problems were not within the scope of the study, this line of investigation was not pursued.

The ThromboLUX method was at a later stage of development, having undergone initial assessment for use with platelet concentrate samples by its inventor (Maurer-Spurej *et al*, 2006; Maurer-Spurej *et al*, 2007; Maurer-Spurej *et al*, 2009; Xu *et al*, 2010). The method was simple to use and the results easy to interpret. However, the results generated were not as expected and differed from those obtained by the developers in Canada. LightIntegra were confident that only a few minor adjustments were needed to make it ready for clinical application so it was decided to continue assessing the ThromboLUX in the subsequent stages of the study.

Studies were carried out to try and understand why the ThromboLUX results generated for this study in Oxford did not show any deterioration as platelet concentrates aged. This pattern of results had not previously been reported by the development team in Canada. By sharing samples with other beta evaluation sites elsewhere in the UK, in Europe and in Vancouver it was established that the problem affects multiple analysers. The insensitivity was not restricted to platelet concentrates produced in one particular country. This argued against subtle manufacturing differences between concentrates produced in Canada and elsewhere that may also affect the ThromboLUX quality score. It was determined, therefore, that the source of the problem most likely stemmed from technical factors related to the methodology or manufacture of the analyser.

Although the tests performed to compare ThromboLUX results across multiple sites were very useful and enabled progress with development of this test, they could have been improved by wider application of appropriate control testing. In all but one case, no alternative tests for assessing the true quality state of the platelets under investigation were carried out. However, the platelets obtained from Colindale in September 2011 were tested by a variety of other methods including HSR, ESC and ROTEM analysis of platelet G value. Although none of these

methods is established as a reliable indicator of platelet quality in isolation, the consensus of these tests, in contrast to the ThromboLUX, suggested a gradual deterioration in platelet concentrate quality over time. The only other experiment that included testing by alternative methods was that carried out on Vancouver platelet concentrates in October 2011. However, the value of the Kunicki scores in this case is limited by the fact that they were not performed prior to 10 days after collection of the platelet concentrates.

LightIntegra initially advised ThromboLUX users that there was a single, centrally defined 'quality cut off value'. This value was sometimes changed with analyser or software modifications but was still assumed to be the same for all instruments. After the sample exchange experiments performed as part of this study made it clear that quite different results were obtained at different sites, LightIntegra decided that an individual, locally defined cut-off value should be established for each instrument/ location combination.

Although the issues regarding sensitivity to poor quality concentrates was not entirely resolved, the instrument upgrade prior to testing the Brentwood concentrates appeared to have led to an increased frequency of low scores at Oxford and the range of scores observed seemed to have widened. It was hoped that this possible improvement would be carried forward into the ageing platelet concentrates phase of the study but unfortunately this did not turn out to be the case.

Although some concentrates showed marginal deterioration of results over the period of testing, others did not and low scores were not consistently observed following artificial induction of poor quality in the laboratory. Results remained higher than those originally reported by the inventor. Due to numerous and regular changes to the analytical system and the data analysis methods by LightIntegra, it was not possible to obtain enough data generated under one single, consistent set of conditions, to attempt to determine a poor quality 'cut off' score with any confidence.

Initial assessment of the ROTEM method appeared promising. The method was sensitive to inappropriate sample storage and results deteriorated as the platelet concentrates aged. Although the sensitivity of the method to subtle changes in platelet concentrate quality was uncertain, the instrument's ability to detect gross quality impairment induced in the laboratory was encouraging. Due to its ease of use and the existing availability of the analysis equipment in many clinical environments it fulfilled the study criteria for an inexpensive and easily applicable test method especially well. However, the inter-batch variability of the ROTEM may limit its useful application. It would be desirable to source a single batch of each reagent in great enough quantities to carry out any further evaluation.

The AWE test also utilised equipment that would already be available in a number of laboratories and fulfilled the ease of use requirements of the study. Furthermore, as an adaptation of an existing reference method that is known to give results that correlate with clinical efficacy of transfusion, it is likely that the AWE test results would reflect transfusion outcome too. The technical performance of this assay was deemed to be acceptable and it was found to be sensitive to laboratory induced poor quality. The results generally decreased as PRP or platelet concentrate samples underwent storage in the laboratory, eventually reaching levels that indicated complete depletion of platelet activity in many cases. This method would undoubtedly benefit from further technical development, some of which would require greater input from Sysmex, but it appeared to show considerable promise.

3.9 *In Vitro* Results Summary

- Reference tests, including platelet count, MPV, HSR, ESC were set up using out of date platelet concentrates
- Ten fresh platelet concentrates were purchased from the NHSBT and tested over 16 days using the following methods: swirl, pH, HSR, ESC, ROTEM and ThromboLUX.

- Four of the fresh platelet concentrates were also tested over 16 days using the AWE Test - an automated HSR-like method performed on a Sysmex coagulometer
- Swirl was apparent in the fresh platelet concentrates for between four and 15 days
- pH reduced from more than 7 to less than 6.5 for each concentrate over the period of testing
- The pH results were intermediate low for the 37^{o}C and 50^{o}C samples but relatively high for the -40^{o}C sample taken from fresh Platelet Concentrate 1
- HSR reduced from between 35% and 55% to less than 15% for each concentrate over the period of testing. However, some concentrates showed 'spikes' in the pattern of results over time, rather than a consistent deterioration
- ESC reduced from between 18% and 30% to less than 10% for each concentrate over the period of testing. However, some concentrates showed 'spikes' in the pattern of results over time, rather than a consistent deterioration
- The iZON impedance analyser was used to generate platelet distribution curves for out of date platelet concentrates that indicated the presence of increasing numbers of small particles as the concentrates aged but reproducibility was poor
- iZON charge measurements were found to be unsuitable for this study
- The between batch CV of the TL 1 analyser was found to be 11.3%
- The within batch CV of the TL 1 analyser was found to be 8.2, 18.8 and 12.6% on three different occasions
- The ThromboLUX analyser was subsequently replaced with a different instrument for further evaluation (TL 2 2)
- TL 2 gave a between batch CV of 10.1
- Instrument 2 was sensitive to quality deterioration caused by storage at -40^{o}C but not to quality deterioration caused by storage at 37^{o}C or platelet concentrate age
- 25 out of date platelet concentrates tested on TL 1 and TL 2 all gave ThromboLUX scores associated with acceptable quality up to 15 days after collection
- Platelet concentrates from the UK, Holland and Canada were tested at Oxford and at other ThromboLUX evaluation sites in an attempt to determine why all results were high
- The ThromboLUX sensitivity to concentrate age and platelet storage temperature were poor and varied significantly between analysers at different sites
- The ThromboLUX results did not change dramatically over the period of testing for any of the fresh platelet concentrates purchased from the NHSBT

- The ThromboLUX gave low (<14) scores for the Platelet Concentrate 1 samples stored at -40^{o}C and 50^{o}C and a borderline (16.1) score for a sample stored at 37^{o}C
- Platelet G measured by the ROTEM decreased with platelet concentrate age and appeared sensitive to quality deterioration caused by storage at -40^{o}C and 37^{o}C
- For most of the fresh concentrates purchased from the NHSBT, the ROTEM results decreased fairly steadily over time. However, there appeared to be a shift in the level of results between testing of PC3 and testing of PC5 that corresponded to an EXTEM reagent batch change.
- The AWE test was set up on the Sysmex CA5100 coagulometer and validated using PRP and platelet concentrate samples
- AWE test results appeared to drop with advanced platelet concentrate age
- The reproducibility of AWE test results was acceptable when compared to other platelet tests
- The AWE test was sensitive to deterioration of platelet quality induced by inappropriate *in vitro* storage conditions, specifically lack of agitation or oxygen exchange
- There was no obvious relationship between AWE test results and platelet concentrate age in the non-expired residual platelet concentrate samples tested in the clinical study
- There was no appreciable effect of sample settling during the test process over a period of at least 24 minutes
- There was no obvious benefit to running a buffer control with each test using the current test settings
- Although the four fresh platelet concentrates tested using the AWE test showed some 'spikes' in the pattern of results over time, all demonstrated definite deterioration by the end of testing

4 CLINICAL TRIAL

4.1 Clinical Trial Introduction

Full ethical approval was granted in April 2015 for the following study:

Study title:	*The evaluation of novel platelet concentrate quality tests and their ability to predict response to platelet transfusion*
REC reference:	*15/SC/0155*
IRAS project ID:	*131997*

This pilot study aimed to demonstrate a method of investigating the predictive value of the selected *in vitro* quality assessment techniques by comparing the results they generated for individual platelet concentrates to efficacy of transfusion. Both clinical bleeding assessments (taken via self-assessment questionnaire) and platelet count increments (calculated from pre and post-transfusion platelet counts) were used to estimate clinical efficacy. The group invited to participate in the study consisted of MDS patients attending the Haematology Day Unit of OUH Trust. This group was chosen because they represented a relatively stable patient population in terms of their clinical condition.

4.2 Clinical Trial Methods

The following tests were carried out as part of the clinical study:

Participants Pre and Post-transfusion Samples

- Automated platelet count and size measurements

Residual Platelet Concentrate Samples

- Automated platelet count and size measurements
- Swirl assessment
- pH
- ESC
- HSR
- AWE test
- ThromboLUX testing
- ROTEM analysis

For each study participant, pre and post-transfusion platelet counts were carried out on a Sysmex XN10 analyser using both impedance and fluorescent methods. The residual platelet concentrate sample was also tested using the XN10 FBC analyser. The platelet counts obtained for both the patient samples and the platelet concentrate were used to calculate CCI using the standard formula shown below, as recently described by Tobian *et al* (2015).

$$CCI = \frac{(\text{PLT count post} - \text{PLT count pre}) \times 10^{11} \times \text{body surface area } (m^2)}{\text{Number of platelets transfused} \times 10^{11}}$$

The body surface area (BSA) was calculated using the Du Bois formula (Du Bois *et al*, 1916):

$$BSA = 0.007184 \times W^{0.425} \times H^{0.725}$$

All platelet concentrate sample dilutions were prepared using Cryocheck™ pooled normal plasma (Precision BioLogic, Canada). Each platelet concentrate sample was diluted to give a platelet count of 300 x 10^3 / µL ± 10% in order to standardise them and compensate for differences in the test results that were entirely due to variation in platelet concentration within the test sample. Diluted samples were used to perform the ESC, HSR, ROTEM and AWE tests.

4.3 Clinical Trial Progress and Individual Participant Results

A retrospective power calculation using the data produced by Maurer-Spurej *et al* (2009) indicated that 71 transfusion episodes were required to identify a correlation of 0.6 between platelet count increments and the ThromboLUX results with 80% power at a 5% significance level. This was not felt to be achievable with the resources available to this study and the initial recruitment target was transfusion episodes, with the hope that additional staff-time and resources would become available to continue expanding recruitment in the future.

It rapidly became clear that recruitment would be problematic under the original conditions. One of the exclusion criteria for the study was 'receipt of a previous platelet transfusion within the past 7 days'. Many MDS patients attending OUH Trust for platelet support were receiving twice weekly platelet transfusions, making them ineligible for the study. It was felt that this group could still provide relevant data for the study and that the measures of platelet efficacy would not be unduly compromised by any platelets that remained in the circulation from earlier transfusions so an amendment to the study protocol was prepared and submitted to IRAS for ethical approval in October 2015. At this point, approval was granted to change the previous platelet transfusion exclusion criteria to 'receipt of a previous platelet transfusion within the past 48 hours'.

Two patients were recruited to the study at around this time. Participant 001 unfortunately suffered a suspected transfusion reaction, causing his platelet transfusion to be cut short and preventing completion of his transfusion efficacy assessment. It was, however, possible to retrieve the remains of his platelet concentrate and undertake testing on it as originally planned. All tests were carried out according to the study protocol for participant 002.

4.3.1 Transfusion episode 001

Patient 001 was a 79 year old male, attending the haematology day unit for weekly platelet transfusions to treat bleeding, secondary to MDS. His blood group was O+ and the group of the

platelet concentrate he received was A+. The platelet concentrate was collected using apheresis, had a volume of 179mL and was 4 days old at the time of transfusion. He weighed 81kg at the time of his visit and was 187cm tall. The available results for participant 001 are shown in Tables 4.1, 4.2, 4.3, 4.5 and Figure 4.1.

Table 4.1: *Reference method test results for study transfusion episode 001*

FBC	PLT F	PLT I	PDW	MPV	P-LCR
Pre Tx	17	17	13.4	11.4	34.6
Post Tx	----------No Result Available----------				
PLT count increase	----------No Result Available----------				
PC	1649	1290	8.4	8.8	14.7
Swirl	Pos				
pH	7.74				
ESC (%)	**ESC**	**ESC 2**	**ESC Mean**		
	12.5	No Result Available			
HSR (%)	**HSR**	**HSR 2**	**HSR Mean**		
	30.1	35.3	32.7		

It was not possible to determine the increase in platelet count for participant 001 as no post-transfusion platelet count was performed and it was not possible to determine, exactly, what volume of his platelet concentrate was transfusion before the transfusion was stopped.

Table 4.2: *AWE test results for study transfusion episode 001*

Test Settings	Test 1	Test 2	Mean
Neat VAR	0.1323	0.1484	0.1404
Neat FIX	0.0755	0.0951	0.0853
Neat VSB	0.1176	0.1186	0.1181
Neat FSB	0.0738	0.0693	0.0716
Diluted VAR	0.0353	0.0264	0.0309
Diluted FIX	0.0202	0.0233	0.0218
Diluted VSB	0.0261	0.0211	0.0236
Diluted FSB	0.0150	0.0164	0.0157

The results of tests that used stir-bars (neat VSB, neat FSB, diluted VSB and diluted FSB) were slightly lower than the results of tests that did not use stir-bars (neat VAR, neat FIX, diluted VAR,

diluted FIX). The results of tests carried out on diluted samples (diluted VAR, diluted FIX, diluted VSB and diluted FSB) are all lower that the results of tests carried out on neat samples (neat VAR, neat FIX, neat VSB and neat FSB). The results of tests carried out using the 80 second fixed time measurements (neat FIX, neat FSB, diluted FIX and diluted FSB) were all lower that the tests carried out using the 15 second variable time measurement settings (neat VAR, neat VSB, diluted VAR and diluted VSB).

Table 4.3: *ROTEM results for study transfusion episode 001*

ROTEM	EXTEM G	FIBTEM G	EXTEM-FIBTEM G	G/PLT
Diluted:	6340	2074	4266	15

Participant 001's ROTEM tests were only carried out on a diluted sample. The results fell within the expected range for non-expired (<7 days old) concentrates as determined by observation of aging platelet concentrates during the previous *in vitro* stage of the study. See Table 4.4.

Table 4.4: *Minimum and maximum ROTEM results obtained for non-expired (<7 day old) platelet concentrates during in vitro stages of the study*

Max EXTEM	18,415	**Max FIBTEM**	3107	**Max E-F G**	16,344	**Max G/PLT**	55
Min EXTEM	3,817	**Min FIBTEM**	1,398	**Min E-F G**	2,405	**Min G/PLT**	10

The results obtained from the ThromboLUX instrument for participant 001 are shown in Table 4.5 and in graphical form in Figure 4.1. When the score calculation was applied to the duplicate test runs, both gave the same score of 18.9. However, as the graphs show, the particle size distributions obtained at the three test temperatures varied somewhat between runs. The proportion of light intensity from the MV fraction over the average distribution was 18%.

Table 4.5 *Duplicate and mean ThromboLUX results for study transfusion episode 001*

TL (1)	TL (2)	TL Mean	MV (1) %	MV (2) %	MV Mean	Beads (1)	Beads (2)	Beads Mean
18.9	18.9	18.9	19	16	17.5	4.8	No Result	4.8

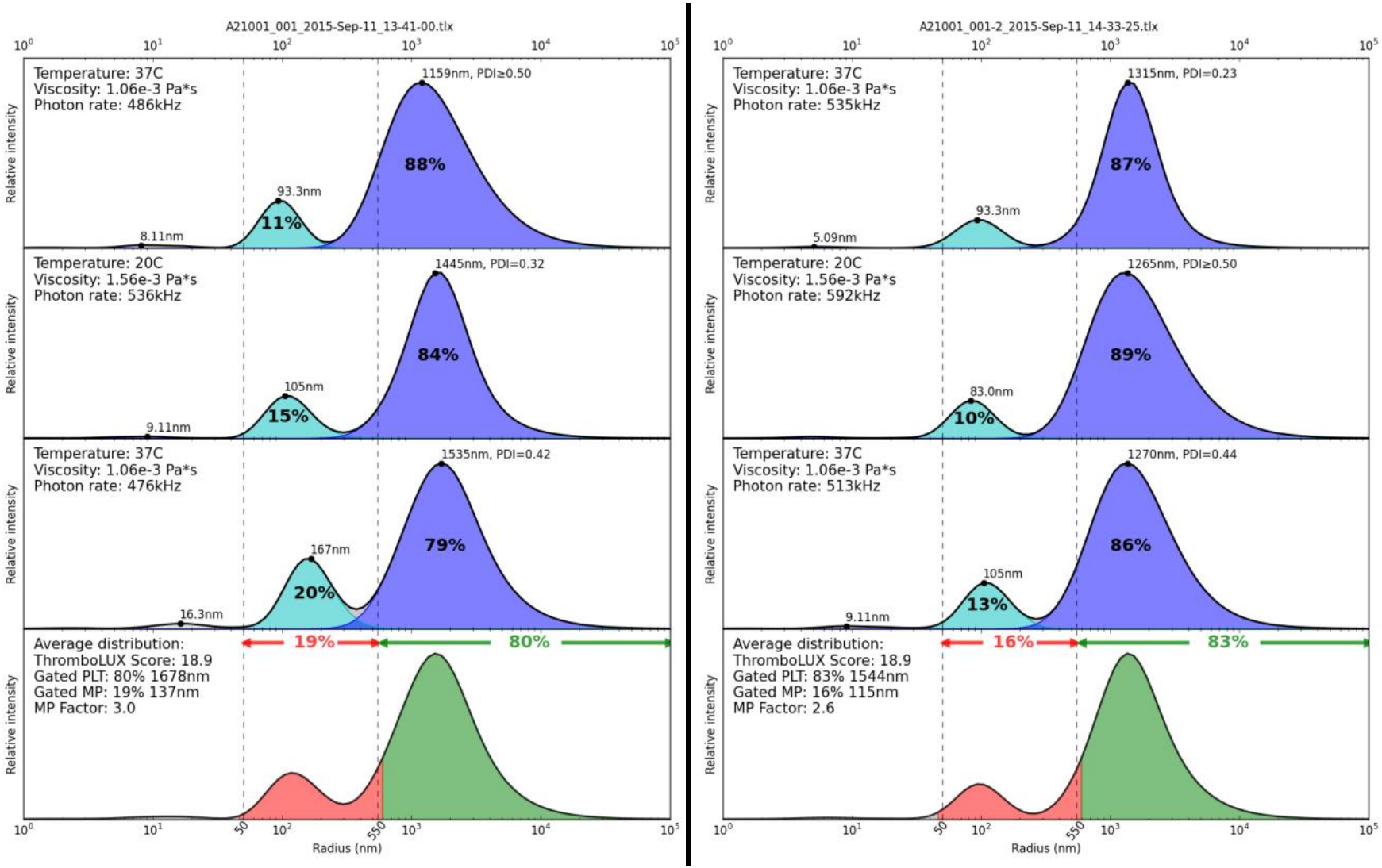

Figure 4.1: *Duplicate ThromboLUX results for study transfusion episode 001. Platelet and MV size distributions are shown as measured first at 37°C (top), then at 20°C (upper middle) and finally at 37°C (lower middle). The average distribution is shown at the bottom.*

4.3.2 Transfusion episode 002

Patient 002 was a 69 year old female, attending the haematology day unit for bi-weekly platelet transfusions to treat bleeding, secondary to MDS. Her blood group was O+ and the group of the platelet concentrate she received was O+. The platelet concentrate was collected using apheresis, had a volume of 197mL and was 4 days old at the time of transfusion. She weighed 79kg at the time of her visit and was 157cm tall. Participant 002's results are shown in Tables 4.6, 4.7, 4.8, 4.9 and Figure 4.2.

