Clinical and Laboratory Measurement to Improve Patient Blood Management: Foundations to the Three Pillar Framework

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This thesis is my own original work. Except where otherwise acknowledged, it represents the work I have performed at John Curtin School of Medical Research and Canberra Hospital.

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Abstract

Transfusion may be a life-saving procedure. However, history shows that the risks of transfusion have been underestimated and transfusion overused. Blood has been considered dispensable and inadequate thought given to its management, not just to prevent transfusion, but to maximise patient outcomes. Voices heralding the need for blood optimisation and conservation throughout the twentieth century have combined to develop a field known as patient blood management.

Patient blood management has been described in terms of three pillars, primarily from a perioperative perspective: optimising the blood pre-operatively; minimising blood loss intraoperatively; and understanding and improving tolerance of anaemia. In this thesis I explore each pillar and argue that improving the way we measure blood-related outcomes has the potential to enhance patient blood management and the approach to transfusion therapy.

Iron deficiency is the most common cause for anaemia worldwide. It is particularly common in pregnancy where it can impact the mother's quality of life, the need for transfusion and potentially have far-reaching effects for children. Despite this, there is a lack of consensus on recognising and treating it during pregnancy. I have explored the use of novel red cell and reticulocyte indices, readily available from automated blood count analysers, as tools for detecting iron deficiency. While effective, these were no better than mean cell volume, although as a screening tool, a higher cut-off value is required. I also argue that there is value is screening with ferritin during pregnancy and show that this is best applied in first trimester.

Red cell transfusion guidelines advocate transfusion based on individual patient needs rather than specific haemoglobin triggers. Measuring this is difficult and most randomised studies have transfused solely based on haemoglobin. I explored tolerance of anaemia in a different light – near infrared. Near infrared spectroscopy can measure tissue oxygenation and provides a potential transfusion trigger. A systematic literature review showed that tissue oxygenation is affected by anaemia and does respond to changes in haemoglobin. However, there was too much heterogeneity

to recommend routine clinical use for transfusion decisions, perhaps as it was most frequently used in acute settings where reduced blood flow is a confounding factor. I therefore explored its utility in chronic anaemia setting and with exercise to see whether poor muscle oxygenation is a limiting factor for activity in anaemia. While there was a measurable impact, there remains too much heterogeneity to identify a transfusion trigger based on tissue oxygenation.

Finally, I have explored alternative strategies for managing thrombocytopenia. Platelets for transfusion are often not kept in rural and regional areas. I have shown through in vitro and transfusion studies that cryoprecipitate improves whole blood haemostasis in thrombocytopenia and should be considered as an option in bleeding patients unable to access platelet transfusions. Cryoprecipitate may be more effective than cryopreserved platelets currently under investigation.

These results highlight the continuing role for research into novel ways to monitor and appropriate target treatment for the blood. They support the concept of research being included amongst a revised model of patient blood management.

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