Table 4.6: *Reference method test results for study transfusion episode 002*

FBC	PLT F	PLT I	PDW	MPV	P-LCR
Pre Tx	3	2	----------No Result Available----------		
Post Tx	27	26	11.3	11.7	36.5
PLT count increase	24	24			
PC	1578	1377	10	10.1	24.2
Swirl	Pos				
pH	7.40				
ESC (%)	**ESC**	**ESC 2**	**ESC Mean**		
	13.0	14.3	13.65		
HSR (%)	**HSR**	**HSR 2**	**HSR Mean**		
	10.6	16.6	13.6		

Table 4.7: *AWE test results for study transfusion episode 002*

Test Settings	1	2	Mean
Neat VAR	0.1256	0.1419	0.1337
Neat FIX	0.0929	0.0873	0.0901
Neat VSB	0.0997	0.1043	0.1020
Neat FSB	0.0715	0.0748	0.0731
Diluted VAR	0.0080	0.0118	0.0099
Diluted FIX	0.0145	0.0154	0.0150
Diluted VSB	0.0047	0.0034	0.0041
Diluted FSB	No Result Available	No Result Available	No Result Available

Again, the stir-bar test results were lower than the non-stir-bar results and the diluted test results were lower than the neat test results. On this occasion however, the diluted FIX result was higher than the diluted VAR result and no diluted FSB result was available.

Table 4.8: *ROTEM results for study transfusion episode 002*

ROTEM	EXTEM G	FIBTEM G	EXTEM-FIBTEM G	G/PLT
Neat:	18262	7358	10904	7
Diluted:	6383	2835	3548	13

As expected, the ROTEM results obtained from the neat sample were considerably higher than those obtained from the diluted sample. However, the G/PLT result was lower. Participant 002's diluted sample results fell within the expected range.

The ThromboLUX scores obtained for concentrate 002 were 15.9 and 17.5. The duplicate runs appear more consistent in terms of size distribution patterns than those obtained for participant 001. The proportion of light intensity from the MV fraction over the average distribution was 7%.

Table 4.9: *Duplicate and mean ThromboLUX results for study transfusion episode 002*

TL (1)	TL (2)	TL Mean	MV (1) %	MV (2) %	MV Mean	Beads (1)	Beads (2)	Beads Mean
15.9	17.5	16.7	6	7	6.5	3.6	No Result	3.6

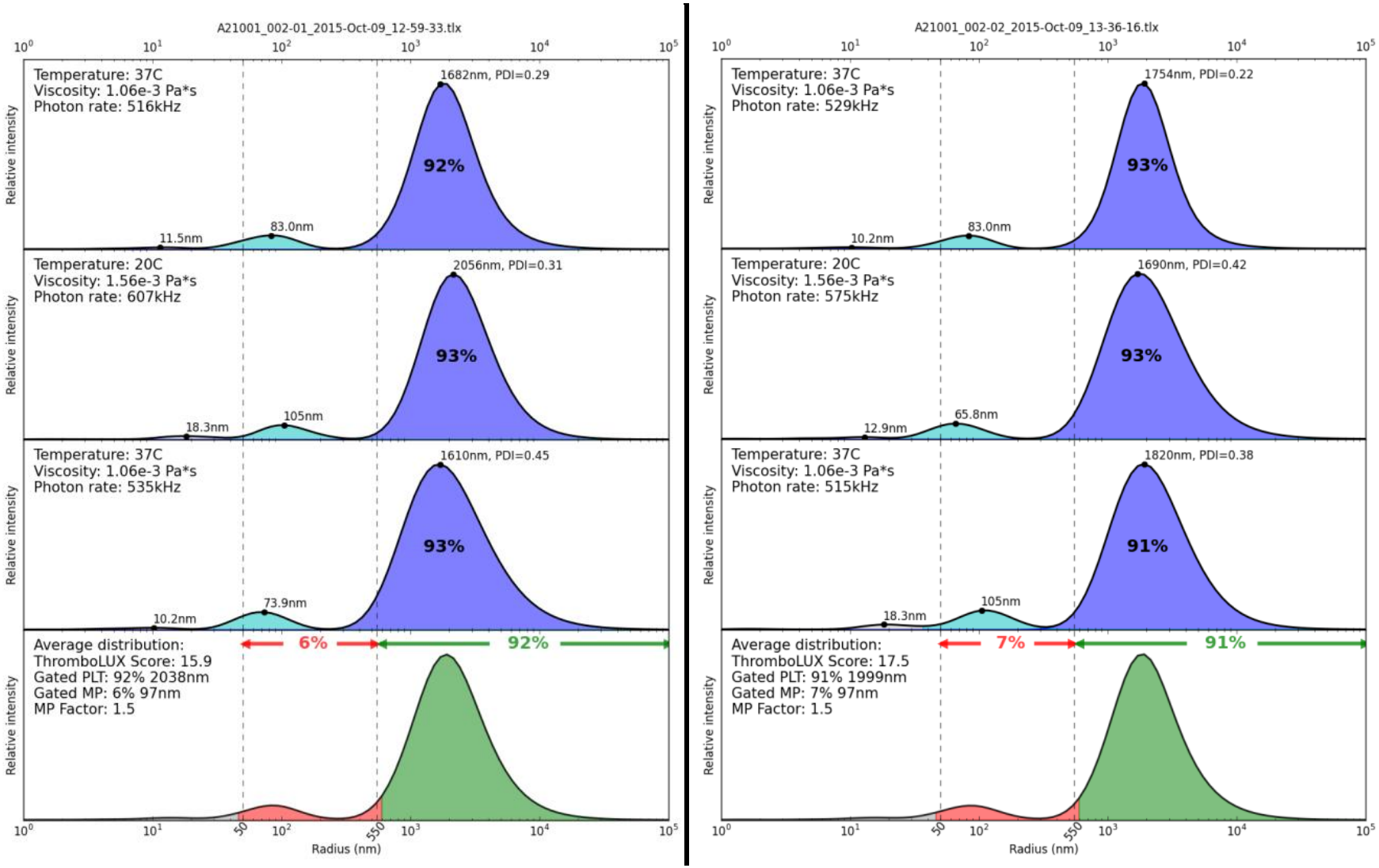

Figure 4.2: *Duplicate ThromboLUX results for study transfusion episode 002. Platelet and MV size distributions are shown as measured first at 37°C (top), then at 20°C (upper middle) and finally at 37°C (lower middle). The average distribution is shown at the bottom.*

Unfortunately, the study began to experience further recruitment issues after this. The clinical contact assisting with identification of potential participants left work temporarily on paternity leave and was then unavailable over the Christmas period. After this, his own research commitments meant that he was not available to search for potential participants for several weeks and there was some delay in communicating this information. A new clinical contact was eventually established and recruitment began again with the enrolment of participant 003 in the spring of 2016 (see below). An additional patient was also approached but declined to be included in the study at this point.

4.3.3 Transfusion episode 003

Participant 003 was a 91 year old male, attending the haematology day unit for bi-weekly platelet transfusions to treat bleeding, secondary to MDS. His blood group was A+ and the group of the platelet concentrate he received was A+. The platelet concentrate was prepared from pooled platelets in additive solution and plasma, had a volume of 305mL and was 7 days old at the time of transfusion. He weighed 72kg at the time of his visit and was 173cm tall. His results are shown in Tables 4.10, 4.11, 4.12, 4.13 and Figures 4.3 and 4.4.

Table 4.10: *Reference method test results for study transfusion episode 003*

FBC	**PLT F**	**PLT I**	**PDW**	**MPV**	**P-LCR**
Pre Tx	2	6	----------No Result Available----------		
Post Tx	39	65	----------No Result Available----------		
PLT count increase	37	59			
PC	1093	845	No Result Available	9.6	No Result Available
Swirl	Pos				
pH	7.24				
ESC (%)	**ESC**	**ESC 2**	**ESC Mean**		
	13	14.3	13.65		
HSR (%)	**HSR**	**HSR 2**	**HSR Mean**		
	10.6	16.7	13.7		

Table 4.11: *AWE test results for study transfusion episode 003*

Test Settings	1	2	Mean
Neat VAR	0.0962	0.1188	0.1075
Neat FIX	0.0639	0.0575	0.0607
Neat VSB	0.0802	0.0859	0.0831
Neat FSB	0.0476	0.0529	0.0503
Diluted VAR	0.0194	0.0238	0.0216
Diluted FIX	0.0177	0.0176	0.0177
Diluted VSB	0.01174	0.01558	0.0137
Diluted FSB	0.0097	0.00871	0.0092

The pattern of AWE test results obtained for the different test settings for participant 003 matches that of participant 001.

Table 4.12: *ROTEM results for study transfusion episode 003*

ROTEM	EXTEM G	FIBTEM G	EXTEM-FIBTEM G	G/PLT
Neat:	2279	539	1740	2
Diluted:	3170	1365	1805	6

Participant 003's neat sample ROTEM results were unexpectedly lower than the diluted sample results. In addition, the EXTEM response graph for the neat samples showed clot retraction, as evidenced by the sudden decrease in observed MCF (see Figure 4.3). Clot retraction can be distinguished from fibrinolysis, which would result in a more gradual tailing off of MCF (Bontekoe *et al*, 2014). The diluted sample results were lower than any observed for non-expired concentrates during the *in vitro* stages of the study.

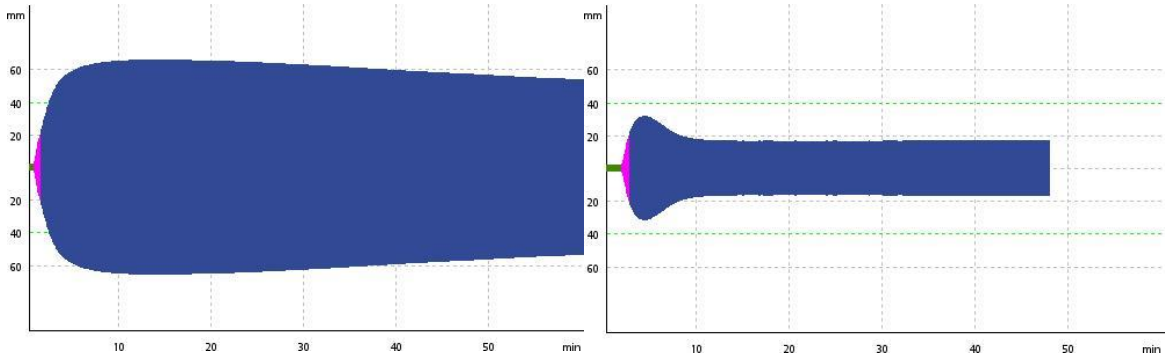

Figure 4.3: *Normal EXTEM response graph (left) and EXTEM response graph for participant 003 showing clot retraction (right)*

The scores generated from them were 19.1 and 13.2. Again, the particle size distribution graphs differ considerably in appearance between the two runs. The proportion of light intensity from the MV fraction over the average distribution was 7%.

Table 4.13: *Duplicate and mean ThromboLUX results for study transfusion episode 003*

TL (1)	TL (2)	TL Mean	MV (1) %	MV (2) %	MV Mean	Beads (1)	Beads (2)	Beads Mean
19.1	13.2	16.2	8	5	6.5	4.9	5.8	5.4

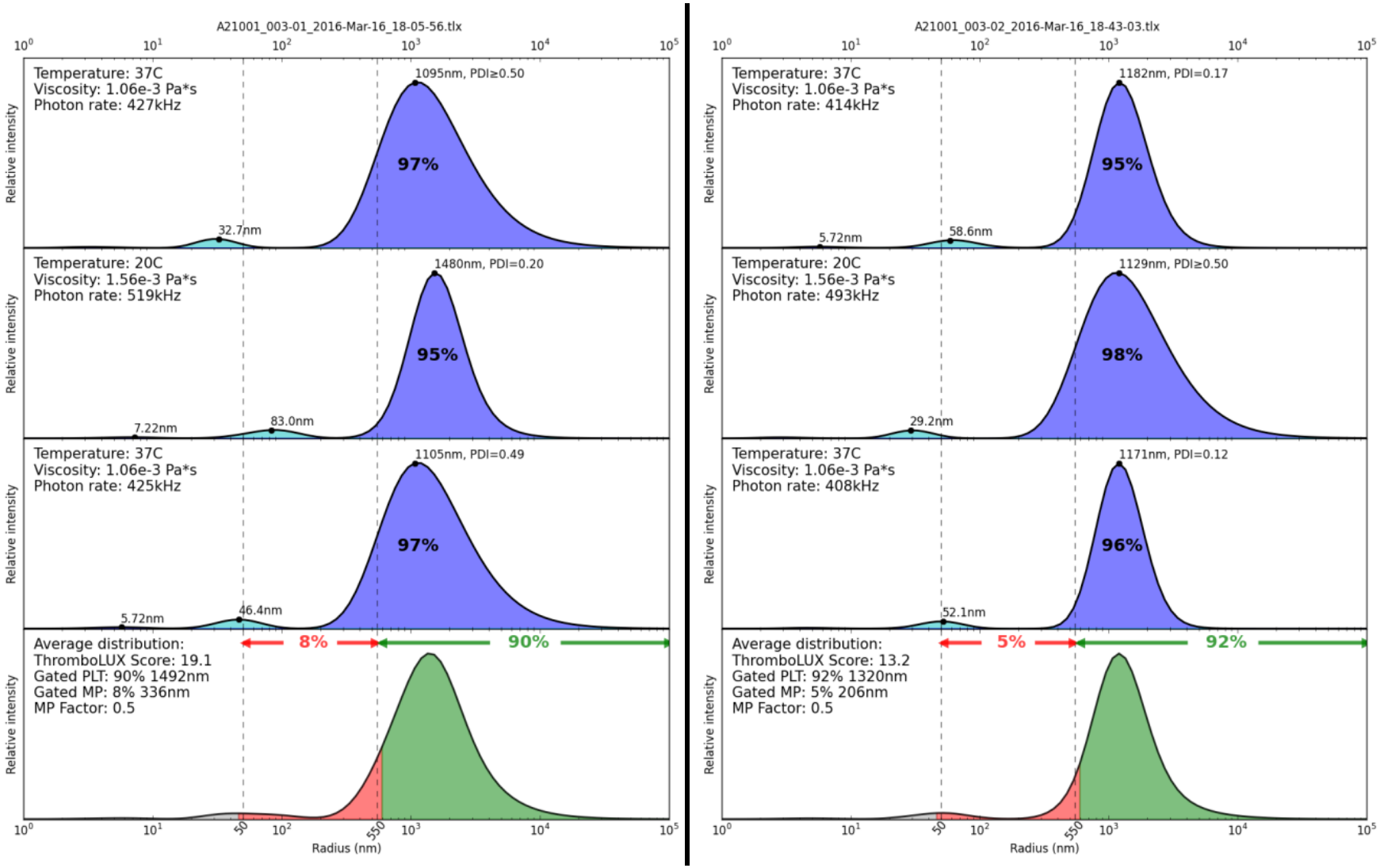

Figure 4.4: *Duplicate ThromboLUX results for study transfusion episode 003. Platelet and MV size distributions are shown as measured first at 37°C (top), then at 20°C (upper middle) and finally at 37°C (lower middle). The average distribution is shown at the bottom.*

4.3.4 Transfusion episode 004

Patient 004 was a 68 year old female, receiving weekly platelet concentrate transfusions as part of her MDS therapy at her local district general hospital. On the day that she participated in this study she had come to the referral centre at OUH NHS Trust for her regular weekly platelet transfusion prior to bone marrow biopsy. Her blood group was O-, as was the group of the platelet concentrate she received. The platelet concentrate was collected using apheresis, had a volume of 207mL and was 5 days old at the time of transfusion. She weighed 66kg at the time of her visit and was 161cm tall. Her results are shown in Tables 4.14, 4.15, 4.16, 4.17 and Figure 4.5.

Table 4.14: *Reference method test results for study transfusion episode 004*

FBC	**PLT F**	**PLT I**	**PDW**	**MPV**	**P-LCR**
Pre Tx	16	18	----------No Result Available----------		
Post Tx	53	57	10.0	10.1	24.7
PLT count increase		39			
PC	1471	1113	7.6	8.6	13
Swirl	Pos				
pH	7.54				
ESC (%)	**ESC**	**ESC 2**	**ESC Mean**		
	13.0	14.9	14.1		
HSR (%)	**HSR**	**HSR 2**	**HSR Mean**		
	24.1	34.7	29.4		

Table 4.15: *AWE test results for study transfusion episode 004*

Test Settings	**1**	**2**	**Mean**
Neat VAR	0.2013	0.242	0.2217
Neat FIX	0.0795	0.0988	0.0892
Neat VSB	0.1868	0.1859	0.1864
Neat FSB	0.0821	0.0836	0.0829
Diluted VAR	0.0348	0.0272	0.0310
Diluted FIX	0.0181	0.0197	0.0189
Diluted VSB	0.02064	0.022	0.0213
Diluted FSB	0.0159	0.0143	0.0151

Again, the pattern of AWE test results obtained for the different test settings for participant 004 matches that of participant 001.

Table 4.16: *ROTEM results for study transfusion episode 004*

ROTEM	EXTEM G	FIBTEM G	EXTEM-FIBTEM G	G/PLT
Neat:	28203	2932	25271	17
Diluted:	10314	1783	8531	28

Participant 004's neat sample ROTEM results were higher than the diluted sample results. All of the diluted sample results were within the expected range for non-expired platelet concentrates. The results obtained for platelet concentrate 004 are, however, considerably increased compared to those observed for concentrates 001 – 003.

Again, there is some variation between the duplicate particle size distributions measured by the ThromboLUX analyser. The calculated scores for the two tests were 20.0 and 16.8. The proportion of light intensity from the MV fraction over the average distribution was 17%.

Table 4.17: *Duplicate and mean ThromboLUX test results for study transfusion episode 004*

TL (1)	TL (2)	TL Mean	MV (1) %	MV (2) %	MV Mean	Beads (1)	Beads (2)	Beads Mean
20.0	16.8	18.4	14	19	16.5	4.5	4.2	4.4

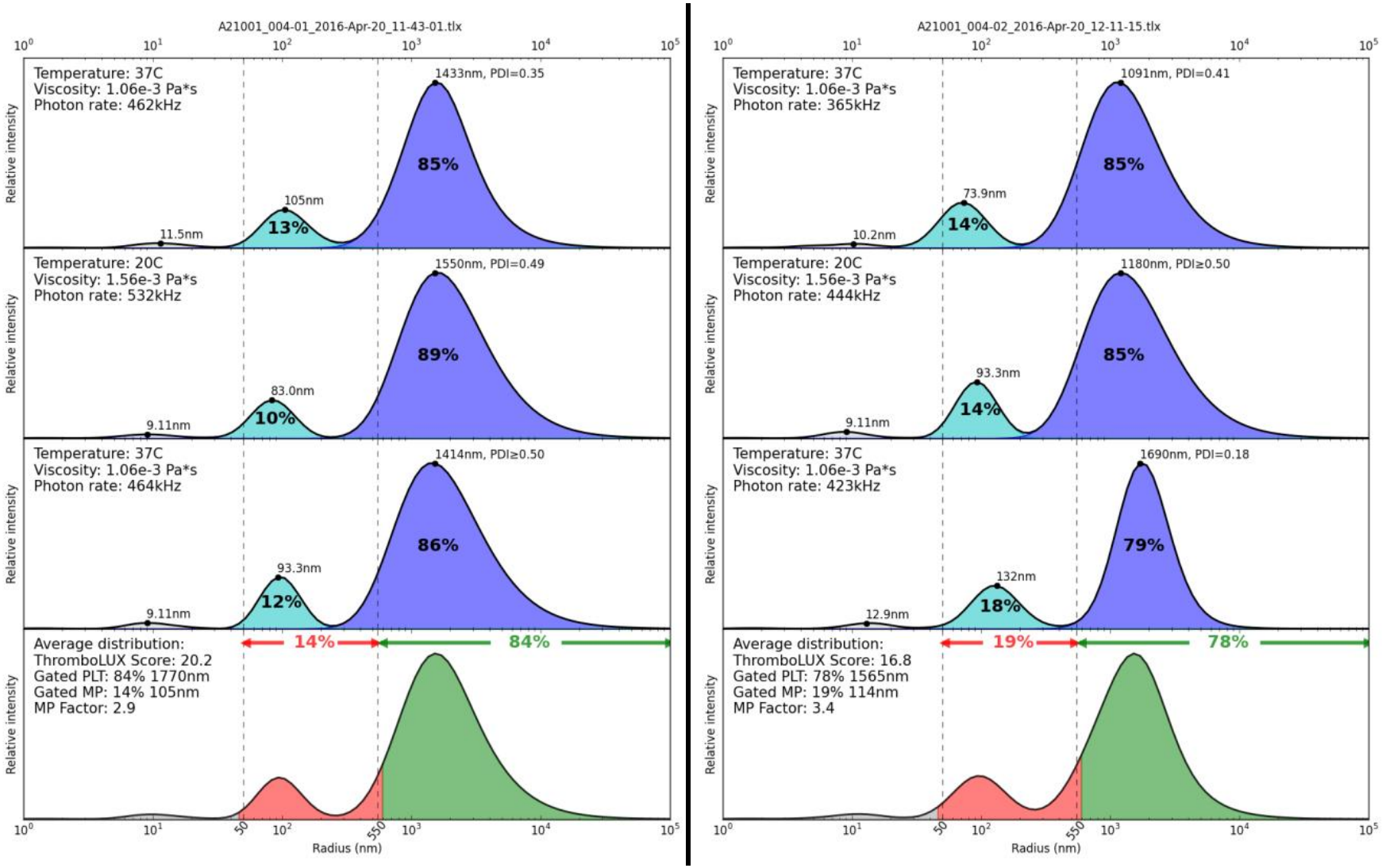

Figure 4.5: *Duplicate ThromboLUX results for study transfusion episode 004. Platelet and MV size distributions are shown as measured first at 37°C (top), then at 20°C (upper middle) and finally at 37°C (lower middle). The average distribution is shown at the bottom.*

Participant 004 was the first study patient to return her bleeding questionnaires. During the time she was filling out her self-assessment bleeding questionnaires, she was hospitalised for a high temperature at one of the nearby district general hospitals. Prior to receiving the platelet transfusion that was included in the study, participant 004 noted a small amount of blood on the tissue after blowing her nose but had no other bleeding symptoms. She had no bleeding symptoms whatsoever for the two days following her transfusion, suggesting a good response to the platelets she received. For the three days following that, she noted increasing amounts of blood upon brushing her teeth and blowing her nose before receiving a further platelet transfusion at the end of the third day. On the next and final day of assessment she still experienced a little bleeding after brushing her teeth and blowing her nose but it was less extensive than the previous day.

4.3.5 Transfusion episode 005

Platelet concentrates 005 and 006 were both transfused to the same individual who had also previously participated in the trial as participant 003. His clinical condition and weight had not changed notably since his previous participation in the study. The platelet concentrate transfused during study episode 005 was prepared from pooled platelets in additive solution and plasma, had a volume of 288mL and was four days old at the time of transfusion. The concentrate and the recipient were both blood group A+. His results are shown in Tables 4.18, 4.19, 4.20, 4.21 and Figure 4.6.

Table 4.18: *Reference method test results for study transfusion episode 005*

FBC	PLT F	PLT I	PDW	MPV	P-LCR
Pre Tx	7	7	----------No Result Available----------		
Post Tx	40	42	13.4	12.2	40.6
PLT count increase	33	35			
PC	906	813	11.3	10.5	28.1
Swirl	Pos				
pH	7.54				
ESC	**ESC**	**ESC 2**	**ESC Mean**		
	5.2	5.7	5.45		
HSR	**HSR**	**Ratio**	**HSR 2**	**Ratio 2**	**HSR Mean**
	25.5	1.7	31.3	1.6	28.4

Table 4.19: *AWE results for study transfusion episode 005*

Test Settings	1	2	Mean
Neat VAR	0.0631	No Result Available	0.0631
Neat FIX	0.0466	No Result Available	0.0466
Neat VSB	0.0561	No Result Available	0.0561
Neat FSB	0.0342	No Result Available	0.0342
Diluted VAR	0.02	0.0202	0.0201
Diluted FIX	0.0168	0.0145	0.0157
Diluted VSB	0.0102	0.0041	0.0072
Diluted FSB	0.00901	0.0084	0.0087

As with the previous AWE tests, the stir-bar results were lower than the non stir-bar results, the diluted sample results were lower than the neat sample results and the fixed end-point results were lower than the variable end-point results.

Table 4.20: *ROTEM results for study transfusion episode 005*

ROTEM	EXTEM G	FIBTEM G	EXTEM-FIBTEM G	G/PLT
Neat:	----------No Result Available----------			
Diluted:	8196	1565	6631	24

There was not enough of the residual platelet concentrate to perform neat ROTEM analysis on platelet concentrate 005. The results obtained for the diluted sample were at a similar level to those observed for concentrate 004 and, again, showed a notable increase from the earlier concentrates included in the study. The proportion of light intensity from the MV fraction over the average distribution was 22%.

Table 4.21: *Duplicate and mean ThromboLUX results for study transfusion episode 005*

TL (1)	TL (2)	TL Mean	MV (1) %	MV (2) %	MV Mean	Beads (1)	Beads (2)	Beads Mean
11.7	8.2	10.0	19	24	21.5	4.0	4.2	4.1

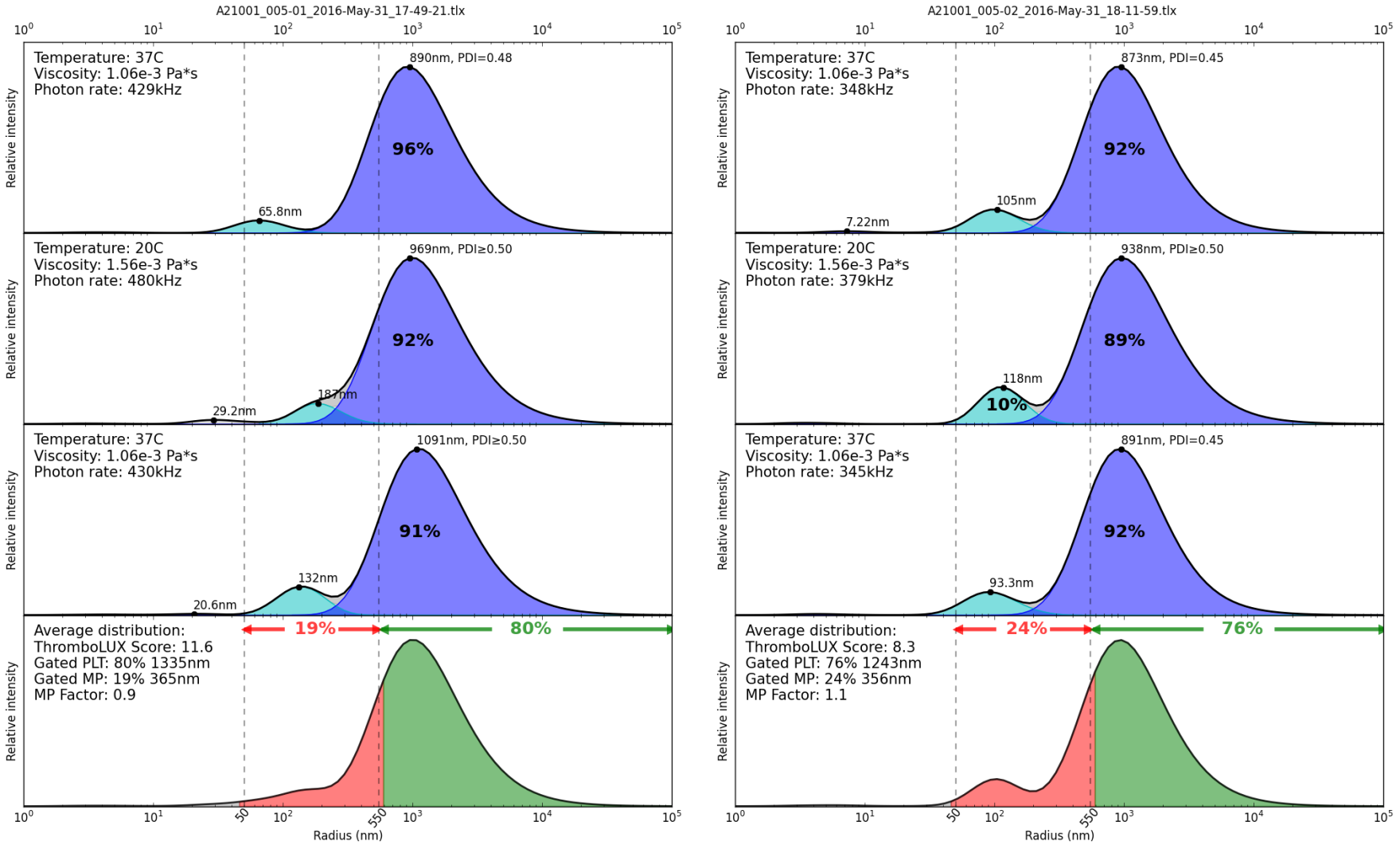

Figure 4.6: *Duplicate ThromboLUX results for study transfusion episode 005. Platelet and MV size distributions are shown as measured first at 37°C (top), then at 20°C (upper middle) and finally at 37°C (lower middle). The average distribution is shown at the bottom.*

The particle size distributions are similar for the two test runs. The calculated ThromboLUX scores were 11.7 and 8.2. These were the lowest scores observed in the study and fall below any of the previously defined cut-off values for 'poor quality' concentrates.

Participant 005 returned a full set of completed self-assessment bleeding questionnaires after this transfusion episode. He reported 'bruised, swollen or painful joints or muscles', 'red / purple spots (known as petechiae / purpura)' and 'one or two bruises, each measuring 2-10cm' on the day he was recruited to the study and these symptoms persisted throughout the monitoring period despite administration of the platelet transfusion. Two days after the transfusion he reported blurred vision, which then persisted until monitoring stopped on day five. On day two the petechiae / purpura also became more widespread before gradually receding to their previous level by day five. The patient noted a new appearance of oral blood blisters on day five, which were not bleeding or painful at that time.

4.3.6 Transfusion episode 006

The results obtained for the final transfusion episode included in the study (006) are shown below in Tables 4.22, 4.23, 4.24, 4.25 and Figure 4.7. Concentrate 006 was obtained using apheresis and had a volume of 234mL. It was four days old at the time of transfusion and was ABO /RhD identical to the recipient (A+).

Table 4.22: *Reference method test results for study transfusion episode 006*

FBC	**PLT F**	**PLT I**	**PDW**	**MPV**	**P-LCR**
Pre Tx	4	4	----------No Result Available----------		
Post Tx	43	45	9.5	10.5	26.3
PLT count increase	39	41			
PC	1355	1074	7.9	8.6	12
Swirl	Pos				
pH	7.6				
ESC	**ESC**	**ESC 2**	**ESC Mean**		
	13.9	14.1	14.0		
HSR	**HSR**	**Ratio**	**HSR 2**	**Ratio 2**	**HSR Mean**
	27.3	1.7	26.5	2	26.9

Table 4.23: *AWE results for study transfusion episode 006*

Test Settings	1	2	Mean
Neat VAR	0.1526	0.1341	0.1434
Neat FIX	0.0699	0.0721	0.0710
Neat VSB	0.1082	0.1125	0.1104
Neat FSB	0.0737	0.0735	0.0736
Diluted VAR	0.0255	0.0429	0.0342
Diluted FIX	0.0257	0.0289	0.0273
Diluted VSB	0.0225	0.02077	0.0216
Diluted FSB	0.01317	0.01338	0.0133

Again, the relative pattern of AWE test results obtained using the different test settings was the same as for the majority of other concentrates.

Table 4.24: *ROTEM results for study transfusion episode 006*

ROTEM	EXTEM G	FIBTEM G	EXTEM-FIBTEM G	G/PLT
Neat:	28614	2905	25709	85
Diluted:	9524	1618	7906	26

The pattern of ROTEM results is as expected, with the neat results substantially higher than the diluted results and the G/PLT result for the diluted sample at around the same level as the results for concentrates 004 and 005. The neat G/PLT result is, however, considerably higher than that obtained for concentrate 004.

Table 4.25: *Duplicate and mean ThromboLUX results for study transfusion episode 006*

TL (1)	TL (2)	TL Mean	MV (1) %	MV (2) %	MV Mean	Beads (1)	Beads (2)	Beads Mean
24.0	21.6	22.8	5	4	4.5	5.3	3.8	4.6

Again, there is very little difference between the particle size distributions obtained for the two test runs. The calculated ThromboLUX scores were 24.0 and 21.6. The proportion of light intensity from the MV fraction over the average distribution on this occasion was 5%.

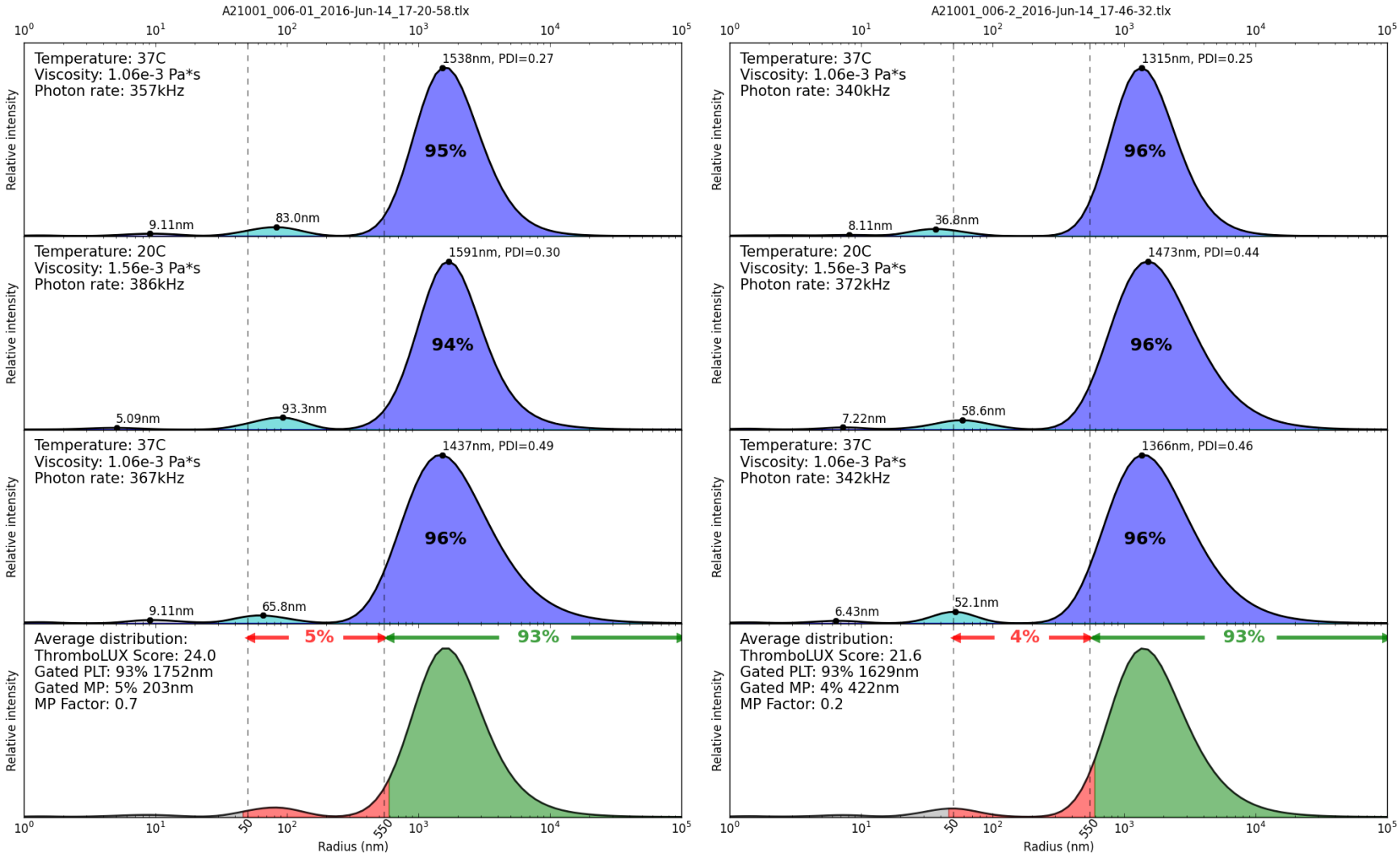

Figure 4.7: *Duplicate ThromboLUX results for study transfusion episode 006. Platelet and MV size distributions are shown as measured first at 37°C (top), then at 20°C (upper middle) and finally at 37°C (lower middle). The average distribution is shown at the bottom.*

A full set of completed self-assessment bleeding score questionnaires were returned by the participant after study transfusion episode 006. As during his previous participation in the study, 'bruised, swollen or painful joints or muscles', 'red / purple spots (known as petechiae / purpura)' and 'one or two bruises, each measuring 2-10cm' were consistent clinical features. On this occasion, the petechiae / purpura were present at a consistent, intermediate severity throughout the course of the study but there were no instances of blurred vision or oral blood blisters.

4.4 Clinical Trial Results for Each Test

The clinical trial results for each of the reference and novel test methods are presented below.

4.4.1 Clinical trial swirl, pH and platelet count results

All six platelet concentrates tested were swirl positive and had pH results that suggested acceptable quality. The differences between the fluorescent, optical and impedance counts (wherever available) appear negligible for all but one of the low platelet counts obtained from the patient samples (see below) but are more marked at the high levels observed in the platelet concentrate samples. On each occasion the platelet concentrate fluorescent count was considerably higher than the impedance count.

It was not possible to determine the increase in platelet count for participant 001 because his involvement in the study was terminated early due to his suspected transfusion reaction. Fluorescent platelet count increases of 24, 37, 37, 35 and 39 x 10^9/L were observed for participants 002, 003, 004, 005 and 006 respectively. Their impedance platelet counts showed increases of 24, 63, 39, 33, 41 x 10^9/L.

4.4.2 Clinical trial CCI results

The overall platelet content of each concentrate was calculated using both the fluorescent platelet count and the impedance platelet count. The total platelet content of each unit, as assessed using the fluorescent count, was 2.95, 3.11, 3.33, 3.04, 2.61 and 3.17 x 10^{11} platelets per concentrate for concentrates 001, 002, 003, 004, 005 and 006 respectively. The equivalent figures calculated using the impedance count were 2.31, 2.71, 2.58, 2.30, 2.34 and 2.51 x 10^{11} platelets per concentrate. See Table 4.26.

Participant 002 had a BSA of 1.798, participant 003 had a BSA of 1.855 and participant 004 had a BSA of 1.697. Participants 005 and 006 were, in fact, the same individual as participant 003 and his weight did not change significantly during the course of the study. The BSA of participants 005 and 006 was, therefore, also 1.855. CCI was calculated using both the fluorescent platelet count results (fCCI) and the impedance platelet count results (iCCI) for each participant. The results are shown in Table 4.26. In each case the impedance CCI was higher than the fluorescent CCI. All CCI results indicated an acceptable response to transfusion (Slichter *et al*, 2005).

Table 4.26: *BSA, Total Platelet Concentrate Platelet Content and CCI values for all participants*

Participant Number	BSA	Total impedance content	Total fluorescence content	Impedance CCI	Fluorescent CCI
001	2.063	2.31 x 10^11	2.95 x 10^11	-	-
002	1.798	2.71 x 10^11	3.11 x 10^11	15,923	13,875
003	1.855	2.58 x 10^11	3.33 x 10^11	42,420	20,611
004	1.697	2.30 x 10^11	3.04 x 10^11	28,775	20,654
005	1.855	2.34 x 10^11	2.61 x 10^11	26,160	24,875
006	1.855	2.51 x 10^11	3.17 x 10^11	30,301	22,822

The iCCI value calculated for participant 003 was more than double that of the fCCI and was considerably higher than all of the other iCCI results observed in the study. This participant was the only one with marked differences between his pre and post-transfusion impedance and fluorescent platelet count results. The impedance platelet count increased from 6 to 65 x 10^9/L

and the fluorescent platelet count increased from 2 to 39 x 10^9/L. This was thought to be the likely cause of his unusual pattern of CCI results. Both the pre and post-transfusion platelet counts performed for participant 003 generated abnormal platelet distribution errors by the impedance method and defaulted to report the fluorescent count. It is possible that small platelet clumps, red cell fragments or microcytes were present in the sample and were counted as platelets during the impedance test, falsely raising the impedance platelet count. When the increment obtained using fluorescent measurements was used to calculate the CCI with the impedance PC platelet count a value of 26,603 was reported, which is much more in line with the iCCI results generated for the other trial episodes.

Participant 003's pre and post-transfusion fluorescent platelet counts were unaffected by the analytical error observed for his pre and post-transfusion impedance platelet counts. These low level impedance counts have been observed to be very similar to the fluorescent counts in other pre and post-transfusion samples, with the main difference between iCCI and fCCI values for the other participants being a consequence of the more divergent impedance and fluorescent counts obtained from the platelet concentrates. This makes it likely that the modified CCI result prepared for participant 003 still represents the 'true' iCCI result that would have been calculated had there been no analytical issue affecting his pre and post-transfusion platelet counts.

4.4.3 Clinical trial ESC results

ESC was tested in duplicate for all samples except concentrate 001. Results of between 12.5% and 14.9% were obtained, with the exception of concentrate 005, which gave results of 5.2% and 5.7%. All duplicate results matched up well, with a difference of <2% in each case and none of the aggregometry traces showed any evidence of technical anomalies. Holme *et al* (1998) quote a reference range of 15-25% for five day old platelets stored under optimal conditions. The discrepancy between this figure and the scores recorded is thought to reflect the less than optimal storage conditions of the concentrate prior to, during and after the transfusion. The

result obtained for concentrate 005 shows clear separation from the other results and may indicate a quality difference with this pack. There is no obvious correlation between the ESC results and the CCI values.

4.4.4 Clinical trial HSR results

The mean HSR results for concentrates 001, 004, 005 and 006 (32.7%, 29.4%, 28.4% and 26.9% respectively) are considerably higher than the results for concentrates 002 and 003 (13.6% and 13.7% respectively) (see Figure 4.8). In all cases, the results are lower than the reference range of 40-80% for five day old platelets stored under optimal conditions quoted by Holme *et al* (1998). Again, this likely reflects non-optimal storage for the duration of the transfusion. The higher results obtained for participant 001 may be due to the fact that participant 001's transfusion was stopped prematurely due to a suspected transfusion reaction. The greater volume of platelet concentrate remaining in the bag and the shorter duration between cessation of agitation and laboratory testing may have contributed to the more 'normal' HSR result obtained.

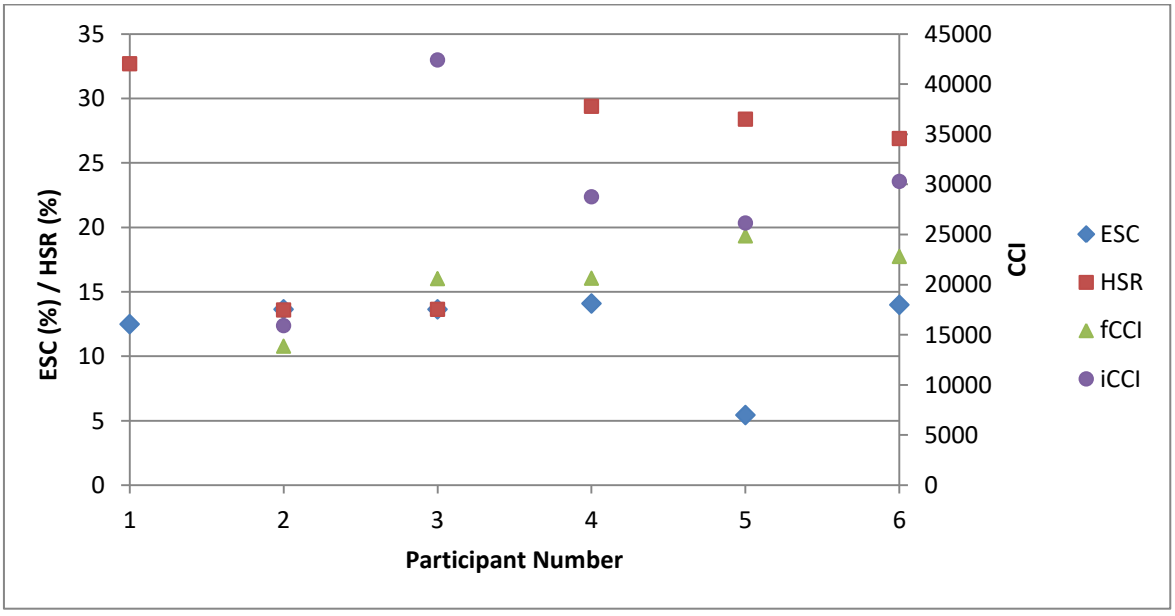

Figure 4.8: *ESC and HSR results for all six platelet concentrates shown alongside the fluorescent and impedance CCI values for each participant*

There was no correlation between the ESC and HSR results (R^2 = 0.0882). Furthermore, the HSR results did not show any clear relationship with the fCCI values (R^2 = 0.5098). However, if the iCCI result for participant 003 (previously identified as being suspect) is excluded, there may be some correlation between the HSR and iCCI results for the remaining participants (see Figure 4.9).

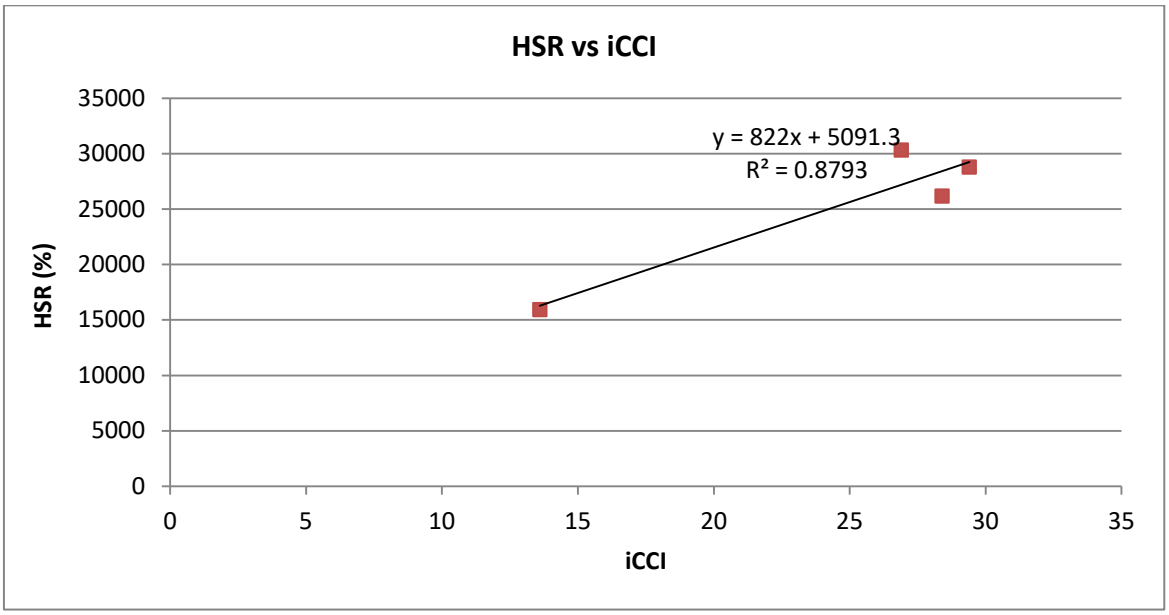

Figure 6.9: *Correlation between the HSR and iCCI results for participants 002, 004, 005 and 006.*

4.4.5 Clinical trial ThromboLUX results

All platelet concentrate samples were tested in duplicate using the ThromboLUX analyser. Results were highly reproducible for the first two concentrates tested at the end of 2015 but became less reproducible in the later stages of the clinical study. There was no change in sample collection, storage practice, test performance technique or analytical environment. It is unclear whether the observed difference is due to coincidence or whether there is some instability inherent to the ThromboLUX system. Although calibration and adjustment of laser alignment is carried out upon installation, routine maintenance is not recommended by LightIntegra. Calibration beads continued to indicate acceptable analytical performance throughout the study. At first glance, there did not appear to be any relationship between the ThromboLUX results and the CCI results (see Figure 4.10).

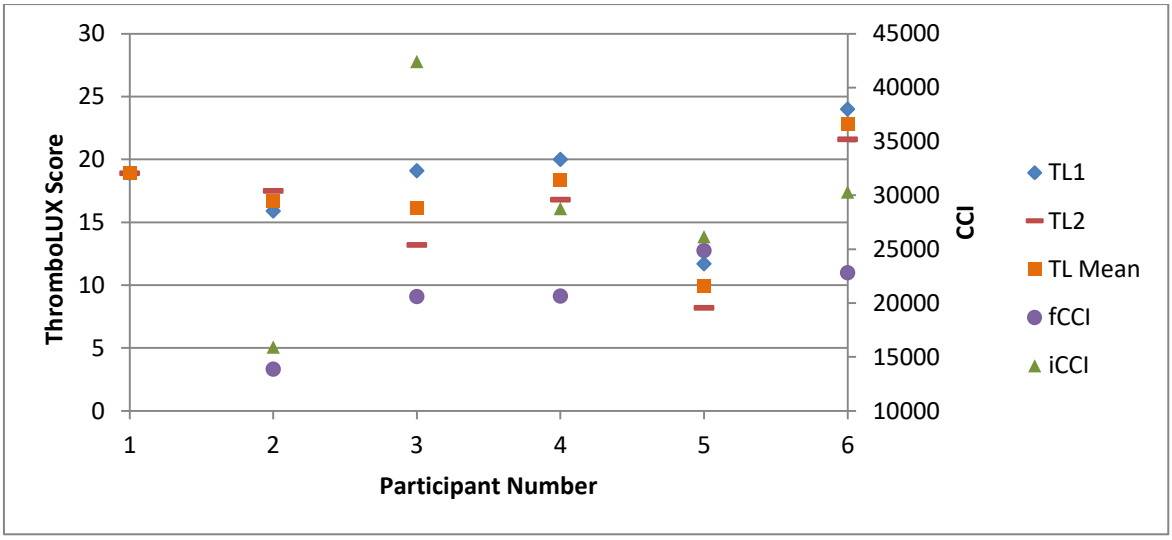

Figure 4.10: *Duplicate and mean ThromboLUX results and CCI values for each participant*

However, if the second ThromboLUX result (and the mean result) is disregarded and the recalculated CCI result as discussed in the CCI results section above is used, the two measurements do seem to reveal an association (see Figure 4.11). As previously noted, the impedance CCI calculated for participant 003 was likely to be spurious, due to inaccuracies in the pre and post-transfusion impedance platelet counts. In addition, this data point was subjectively identified as an outlier on all graphs prepared to study correlation between the ThromboLUX results and CCI.

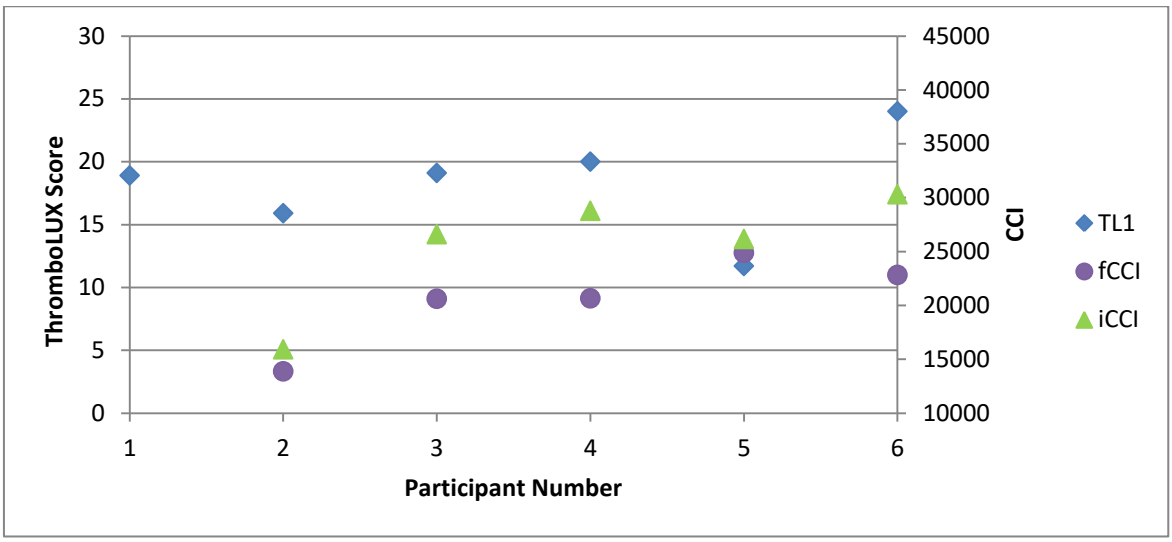

Figure 4.11: *First measurement ThromboLUX results (TL1) and CCI values for each participant*

The concentrate 005 results were clear outliers in the correlation graphs prepared for the ThromboLUX and CCI results. See Figure 4.12.

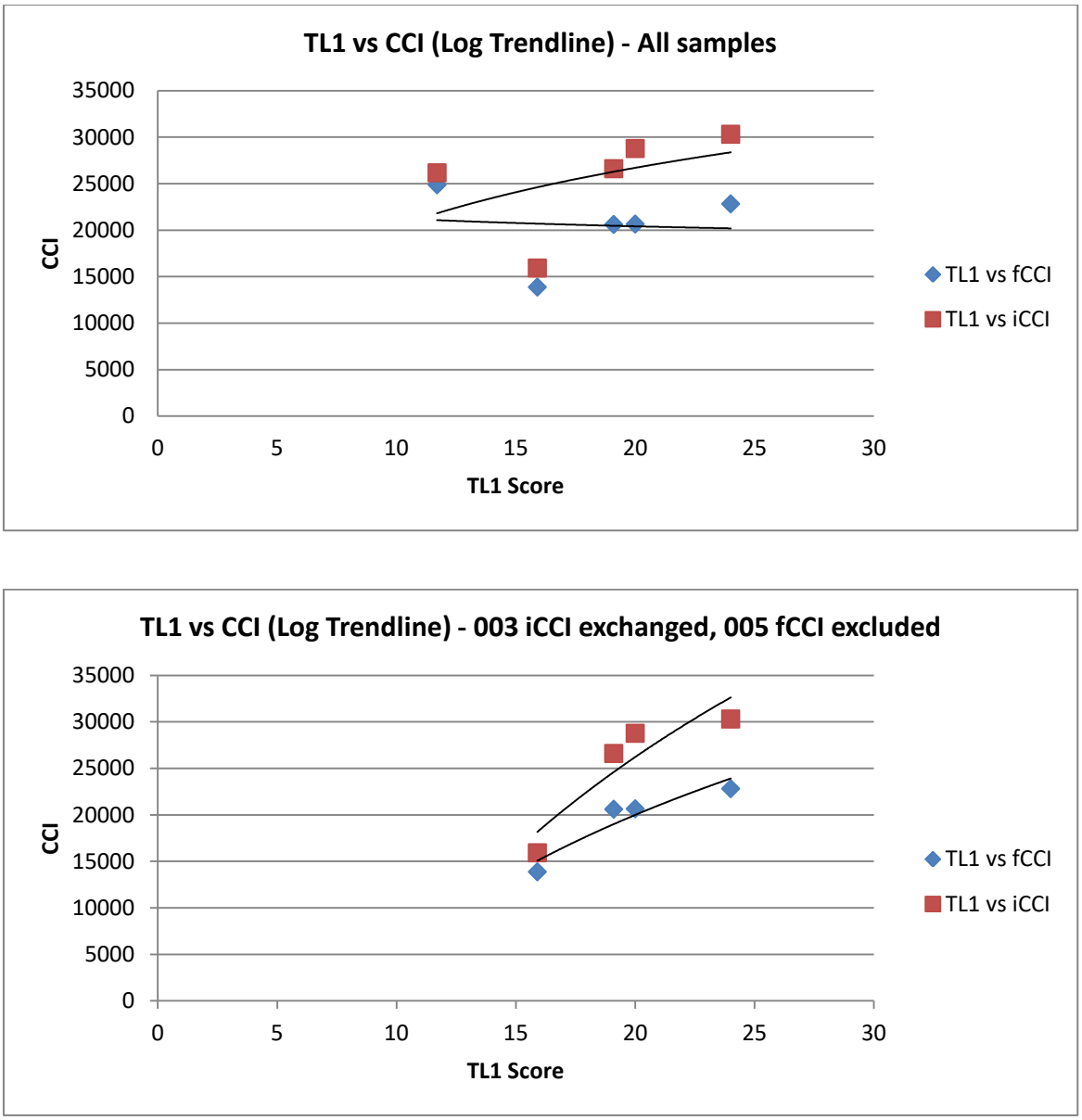

Figure 4.12: *TL1 vs. CCI results for all concentrates (top) and using the recalculated iCCI result for concentrate 003 and with concentrate 005 excluded (bottom)*

The ThromboLUX result for concentrate 005 was below any previously defined quality 'cut-off' values. In addition, this concentrate gave a low ESC result and did not appear to produce any significant clinical benefit according to the clinical bleeding assessment. This transfusion episode did not give low CCI values but the impedance and fluorescent results did have a non-typical

relationship. All other iCCI results were markedly higher than the fCCI results obtained for the same concentrate. The iCCI and fCCI results obtained for platelet concentrate 005 showed relatively little separation, suggesting the possibility that either the fluorescent platelet count result for concentrate 005 was suppressed or the impedance platelet count result was increased. There is no indication in the platelet count results of any errors or anomalies so it is thought to be most likely that this outlying result was caused by factors related to the platelet concentrate condition. Correlation between CCI and ThromboLUX scores improved dramatically if the result for concentrate 005 was excluded. See Table 4.27.

Table 4.27: *Correlation (R^2 values) between TL1 results and calculated CCI for each platelet concentrate. Impedance CCI (iCCI) and fluorescent CCI (fCCI) results are shown for the entire dataset (the dubious result for concentrate 003 has been exchanged) and for each dataset with the concentrate 005 results excluded. All correlations of >0.80 are shown in bold. Logarithmic trend lines were used.*

CCI	Correlation
fCCI	0.0066
fCCI (Outlier 005 Excluded)	**0.8758**
iCCI (Dubious 003 Result Exchanged)	0.1939
iCCI (Dubious 003 Result Exchanged, Outlier 005 Excluded)	**0.8351**

It was not possible to determine a definite 'quality cut-off score' with the latest version of the analyser used in this study, which was modified shortly before the clinical trial began. All platelet concentrates, except for concentrate 005, gave average scores of more than 16 – a level higher than any of the 'cut-off' values employed in previous phases of the study. Platelet concentrate 005 had a considerably lower ThromboLUX score than all the other concentrates tested (average 9.95). This result would have been below any of the previously defined 'cut-off' values associated with this test method.

During this phase of the study the relative light intensity of the MV fraction was made routinely available in the ThromboSight software. The results obtained for the six clinical trial samples fell into two neatly disparate groups. Three samples gave results of between 5 and 7 and the other three samples gave results ranging from 17 to 22. These MV intensity results did not show any sign of correlation with any of the other tests performed. However, it could potentially be of note that the three patients who received the higher MV concentrates were all qualitatively linked by their clinical features. Participant 001's transfusion was halted due to a suspected transfusion reaction, participant 004 was hospitalised due to fever in the days following her transfusion and participant 005 experienced very little clinical benefit from his platelet transfusion.

4.4.6 Clinical trial ROTEM results

When considered as a whole, the ROTEM results do not show a clear relationship with either fluorescent or impedance CCI. See Figure 4.13.

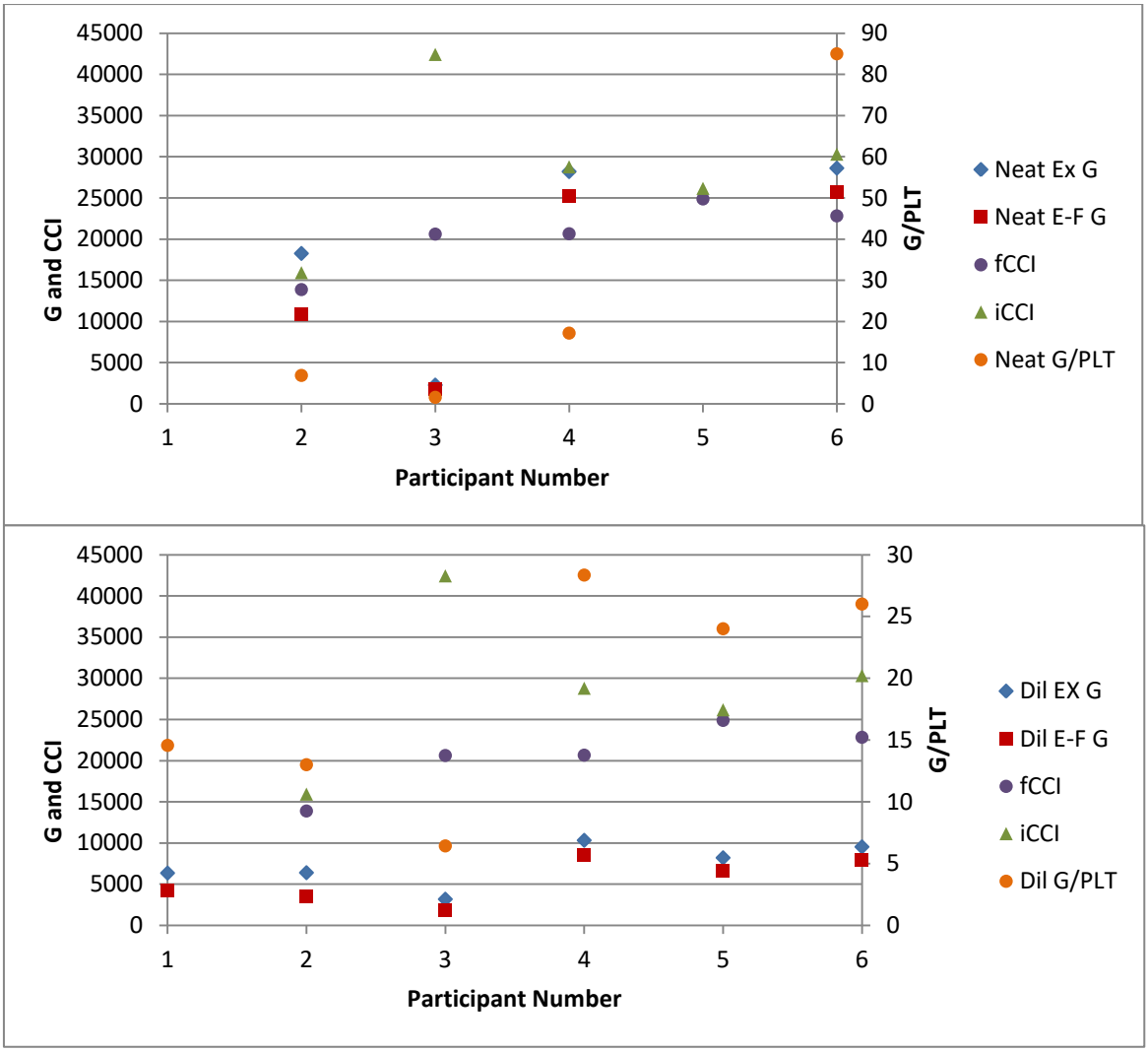

Figure 4.13: *EXTEM, EXTEM – FIBTEM and G per platelet results obtained from neat (top) and diluted (bottom) samples shown alongside fluorescent and impedance CCI values*

All results were considerably lower for concentrate 003 than the other concentrates and than expected. As concentrate 003 had given abnormal result graphs and unexpectedly lower results for the neat sample than for the diluted one, a sample anomaly was suspected. Upon exclusion of concentrate 003, the remaining results show better correlation with the CCI (see Figure 4.14).

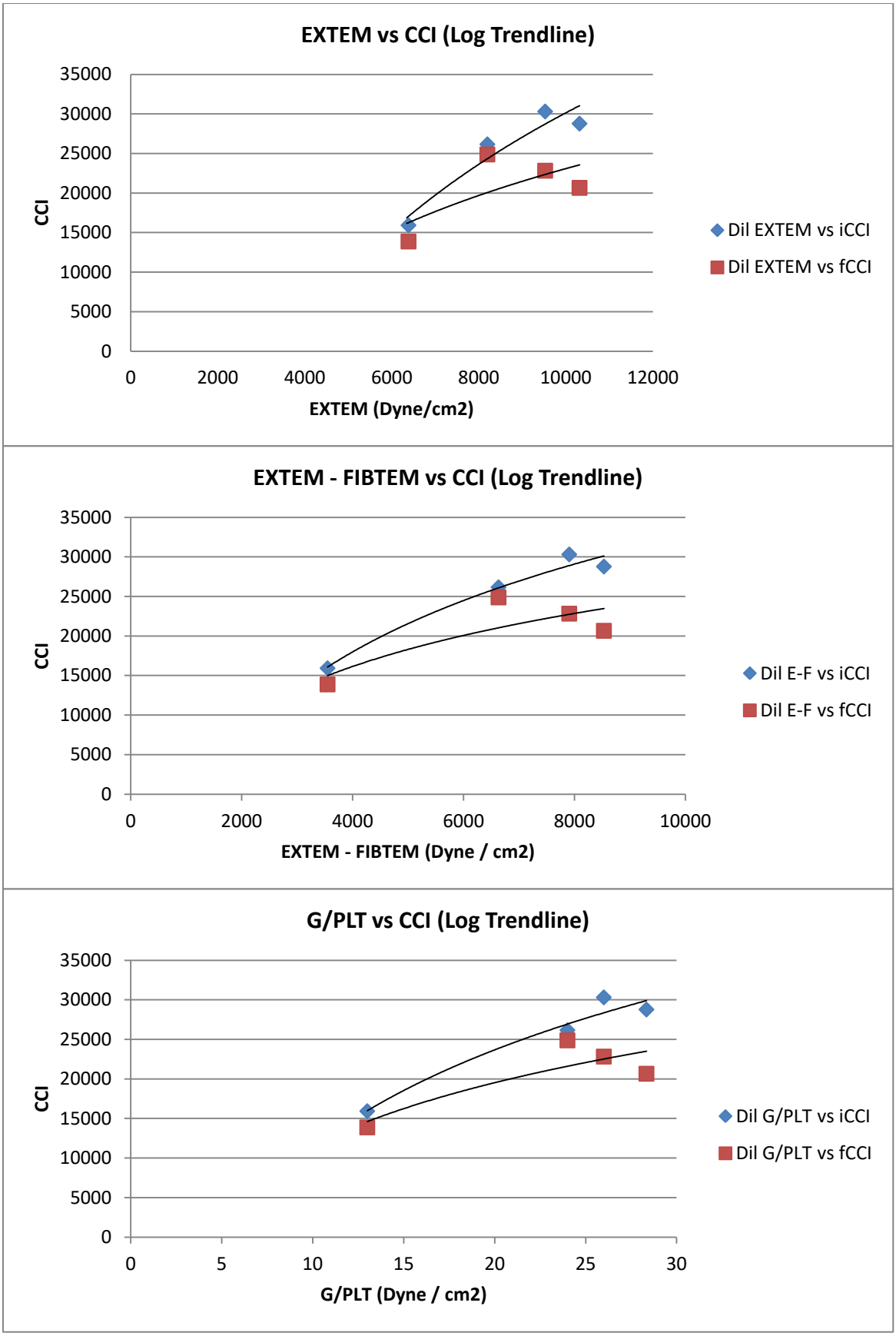

Figure 4.14: *Correlation graphs for diluted EXTEM, EXTEM – FIBTEM and G per platelet vs. CCI*

Good correlation figures were obtained for the neat samples once concentrate 003 was excluded (see Table 4.28). However, these datasets only include three study episodes each because there was not sufficient sample to run a neat test for concentrate 005.

Table 4.28: *Correlation (R^2 values) between EXTEM, EXTEM – FIBTEM and G/PLT results and calculated CCI for each platelet concentrate. Fluorescent CCI (fCCI) results are shown for the entire dataset and with the result for concentrate 003 excluded. Impedance CCI results are shown with the dubious result for concentrate 003 exchanged and with the result for concentrate 003 excluded. All correlations of >0.80 are shown in bold. Logarithmic trend lines were used.*

CCI	NEAT			DILUTED		
	EXTEM	**E - F**	**G/PLT**	**EXTEM**	**E - F**	**G/PLT**
fCCI	0.0006	0.0146	0.1604	0.0639	0.1747	0.1665
fCCI (Concentrate 003 Excluded)	**0.9581**	**0.9537**	**0.8138**	0.4519	0.6503	0.716
iCCI (Dubious 003 result exchanged)	0.0025	0.0385	0.1758	0.0713	0.1808	0.1473
iCCI (Concentrate 003 excluded)	**0.9953**	**0.9936**	0.6966	**0.9026**	**0.9696**	**0.955**

Unfortunately, it was necessary to change lots of EXTEM reagent during the study. Recruitment was not fast enough to complete the study before the original lot expired and a new lot was used for study episode 004 onwards. A shift in results is apparent in the data (see Figure 4.13)

4.4.7 Clinical trial AWE test results

The average AWE test results for all six concentrates are shown in Figures 4.15 (neat samples) and 4.16 (diluted samples). Every concentrate was tested in duplicate using each of four test settings for the neat samples and the same four test settings for the diluted samples. For the neat samples, the results for concentrates 003 and 005 are reduced compared to those obtained for the other concentrates because these were pooled platelet concentrates, with lower platelet concentrations than all of the other (apheresis) concentrates included in the study.

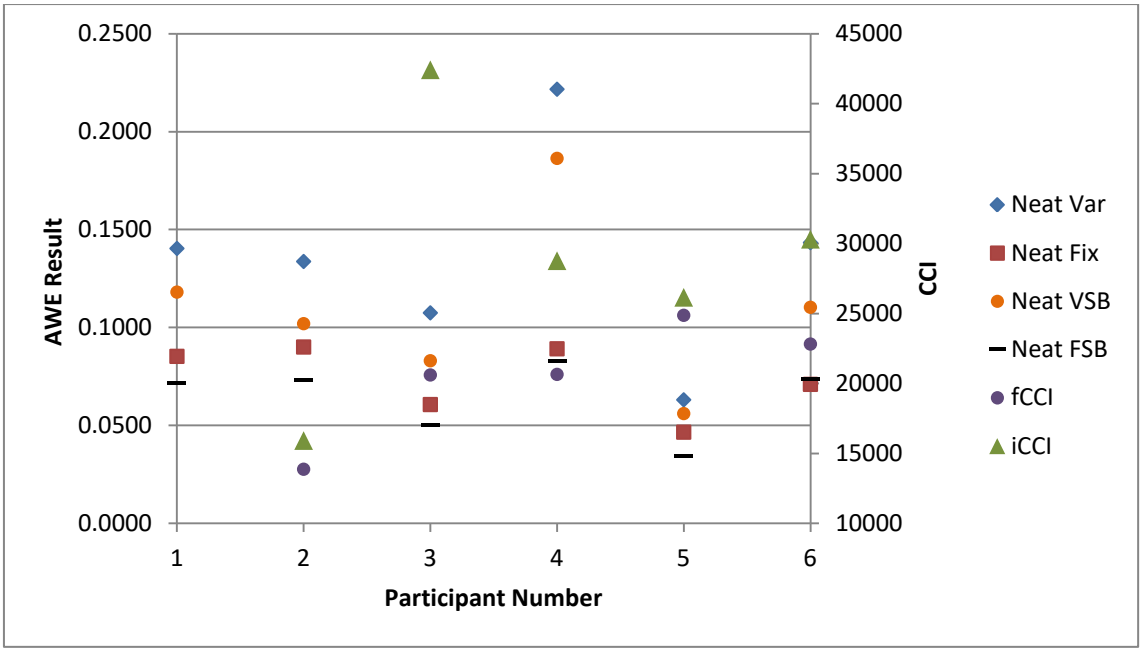

Figure 4.15: *AWE Test results obtained from neat samples using a variety of test settings and shown alongside the fluorescent and impedance CCI values for each participant*

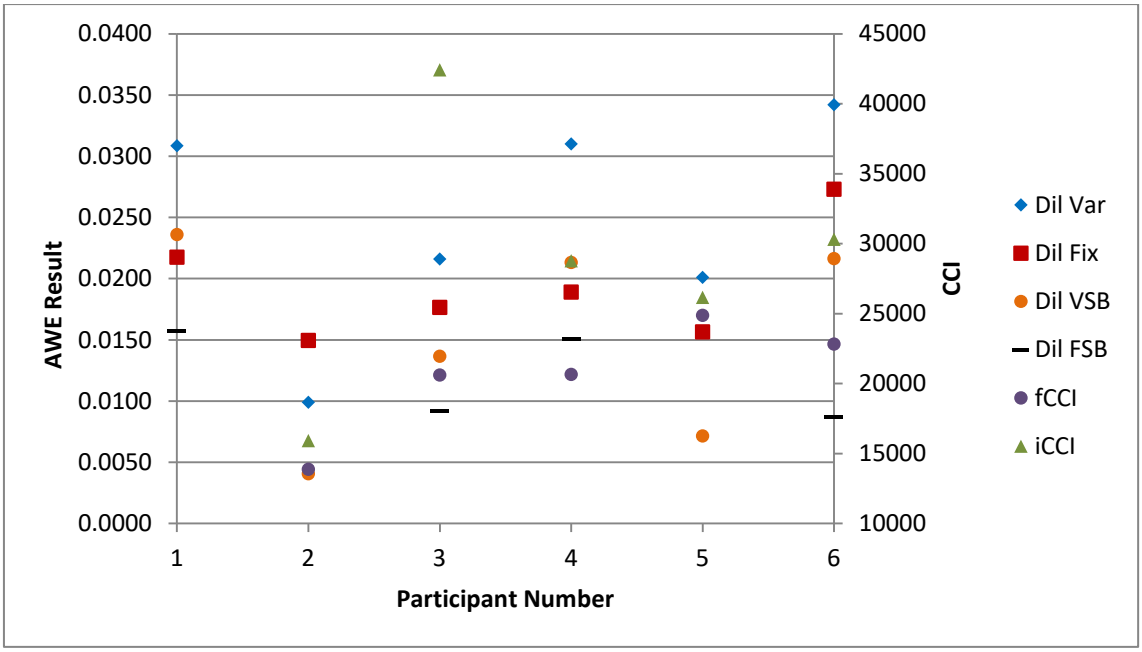

Figure 4.16: *AWE Test results obtained from diluted samples using a variety of test settings and shown alongside the fluorescent and impedance CCI values for each participant*

The results obtained using the diluted samples show better correlation with CCI than those obtained using neat samples. For the diluted samples, correlation was usually better if a logarithmic trend line was used. See Table 4.29. Of the four test settings investigated, the diluted VAR settings show the best correlation with both iCCI and fCCI.

The impedance CCI for concentrate 003 lay outside of the general pattern of results for all sample and measurement variations. When it was excluded from analysis, the correlation of the diluted VAR results with iCCI increased from 0.3461 to 0.9723. The correlation of iCCI with AWE results using all other test settings also improved substantially. The iCCI result for concentrate 003 was also exchanged for one calculated using the fluorescent pre and post-transfusion platelet counts with the impedance concentrate count as described for the ThromboLUX and ROTEM tests (see 'Exchange 003' results in Table 4.29). Improved correlation was maintained after the substitution.

Table 4.29: *Correlation (R^2 values) between neat and diluted AWE results and CCI for each concentrate. Results are shown for the entire dataset, for each dataset with one outlying result excluded (Exclude 003 / 005) and for the impedance CCI datasets after the iCCI result for concentrate 003 was recalculated (Exchange 003). All correlations of >0.80 are shown in bold. Logarithmic trend lines were used.*

	NEAT				DILUTED			
	VAR	**FIX**	**VSB**	**FSB**	**VAR**	**FIX**	**VSB**	**FSB**
iCCI	0.0029	0.1516	0.0046	0.0526	0.3461	0.1195	0.4211	0.0967
iCCI (Exclude 003)	0.0207	0.0905	0.0428	0.0006	**0.9723**	0.5311	**0.8241**	0.0967
iCCI (Exchange 003)	0.0152	0.0999	0.0310	0.0026	**0.9632**	0.5046	**0.8251**	0.6813
fCCI	0.1611	0.5467	0.1057	0.2924	0.5467	0.1609	0.2828	0.1889
fCCI(Exclude 005)	0.0265	0.3149	0.0335	0.0100	**0.9330**	0.6929	**0.9345**	0.1889

To a lesser degree, the fluorescent CCI obtained for participant 005 (previously identified as potentially poor quality by the ESC and ThromboLUX results) also lay outside of the general pattern of results. When the fCCI value for participant 005 was excluded from the analysis, the correlation of the diluted VAR results with iCCI increased from 0.5427 to 0.8699 and the correlation of fCCI with AWE results using all other test settings also improved. See Figure 4.17.

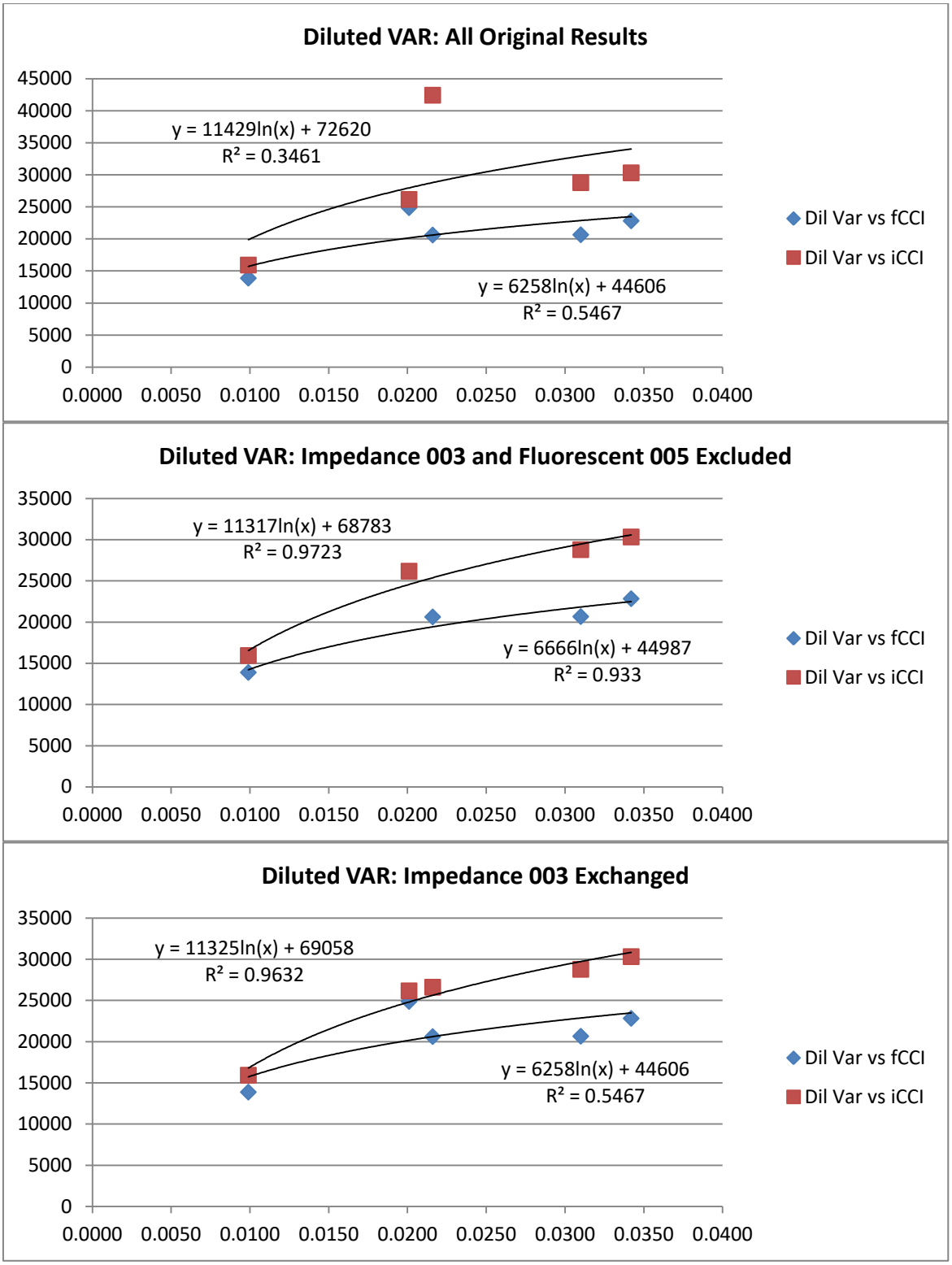

Figure 4.17: *Correlation between diluted VAR tests and CCI. The graphs show the original results for all study participants (top), all results except the impedance results for participant 003 and the fluorescent result for participant 005 (middle) and the results for all study participants after participant 003's impedance result has undergone re-calculation using his fluorescent pre and post-transfusion platelet counts (bottom).*

Of all the AWE results, the diluted VAR results showed the best correlation with the HSR results, however, this was not very good. The correlation improved from 0.5402 to 0.7888 if the result for concentrate 003 was excluded from the analysis. See Figure 4.18.

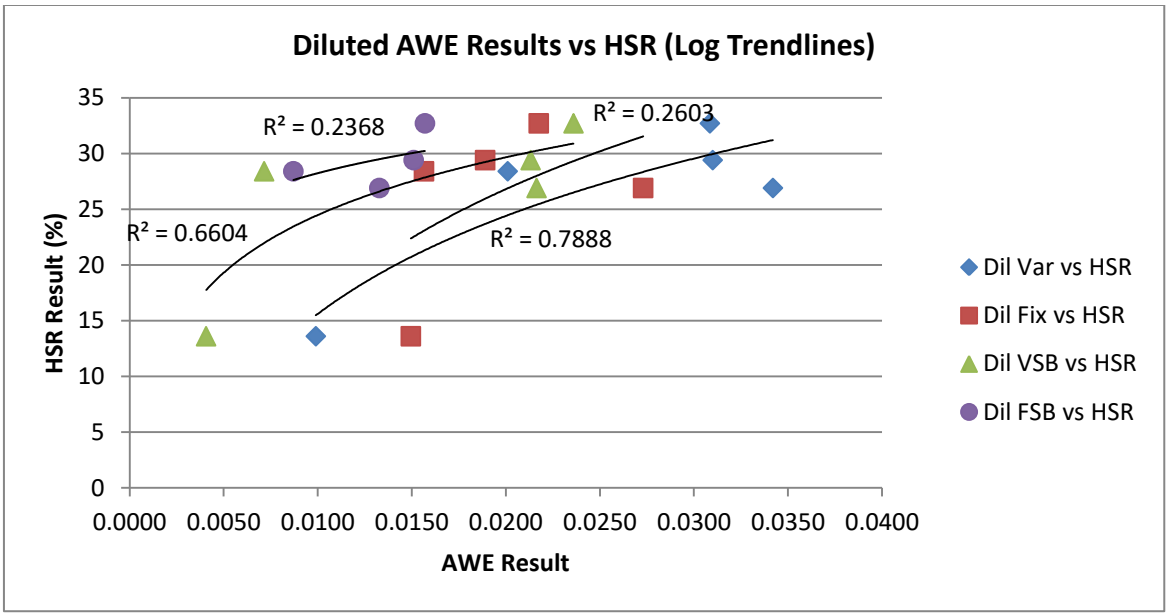

Figure 4.18: *Correlation between AWE and HSR results*

There was also a possible slight correlation between the neat FSB result and the neat EXTEM and EXTEM – FIBTEM results. See Figure 4.19. However, only four results are available for each of these ROTEM parameters so any potential association is a little dubious.

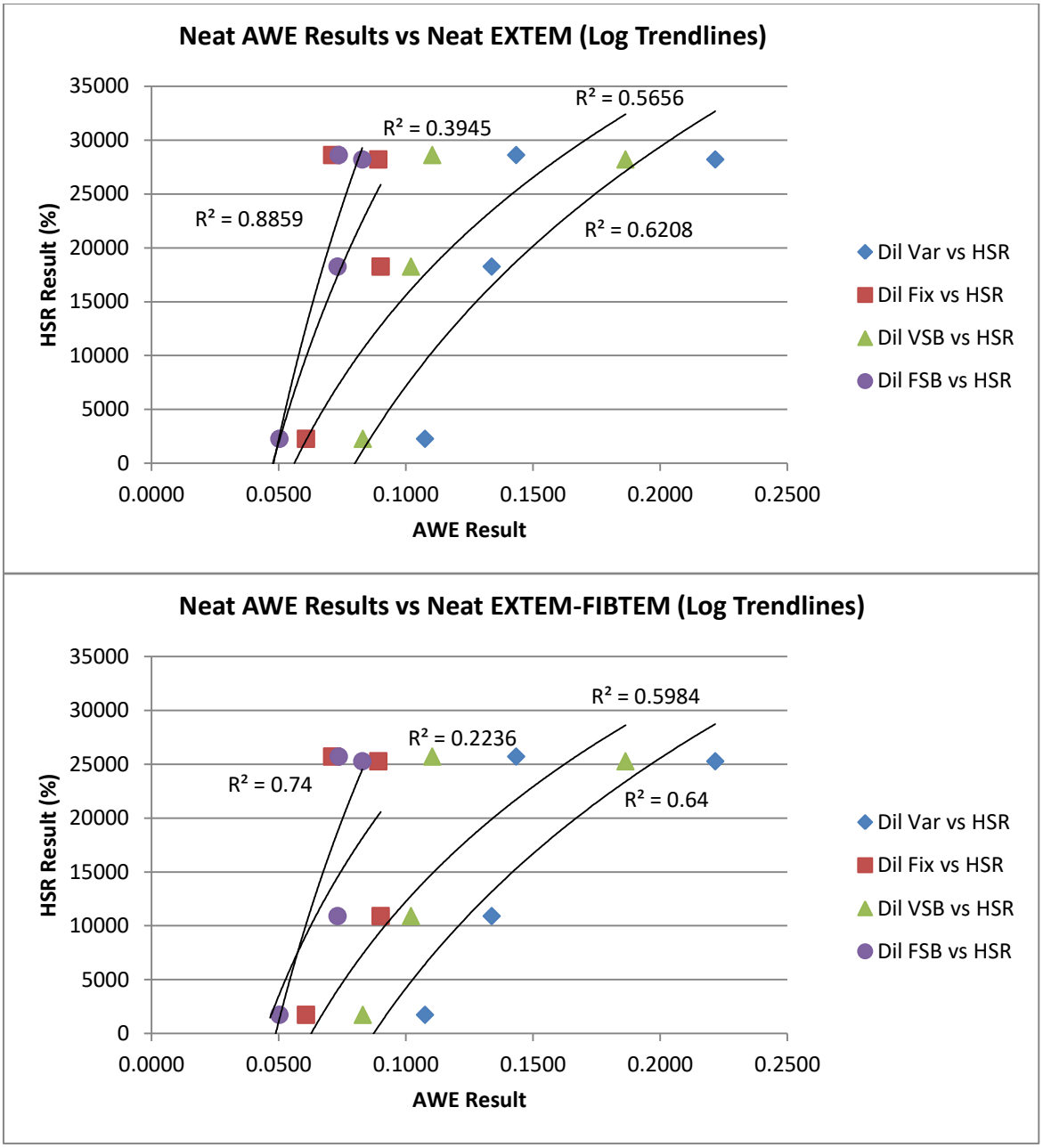

Figure 4.19: *Correlation between neat AWE and neat EXTEM results (top) and neat AWE and neat EXTEM-FIBTEM results (bottom)*

Correlation between neat diluted AWE results and the ThromboLUX results (mean or TL1 results) was poor but seem to show general trend in the same direction. See Figure 4.20.

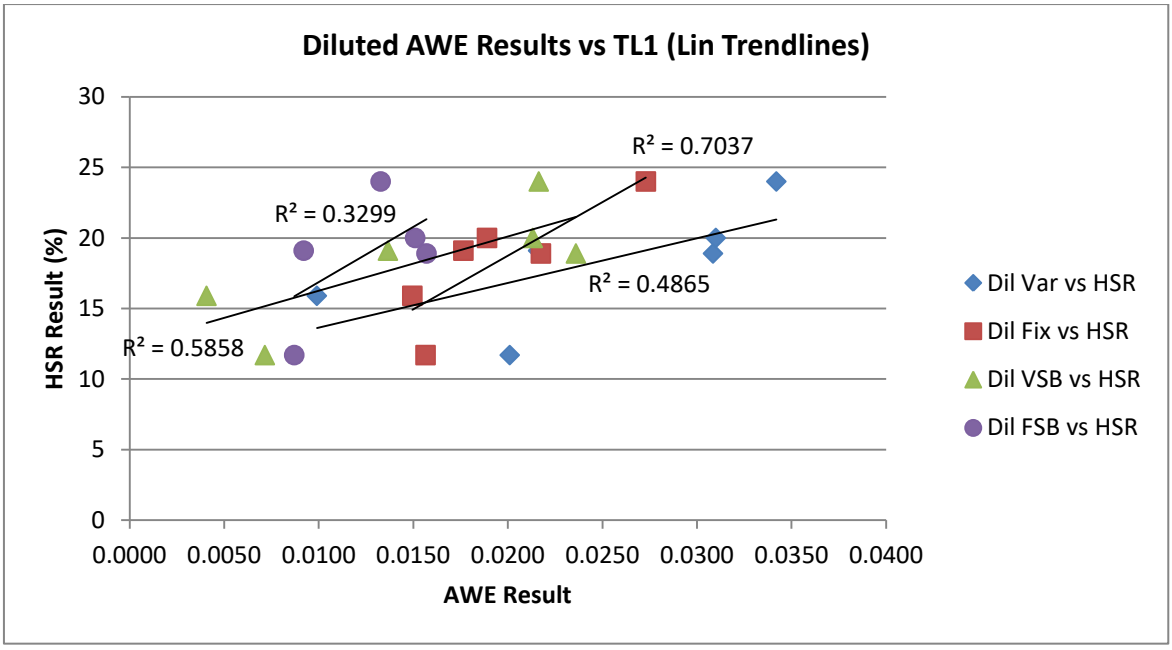

Figure 4.20: *Correlation between diluted AWE results and ThromboLUX (TL1) results*

No correlation was observed between TL mean or TL1 results and ROTEM results for neat or diluted samples.

4.5 Clinical Trial Discussion

Performing a clinical trial to assess platelet quality assessment methods with sufficient numbers to achieve statistical significance was known to be outside the scope of this study. It was felt, however, that performing a pilot study to work through the analytical and organisational aspects of an appropriate study design would be a novel undertaking of benefit to the field.

The pilot study enabled development of the technical aspects of the trial, aiding improvement of protocols for retrieval of the residual platelet concentrate and completing testing in an efficient and timely fashion to ensure results were generated using as 'fresh' a specimen as possible. Methods of participant identification, patient approach and consent, were refined and new contacts were made amongst the Clinical Haematology medical and nursing staff.

All of the study patients recruited experienced adequate increments at 1 hour post-transfusion, which are defined by Askari *et al* (2002) as a CCI of >7000. The fluorescent and impedance platelet counts obtained on the Sysmex XN analysers appear to be effectively interchangeable at the low levels observed in the patient samples but the fluorescent counts are considerably higher at the levels observed in the platelet concentrate samples. There is very little information available in the literature regarding the relative accuracy of the two methods at high platelet counts so it is difficult to be sure which CCI measurement is most representative of the patient's true increment. It may transpire that the relationship between the calculated iCCI and fCCI parameters is itself informative regarding platelet function.

Every concentrate was swirl positive and had pH values well above 7, making it unlikely that the quality of any of them was very poor. However, the ESC and HSR results for all concentrates were below the normal ranges stated by Holme *et al* (1998) for fresh platelet concentrates stored under optimal conditions. The ESC and HSR tests are known to be more sensitive to deterioration of platelet concentrate quality than swirl assessment or pH measurement so these observations are not necessarily contradictory (Holme *et al*, 1998. Shivastava, 2009). It is likely that the lack of agitation during the transfusion and laboratory processing and the sub-optimal oxygen exchange conditions for the platelet concentrate sample in the lines of the transfusion giving set and laboratory sample pot account for the reduction in ESC and HSR results.

The duplicate ThromboLUX results for PC 003, 004, 005 and 006 were discrepant. In all cases, the second test gave a lower result than the first one. It is possible that the platelet quality becomes progressively compromised as the sample is stored in a capped, airtight container during laboratory processing. This does not explain why some concentrates give discrepant results while others do not appear to. The durations of storage before testing were similar for all concentrates. It is possible that environmental factors (e.g. temperature in the laboratory) or concentrate

specific factors (e.g. duration for which the concentrate was removed from agitation prior to transfusion) influences the degree of discrepancy observed.

Although by no means conclusive, there does appear to be some relationship between the ThromboLUX score (if only the first test result is considered) and the CCI values obtained for the participants. The only concentrate that did not fit into this relationship was concentrate 005, which had been independently identified as potentially poor quality or 'odd' by the ESC test and an abnormal fCCI / iCCI relationship. It is unclear why the second ThromboLUX measurements for each concentrate differ so much from the first. It seems most likely that storing the sample in a capped container without adequate oxygen exchange or agitation even for a short period has a dramatic effect on the ThromboLUX score. If this effect is due to lack of agitation, it could imply a similar degree of previous deterioration that began before the sample was collected due to lack of agitation during the transfusion process.

It is not clear why the ROTEM result for concentrate 003 was so much lower than those obtained for the other concentrates, or why the relationship between the neat and diluted sample results was non-typical. If this result is excluded, there is a degree of correlation between the remaining results.

The results for concentrates 001 to 003 are substantially lower than those for 004 to 006. This is likely due to the change in EXTEM reagent batch that occurred at this point. As previous stages of this study (the Aging Platelet Concentrate Study – 5.3.6) have noted changes in results between reagent batches, it was highly desirable to use a single batch of reagents for all testing within a particular set of experiments. In practice, this was not possible due to the short shelf-life of the reagents provided by TEM. This centre's relationship with TEM is relatively new and small-scale. It is hoped that the promising results of this study would aid future requests for the supply of long dated products.

The AWE test results generated in the clinical phase of this study add to previous suggestions that this test has potential for future development. The diluted VAR test settings appear to give the most clinically significant results at present. Further investigation is required to further investigate this relationship with greater study numbers, to compare with 24 hour increments and possibly to apply different variations of the current test settings. However, the possibility of performing the test without using specialised Stir-Bar cuvettes would decrease the costs of the assay and enable samples to be ran routinely without loading non-standard consumables. An additional priority for future improvement of the AWE test is the development of a suitable means of quality control testing.

Participant 004s transfusion appeared to prevent the manifestation of any clinical bleeding symptoms in the days immediately following her transfusion but she experienced the onset of some minor clinical bleeding after three days. Participant 005 had a higher baseline level of clinical bleeding that did not appear to be improved by this transfusion. However, when the same patient participated in the trial again as participant 006 his clinical bleeding symptoms appeared to be marginally less severe following transfusion. It is tempting to link the apparent lack of clinical effect observed after transfusion 005 to the low ESC and ThromboLUX results and would be very interesting to expand this clinical bleeding assessment aspect of the clinical trial to determine whether this pattern is repeated.

Concentrate 005 was identified as possibly poor quality by several of the laboratory methods used in this study and also by the clinical bleeding assessment. The CCI values for this concentrate, however, were not notably reduced. The transfused platelets may have survived for long enough to give a normal 1 hour CCI but been defective enough to perform poorly in some of the platelet quality tests and *in vivo*. Had the study incorporated 24 hour increment, it may have been found that the platelets in concentrate 005 had reduced survival and gave a low 24 hour CCI. Incorporation of a 24 hour CCI would be a great benefit to any future study.

It is notable that the two concentrates that gave outlying or spurious results and the only concentrates for which there was any suggestion of poor quality were the only two pooled buffy coat concentrates included in the study (concentrates 003 and 005). Although the numbers included in this study are far too low to make any conclusions, this raises the possibility that the quality of pooled concentrates, as assessed using routinely applicable *in vitro* assessment techniques, is generally lower than that of apheresis concentrates. Alternatively, there could be some factor inherent to pooled concentrates, perhaps something related to the platelet donation processing procedure, which causes these concentrates to respond differently in the laboratory without affecting their clinical efficacy. Ideally, the *in vitro* assessment of pooled and apheresis platelet concentrate quality would be studied in separate studies or in studies large enough to compare the two groups and look for differences between them.

In summary, all technical aspects of the clinical trial worked broadly as expected from previous stages of the study, although no clear and definite relationship was observed between transfusion efficacy and any of the measures under investigation. The major difficulty was recruitment, which was problematic largely because the study was performed remotely from the clinical location of potential participants. Several steps were taken to combat this, including modification of exclusion criteria and working more closely with the medics based on the Haematology Day Unit.

4.6 Clinical Trial Summary

- Ethical approval was granted for a pilot study that aimed to assess the ability of *in vitro* platelet concentrate quality assessment tests to predict transfusion outcome, as determined by CCI and bleeding score, in MDS patients
- The tests carried out on the residual platelet concentrate sample were platelet count, swirl assessment, pH measurement, ESC, HSR, AWE, ThromboLUX testing and ROTEM analysis

- Recruitment was problematic but six participants were recruited to the study
- Two pooled platelet concentrates and four apheresis concentrates included in the study
- Participant 001's transfusion was stopped early due to a suspected transfusion reaction so it was not possible to calculate CCI values but these are available for all other participants
- Impedance and fluorescent platelet count results similar for patient samples but fluorescent counts higher than impedance counts for platelet concentrate samples
- CCI was calculated using both impedance and fluorescent platelet counts
- All CCI results suggest acceptable response to transfusion
- Participant 003's iCCI result (and possibly participant 005's fCCI result) was dubious
- All residual platelet concentrate samples were swirl positive at the time of testing
- All pH results suggested acceptable platelet concentrate quality
- All of the ESC results obtained in the clinical study were lower than those previously reported for acceptable quality platelets that had been stored appropriately
- All of the HSR results obtained in the clinical study were lower than those previously reported for acceptable quality platelets that had been stored appropriately
- ESC and HSR did not show any clear relationship either with each other or with the CCI values
- The reproducibility of the ThromboLUX results declined over the period of the study
- TL1 results showed good correlation with CCI when concentrate 005 was excluded from the analysis and the recalculated iCCI result for concentrate 003 was used
- Concentrate 005 was identified as poor quality by the ThromboLUX analyser and the ESC result. It was also identified as non-typical by its platelet count / CCI results
- It was not possible to determine a definite 'quality cut-off value' for the ThromboLUX
- The ThromboLUX % MV intensity results fell into two disparate groups but did not show correlation with any of the other quantitative test results
- Apparent shift in ROTEM results with lot change between participants 003 and 004
- Participant 003's ROTEM results were lower than expected for non-expired platelet concentrates
- There was a possible relationship between the ROTEM result and CCI values after the outlying result for concentrate 003 was excluded

- Diluted sample AWE tests showed better correlation with the CCI values than neat tests
- Diluted VAR settings showed the best correlation with both iCCI and fCCI
- Relatively good correlation was observed between AWE results and CCI values after exclusion of dubious CCI results (the 003 iCCI result and the 005 fCCI result)
- There were some possible loose associations between the AWE results and the HSR, ROTEM and ThromboLUX results
- Three out of the six participants returned their bleeding questionnaires
- Participant 004's bleeding questionnaires suggest good initial response to the study transfusion, followed by gradual worsening of bleeding symptoms until another transfusion was administered five days after the study transfusion
- Participant 005's questionnaire shows little clinical response to platelet transfusion, although bleeding symptoms continued to worsen throughout the period of monitoring
- Participant 006 (who was the same individual as participant 003 and 005) experienced some clinical benefit from his platelet transfusion on this occasion

5 DISCUSSION

5.1 General Observations

Platelet concentrate quality testing is a fairly specialised undertaking and, therefore, not something that gains a lot of commercial interest. Potentially useful testing techniques are often designed for different purposes and require a significant amount of modification in order to apply them to platelets. Furthermore, platelets are quite complex cells, with a variety of different functions, not all of which are completely understood. This means that the way they react during different in vitro testing is not always easy to predict or interpret. This general observation has been borne out during all phases of the study.

It is possible to pick out individual concentrates that do not fit into the general trend when observing a group of results (e.g. 003 and 005 from the clinical trial – sections 4.3 and 4.4). However, the ability to do this is of very little use in clinical practice as, in most cases, there would only be a single result available in isolation from both other concentrates and other testing time points for the same concentrate.

5.2 Pooled vs. Apheresis Concentrates

Pooled platelet concentrates generally have lower platelet concentrations and higher overall volumes than apheresis ones, as observed in the *in vitro* stage of the project (section 3.3.1). Although the greater volume of the pooled concentrates compensates for the reduced platelet concentration in terms of total number of platelets transfused to the patient, there is no comparable compensating effect in many of the available laboratory tests. The practice of diluting platelet concentrate specimens in normal plasma to give a standardised count compensates for these differences to a degree and may, additionally, mimic the revitalising effect

of normal plasma that has previously been described for post-transfusion platelets (Berger *et al*, 1998; Ishikawa *et al*, 1987; Miyaji *et al*, 2004; Rinder *et al*, 2003).

The number of samples tested during this project was, for the most part, not sufficient to separate the results for pooled and apheresis platelet concentrates into different subgroups for analysis. As supply of suitable test samples was often problematic, it was not possible to restrict testing to a particular type of concentrate. However, pooled and apheresis concentrates are widely accepted to differ in several respects, even if the clinical relevance of these differences are not always clear (Reviewed in van der Meer, 2013; Ali, 2011). Even concentrates of the same type produced using different collection systems are found to differ in their biological characteristics. Macher *et al* (2010) describe differences in glucose, pH, lactate and lactate dehydrogenase (LDH) between apheresis concentrates produced by different collection systems.

Observations of differences between the mechanisms in which the two types of concentrates deteriorate with age further complicate this picture. Paglia *et al* (2014 and 2015) describe a complex platelet concentrate deterioration process that involves discrete phases dominated by different biochemical processes. Crucially, they found the phases of deterioration and the times at which the shifts occurred to differ between apheresis and pooled buffy coat concentrates to differ. Added to this complexity, a large number of different additive solutions and anti-microbial treatments are under development, all of which have the potential to affect platelet quality and deterioration characteristics in their own, as yet undefined ways (Bergmeier *et al*, 2003; Cardigan *et al*, 2008; Deyhim *et al*, 2015; Kostelijk *et al*, 2000; Leitner *et al*, 2016; Lin *et al*, 2004; van der Meer, 2016; Osman *et al*, 2016; Ostrowski *et al*, 2011; Pienimaeki-Roemer *et al*, 2014; Védy *et al*, 2009; Wagner *et al*, 2009, Zhuang *et al*, 2014).

Added together, these factors make the need for a simple, easily applicable measure of platelet quality all the more desirable but have the potential to greatly complicate the development and assessment of tests in the future. The best approach wherever possible would be to ensure that

all concentrates tested were produced using the same methodology. However, this would greatly restrict the ability of smaller teams to carry out platelet concentrate research and effectively limit it almost entirely to blood service laboratories and institutions able to source large enough numbers of specific types platelet concentrates from them. The studies carried out under such circumstances would likely be of a higher quality but ideas and innovations that may be generated during the undertaking of more numerous, smaller studies could be passed over. Ultimately, this is a question of research ethics that should be taken into consideration at the point of blood donation.

5.3 Control Tests

A wide variety of control methods were available for potential inclusion in the study but this was drastically reduced by selecting only those that had previously been found to correlate with clinical efficacy. It would have been helpful to include the Kunicki score in the study control panel but this was not possible as the specialist experience or image capture equipment deemed necessary to make this highly subjective method work reliably was available. This was unfortunate as being able to observe the quality of the platelets visually may have helped identify problems with the results of other control and experimental methods, as well as aiding identification of platelet samples in which the quality was not as expected.

The swirl and pH tests were not initially included in the study due to their reported insensitivity to the low levels of poor quality likely to be found in clinically available concentrates. Both were added to the study test repertoire to aid identification of very poor quality concentrates tested in the ThromboLUX. As expected, the platelet concentrates tested as they aged in the laboratory did not show abnormalities in these parameters until relatively late and all concentrates tested in the clinical trial gave normal results for both tests. However, the swirl test in particular is a simple,

inexpensive method that could be widely applied immediately. The sensitivity may be low but it could, at least, be used as a quick check for severely compromised units.

The ESC and HSR were more informative and showed clear deterioration of results as platelet concentrates aged. However, neither showed a convincing relationship with CCI in the clinical phase of this study, raising the possibility that the retrospective sampling method employed was not optimal. However, the reproducibility of both tests is known to be poor so it is quite possible that clearer relationships would become apparent if more samples were tested.

Despite these drawbacks, the ESC and HSR assays are widely used in assessment of platelet concentrate quality for research and production development (Kiminkinen *et al*, 2016; Kraemer *et al*, 2015; Ringwald *et al*, 2012; Sandgren *et al*, 2012; Sandgren *et al*, 2015). Even though neither is optimal in terms of technical performance and correlation with platelet transfusion outcome, their automation would likely offer benefits in terms of making them more easily applicable, better standardised and more readily performed by a wider staff group. Straightforward automation of the standard HSR / ESC methods would probably lead to improvements in precision and accuracy of results by reducing the potential for human error in pipetting and timing of test steps. Although analysis of data by the analyser is more complex because it requires additional steps, the relative stability of reagents required for these assays makes them more readily automatable than the automated platelet aggregation panel that Sysmex now offers for use in the diagnosis of platelet function disorders as described by Lawrie *et al* (2014).

5.4 iZON Instrument

As a technique that had not initially been designed for studying platelets, the iZON analyser did appear to have some potential for future use. It was shown that the test could be potentially

applied to the measurement of platelets and MV in ageing platelet concentrates. However, it was quickly found to require a substantial input of time and expertise that were felt to be beyond the scope of this study. In addition, there was no reason to believe that it would produce information of use for the rather specific purpose of studying platelet concentrate quality. It would be preferable to establish the features and limitations of this method using a better characterised sample type, such as fresh PRP, to gain an understanding of the expected 'normal' results. It could be compared with other impedance platelet counting / sizing techniques and a reference range for platelet charge could be established. The use of fresher samples would, hopefully, enable easier acquisition of a sufficient number of data points that were less likely to be affected by selection of the less activated platelets in the sample. Test results from ageing platelet concentrate samples, which are relatively poorly characterised and difficult to process, as well as platelets stored under non-physiological conditions could then, perhaps, be better interpreted against this benchmark.

5.5 ThromboLUX Analyser

The ThromboLUX analyser was at a different stage in development, having been extensively tested and trialled by its developers in Canada as a prototype. It was anticipated that this instrument would need a minimal amount of in house validation, before an extensive *in vitro* study of ageing platelet concentrates was carried out, followed by a clinical trial. Funding was secured for these investigations and plans were made to undertake them alongside NHSBT trials and projects at various centres. However, the ThromboLUX analyser did not perform as expected from the outset.

Since the beginning of this study the ThromboLUX analyser has been evaluated in a number of laboratories internationally. It appeared to show great promise in terms of its ease of use and

clinical relevance as evidenced by the large scale clinical trial carried out by the developer using a prototype instrument (Maurer-Spurej *et al,* 2009). Unfortunately, this success has not been replicated during the beta evaluation phase of the second generation instrument. Light scattering measurements require precise alignment of the sample in 3 dimensions. The prototype ThromboLUX used capillary tubes inserted into a sample holder by hand to place the sample into the light path. The second generation ThromboLUX first received for use in this study had undergone a radical redesign, including the introduction of a sample presentation system that used disposable sampling sets manufactured and sold by LightIntegra. This change in the analyser set up may have introduced a degree of variability into the alignment of the sample in the light path, potentially affecting the DLS results recorded by the instrument. The third and fourth ThromboLUX instruments used in this study (from the beginning of the sample exchange experiments described in chapter 3 onwards) reverted to using a syringe and capillary tube system but the internal optical elements were not otherwise reengineered.

The ThromboLUX has been found unable to identify bacterially contaminated platelet concentrates (Taha *et al*, 2016), not to correlate with the ESC and HSR assays (Kraemer *et al*, 2015) and insensitive to inappropriate storage of platelets in EDTA (Raczat *et al*, 2014). The publication bias towards scientific studies with positive results is widely known (Dirnagl *et al*, 2010), making the appearance of negative ThromboLUX evaluations in the literature all the more relevant. The ThromboLUX appears to have some potential utility as a method for the quantitation and sizing of PMV and this feature has been of prominent focus in recent publications by the development team (Labrie *et al*, 2013; Maurer-Spurej *et al*, 2016).

The inability of the ThromboLUX to reliably identify aged or inappropriately stored samples by returning a low score caused considerable concern and was, ultimately, a problem that was never resolved. LightIntegra were unable to explain this observation and were hesitant to advise whether other centres were experiencing the same problems. They maintained that they had not

experienced these features in their initial trials of the instrument but these were performed on a previous, prototype version of the analyser, rather than the mass produced second generation version that was on loan for the current study. This issue clearly affects the potential clinical utility of the ThromboLUX analyser as a pre-transfusion quality screen because inappropriate storage or accelerated age related deterioration of platelet concentrates are both issues that could, theoretically at least, occur quite commonly in the hospital transfusion laboratory environment.

Several changes and upgrades to the ThromboLUX equipment have slowed the progress of this study without leading to significant improvements to results. Significant technical changes to the analyser and the way it is set up, in addition to alterations to the data processing algorithms have prevented accumulation of large sets of data relating to individual analyser / software combinations. After each change, LightIntegra advised that previous problems with sensitivity had been resolved and results up to that date were no longer representative of the current system. It can be clearly seen, in retrospect, that there were no sudden and significant improvements in the performance of this test but that does not mean that there were not changes in the way it worked so combining all of the smaller datasets remains erroneous.

In order to establish a new local cut-off value for each ThromboLUX machine / location combination, it will be necessary to test a relatively large number of concentrates at different time points to determine the range of results that can be expected for concentrates of varying quality throughout the ageing process. In order to ensure that they relate to post-transfusion platelet efficacy, the ThromboLUX scores of transfused platelet concentrates should also be compared to transfusion outcome to determine whether the new cut-off value is of any clinical relevance. Clearly this will be impractical in the vast majority of cases. Furthermore, it is unclear how great the changes to an analyser or its location must be to invalidate a previously defined cut-off value. Will routine analytical upgrades or moving the analyser to a different room in the

same laboratory necessitate extensive testing to establish a new cut-off value? These are questions that must be addressed by LightIntegra before the ThromboLUX could be safely introduced into clinical practice.

Despite the sensitivity concerns surrounding the ThromboLUX, it did appear to show some correlation with CCI and detected a suspected poor quality platelet concentrate in the clinical trial (see section 4.4.5). It is quite possible that the analyser is able to identify some types of impaired quality but not others, for instance it may detect some aspects of inter-donor variability in platelet quality that can lead to differences in clinical response to transfusion. It may be of use as part of a panel of methods employed in a research and development setting, if not the exclusive screening tool it was initially marketed as. Alternatively, further characterisation of how, exactly, the raw ThromboLUX measurements relate to platelet function and quality may facilitate development of the analytical system or result interpretation algorithm to enable the instrument to fulfil its original brief.

The availability of relative MV contribution to light scattering intensity may be of interest to future researchers. The data obtained during the clinical trial appeared to show the possibility of a relationship between the MV level within a platelet concentrate and clinical transfusion outcomes. However, this interesting measurement feature was, unfortunately, made available too late for us to fully characterise and utilise within this study.

5.6 ROTEM Analysis

The ROTEM results have consistently shown deterioration with platelet concentrate age and the method is sensitive to temperature induced platelet damage. Changes are large enough to show clear distinction between different time-points and seem to correlate, to a degree, with control tests. This method is an easy to use, established, standardised, commercially available CE marked

test that is widely used for routine diagnostic purposes. As such, it appears eminently suitable to apply to the problem of measuring platelet concentrate quality. In addition, the 'global' nature of thromboelastometry tests means that this method has a good chance of relating to post-transfusion platelet function *in vivo*.

McNulty *et al* (1997) harvested platelets from volunteers and then added them to blood samples to investigate whether they had any effect on TEG measurements including the maximum amplitude (MA), alpha-angle and R-time. Addition of platelets treated with liquid nitrogen to diminish quality resulted in increased MA comparable to that measured in samples treated with appropriately stored (presumably higher quality) platelets. On the basis of this observation the authors predicted that platelet transfusion would lead to increased clot strength if their blood was tested immediately after treatment. This raises the possibility that thromboelastography results obtained for platelet concentrate samples would be primarily determined by the number of platelets present, rather than their functionality. In this study however, platelet G was found to decrease as concentrates aged independent of platelet concentration and clotting factor concentrations (using samples diluted to a standard count in a normal plasma diluent).

The maximum clot firmness (MCF) and MA parameters reported by the ROTEM and TEG instruments respectively are influenced by both the elasticity and viscosity of the clot containing sample. The platelet component of clot strength is better represented using measures of the force with which the forming clot resists movement of the pin which are more representative of clot elasticity (CE). The direct measurement of this force is reported in the ROTEM software as the 'G' value in units of dyne/cm^2 (Solomon *et al*, 2015). The relationship between clot amplitude and force / elasticity measurements is not linear. Because of this, subtracting FIBTEM MCF values from EXTEM MCF values does not give a result that is representative of the platelet contribution to the clot. Force / elasticity based measurements must be used for assessment of platelet function (Solomon *et al*, 2015).

More recently, a number of studies have used thromboelastography or thromboelastometry as a measure of platelet concentrate quality under various circumstances (Bynum *et al*, 2016; Ostrowski *et al*, 2011; Ponschab *et al*, 2015; Svendsen *et al*, 2007). However, these studies did not examine the relevance and reliability of the method and no justification given for its use. Two recent studies have investigated the applicability and relevance of the TEG system (Bontekoe *et al*, 2014) and the TEG and ROTEM systems (Arbaeen *et al*, 2016) to assessment of platelet concentrate quality. Both Arbaeen *et al* (2016) and Bontekoe *et al* (2014) used amplitude based measurements, rather than force based measurements for their analysis. Both of these studies may benefit from reanalysis of the data using parameters that better reflect clot elasticity.

Bontekoe *et al* (2014) tested 19 apheresis platelet concentrates on days 1, 5, 8 and 12 after collection from the donor with the TEG system and found changes over storage. They used Octoplas as a MV free diluent and kaolin and collagen activators. MA was chosen as the primary measurement. The authors note that MA was not very sensitive to changes in platelet count within the range of 100 – 1000 x 10^9/L so very dilute samples were used in an attempt to increase the sensitivity of the method to differences in platelet quality. Again, little distinction is made between platelet quantity and platelet quality. Rather than diluting to a standard platelet count, 1 in 10 dilutions were used for all samples in order to ensure that fibrinogen levels introduced in the diluent were relatively constant. This was a valid option due to the design of this study, which used apheresis concentrates produced with the same technology meaning the range of initial platelet counts was small.

Clot retraction was observed in several of the samples tested by Bontekoe *et al* (2014). This manifests as a sudden reduction in the clot amplitude that can be visualised on the graph as the clot suddenly detaches from the pin. It can be distinguished subjectively from fibrinolysis, which appears as a more gradual decrease in clot amplitude. Bontekoe *et al* (2014) noted that clot retraction became less prominent as platelet quality declined. Clot retraction was noted in the

clinical trial sample from participant 003 in this study (see Figure 4.3). This feature may be an interesting target for future investigations of the relationship between thromboelastography / thromboelastometry and platelet concentrate quality.

Bontekoe *et al* (2014) found accurate pipetting in terms of volume and timing to be critical to the reproducibility of TEG results. This is one possible benefit to the ROTEM system, which has an integrated pipette, controlled by the analyser software. Liquid is drawn up by an automated vacuum system rather than manually controlled plunger and the test timings follow on screen prompts. If critical test timings are not met the test is cancelled and must be restarted. These features may lead to the generation of more reliable test results with the ROTEM system.

This study used the standardised and commercially available EXTEM and FIBTEM reagents supplied by ROTEM, which use TF as an activator with and without the cytochalasin D platelet inhibitor respectively. Bontekoe *et al*, (2014) used kaolin and collagen and Arbaeen *et al* (2016) used kaolin. It may be of interest for future studies to investigate the effects of a wider range of platelet activators within thromboelastography / thromboelastometry systems but the use of a widely applied and validated reagent combination seems reasonable in the present study.

Arbaeen *et al* (2016) used both the TEG and ROTEM systems to assess eight platelet concentrates on days two, five and eight after collection. Again, amplitude based measurements, rather than force based ones, were used. They investigated the effect of using diluents with high of low MV levels and found that use of MV enriched diluent led to increases in the TEG MA value. The CryoCheck normal plasma used for this study was not specifically low in MV but was unlikely to be as high as the MV enriched diluents used by Arbaeen *et al* (2016). Also, only a single batch of normal plasma diluent was used in this study so any MV effect would be consistent across all tests.

Arbaeen *et al* (2016) also tested samples diluted to give a range number of different platelet concentrations (400, 300, 200 and 100 x 10^9/L) and found the MA to decrease as platelet concentrate decreased but presented no convincing reason for favouring one dilution over another. The diluted sample ROTEM tests presented in this thesis were all carried out on samples standardised at counts of 300 x 10^9/L ± 10%. This level was used as it was representative of a normal platelet count at the level comfortably within the range the analyser would be expected to encounter and measure reliably in its usual application as a whole blood measurement tool. Also, 300 x 10^9/L is the platelet concentration recommended for HSR and ESC testing, suggesting that this level enables the platelets to react in a way representative of their overall quality. Although the justifications for the concentration used in this study remain valid according to the evidence that is currently available, a more comprehensive study of the platelet dilution level that results in the most clinically relevant ROTEM test results could be of future benefit to the field.

The fact that MA decreased as platelet biomass decreased in the more diluted samples was given as justification for its use as the primary measurement by Arbaeen *et al* (2016). However, the MA does not separate the effects of viscosity (related to platelet biomass) and elasticity (related to platelet function) and is therefore unable to distinguish reliably between changes in platelet concentration and platelet activity within a platelet concentrate sample.

In contrast to Bontekoe *et al* (2014), they did not observe any significant deterioration of the TEG results as the platelets aged. This may have been because they concluded testing two days earlier in the platelet storage period or may have been linked to some other methodological difference between the two studies. In the fresh platelet concentrate study performed in Oxford, deterioration of ROTEM results by day ten after collection was clear with the initial lot of EXTEM reagent but less so with the second batch. Reduction of thromboelastometric potential did not become obvious for all concentrates tested with the second batch of EXTEM reagent until day 14

after collection (see Figure 3.26). Arbaeen *et al* (2016) found the TEG system MA measurement to be sensitive to artificially induced poor platelet quality caused by lack of agitation, reduced oxygen permeability of container and inappropriate storage temperature (30^{o}C) mirroring the reduced ROTEM G results observed for inappropriately stored samples in this study.

The results generated in the clinical pilot study hinted at a possible relationship between the ROTEM results and transfusion efficacy. However, several factors complicated this and would need to be addressed in future studies. The first was the lot specific effect on results discussed previously. It may be possible in future studies to standardise the ROTEM results obtained for different lot numbers, perhaps by presenting them as a ratio of a 'normal' value. A 'normal' control sample (e.g. fresh PRP taken from a volunteer) could be tested five or ten times using the ROTEM for each new batch of EXTEM and FIBTEM. The platelet concentrate G results could then be expressed as ratios of the average normal control results for the appropriate test. The FIBTEM ratio could be subtracted from the EXTEM ratio to give a platelet only ratio. Sourcing a suitable and representative 'normal' platelet quality control sample is a challenge during the application of any platelet quality test but overcoming it could potentially reap great benefits.

In addition to the problem of standardising results across different reagent lots, at least one concentrate (003) was found to give atypical results for no discernible reason. It may be that the very low results, the atypical relationship between neat and diluted sample results and the clot retraction observed for this sample provide usable information about platelet concentrate quality but the factors leading to these unusual results are not yet understood. It is also not clear whether the ROTEM is sensitive to the low level impairment of platelet concentrate quality that is more likely to found in platelet concentrates for clinical use than severe quality deterioration.

5.7 AWE Test

The AWE test also appears to have some potential as a routinely applicable laboratory marker of platelet concentrate quality. The HSR test is one of the few laboratory measurements that have consistently been shown to correlate with the clinical efficacy of platelet concentrates (Holme *et al*, 1998; Shivastava, 2009) and the AWE test showed the best correlation with CCI out of all the experimental methods assessed in the clinical study. As an automated and simplified version of the HSR, the AWE test is potentially quicker and easier to perform, less prone to operator error and more standardised in terms of technical performance. However, the number of samples tested during this project is too low to draw any firm conclusions, particularly when the results generated for particular samples were questionable and needed to be excluded.

The precision of the AWE test was acceptable when compared to that of other platelet quality tests and the test results do not appear to undergo any significant changes due to settling of the samples during processing on the analyser. All of the samples tested showed deterioration when they were stored in sub-optimal conditions, with the exception of a single sample stored for one day only that may potentially have undergone a degree of revival due to the addition of normal plasma. There is not yet enough data to favour any particular test setting over the others. If it is proven that stir-bar cuvettes are not needed the cost of the assay would be reduced and the inconvenience of loading non-routine consumables entirely removed. See section 3.7.1.

The HSR test is performed in duplicate using water for the ‘test’ sample and Owren’s buffer for the ‘control’ sample. The isotonic buffer is not taken up by the platelets and so does not trigger any ‘recovery’ response during which it is pumped back out. The change in OD due to dilution when a volume of water or buffer is added to a sample varies from specimen to specimen depending upon the colour of the original sample. In the HSR test, this initial change in OD is measured and taken into account during calculation of results to effectively blank the specimen and compensate for differences in specimen colour that may affect the degree of change in OD.

This enables the results to be expressed as a percentage 'response' – the percentage by which the OD of the test sample corrects towards that of the control sample. It is likely that this practice increases the accuracy of the HSR result.

The Sysmex analysers are only able to report measurements across one time span so the initial decrease in OD due to the addition of diluent (either water or buffer) is not recorded or included in the calculation of results. Rather, the absolute value of the OD change from the point of maximum water uptake to various endpoints during the recovery phase is considered. It would be preferable to express results as a percentage recovery and control for variations in concentrate colour but that would require considerable changes to the Sysmex CA5100 software that are currently beyond the scope of this study.

A possible avenue for further study would be automation of the ESC test on a similar platform. All of the same potential benefits would apply to ESC automation as apply to the HSR and data processing could potentially be simpler within the existing setup. It is possible that the two tests, performed side by side, may give more information in combination than either could individually.

5.8 Future Clinical Trial

The next logical step to build on the progress made so far by this study would be to perform a larger clinical trial that would reach clinically significant numbers. This would enable investigators to determine whether the possible associations detected between transfusion efficacy and the experimental laboratory tests assessed in the pilot study achieve significance with greater numbers. A retrospective power calculation using the data produced by Maurer-Spurej *et al* (2009) indicates that 71 transfusion episodes are required to identify a correlation of 0.6 between platelet count increments and the ThromboLUX results with 80% power at a 5% significance level. Any future study should aim to achieve a sample size of at least 70 transfusion episodes.

Recruiting the numbers required would be more easily achieved if any future trial was carried out in closer partnership with the clinical side of the service as recruiting patients was a major stumbling block for the pilot study. It might be possible to include inpatients in future study but it is not certain that this would be desirable as inpatients are clinically less stable and would be more likely to experience changes to their platelet count due to non-study related factors such as treatment or rapid clinical deterioration. It may be preferable to extend the study to other centres (although this would make it difficult to standardise laboratory testing) or other participants with different diagnoses (although this would make it difficult to standardise the participant's clinical condition).

The 1 hour post-transfusion increment measures platelet recovery, indicating how many of the transfused platelets were taken up into the patient's blood stream and avoided immediate removal due to mechanical or biological mechanisms. A 24 hour increment is sometimes used to give additional information about platelet survival and can be a useful indicator of reduced platelet quality that reduces the lifespan of the transfused platelets (Slichter *et al*, 2005). Unfortunately, it was not possible to measure 24 hour increments in this study as the patient population consisted of outpatients who were not available for repeat venepuncture on the day after their platelet transfusion. Using Haematology inpatients as a study group would likely make it easier to obtain additional participant platelet counts that could be used to calculate 24 hour increments but may not be desirable for the reasons discussed above.

At 50%, return of self-assessment bleeding questionnaires was quite poor in this pilot study. A post-transfusion contact step may have encouraged better compliance with this part of the trial. Future studies should incorporate a telephone interview at two days post-transfusion to assess clinical bleeding and check for any problems completing the questionnaires.

There is some question over whether the giving set samples used in the pilot study were representative of the whole concentrate. It would be preferable to take a sample from the

concentrate just before the transfusion was begun, although this would be more difficult in ethical and organisational terms. It would be necessary to convince an ethics panel that all risks of contaminating the concentrate and therefore exposing the participant to infection had been nullified, possibly by using a closed system for sampling (Védy *et al*, 2009). In addition, the first patient contact in the pilot study design was on the morning of transfusion when the patient arrived at the day unit. Potential participants were able to reflect on whether to participate or not in the time during which their transfusion was carried out before sampling at the end of the transfusion. Sampling at the beginning of the transfusion would necessitate an earlier patient contact step. This could be achieved if a member of the clinical team spoke to potential participants during a prior appointment so that, if the participant was agreeable, consent could be obtained when they arrived at the Haematology Day Unit on the day of their transfusion.

If prospective sampling is not possible, the next best thing would be to retain agitation and good oxygen transfer conditions in the platelet concentrate sample until the laboratory analysis is performed. It would be comparatively simple to transfer platelet concentrate samples to breathable containers and ensure they undergo a period of upon arriving in the laboratory to ensure adequate re-suspension of all platelets in the sample prior to analysis. Specimens could also be stored on an agitator between sampling for tests.

In future clinical studies, it would be advisable to store stained blood films from the pre and post-transfusion platelet count samples taken from the participants. These films would have allowed the validity of platelet counts to be checked and aided investigation of any suspected problems with the results. This would have been very useful in the pilot study for participants 003 and 005.

5.9 Novel Contribution

Several aspects of this study have been novel contributions to the field. No attempt has been made elsewhere to analyse platelet samples using the iZON system. Although development of a

fully optimised and reliable platelet analysis method was ultimately deemed to be outside of the scope of this study the basic application was proven to have potential (see Figure 3.1).

The clinical section of this project represented the first trial of the ThromboLUX analyser outside of the original development team (Maurer-Spurej *et al*, 2009). This study uniquely included clinical bleeding assessments, an outpatient population with a specified haematological diagnosis (MDS) and a range of other platelet concentrate quality tests. For the first time, the ThromboLUX score was performed alongside pH, HSR, ESC, ROTEM analysis and the new AWE test.

This was the first occasion on which both impedance and fluorescent platelet count CCI values were available for platelet concentrates generally and for comparison with ThromboLUX results in particular. Fluorescent platelet concentrate platelet counts were observed to be consistently higher than impedance platelet concentrate platelet counts (see Tables 4.1, 4.6, 4.10, 4.14, 4.18 and 4.22). The availability of two different types of CCI values enabled identification of two possible outlying results (one for each type of CCI value) and exclude them from data analysis, revealing a possible relationship between the ThromboLUX score and both CCI measurements (see Figure 4.12 and Table 4.27), the ROTEM results and iCCI (see Figure 4.14 and Table 4.28) and the AWE test results and both CCI measurements (see Figure 4.17 and Table 4.29).

Previous assessments of the utility of thromboelastography in platelet concentrate quality assessment have concentrated mainly on the TEG instrument, have been restricted to *in vitro* analysis and have used clot amplitude based measurements (Arbaeen *et al*, 2016; Bontekoe *et al*, 2014). The use of the ROTEM instrument in a clinical trial here is novel, as is analysis of force based measurements that better relate to clot elasticity. The standard reagent combination of EXTEM and FIBTEM reagents developed and optimised for routine clinical diagnostic use had not previously been used. This study has uniquely demonstrated a substantial difference in the results generated for platelet concentrate samples using different EXTEM reagent batches (see Figure 3.26).

The AWE test was exclusively developed for use in this study and appears to show some potential for the measurement of platelet concentrate quality (see Figure 4.17 and Table 4.29). There is no published literature relating to any previous attempt to automate any variation of either the HSR or ESC test and the concept appears to be entirely novel to this study. Although there is much scope for further development and investigation of this methodology, the work presented here forms a sound basis to build upon.

5.10 Conclusion

Several of the experimental test methods investigated through the course of this study show some potential for utility as a widely applicable screening test of platelet concentrate quality. More testing is required for any statistically significant conclusions to be made about any of them. However, the ground work for designing an appropriate clinical trial has been laid. It seems likely though, due to the difficult nature of platelet testing in general, that any single screening test would be at most the first line investigation, with concentrates that give abnormal results requiring further investigation or confirmation.

Ultimately it may be found that problems with different platelet function pathways can only be detected using different techniques and the goal of a single, easily applicable screening test is not achievable. Rather, a carefully selected panel of tests, each targeted at a different aspect of platelet function may be required. This does not necessarily mean that routine or at least more easily carried out testing will not be possible in the future. With the rapid development, automation and miniaturisation of laboratory techniques, tests that were once considered specialised are now regularly carried out on a routine or even point of care basis. This trend is likely to continue in the future and may eventually make even complex platelet concentrate quality testing commonplace.

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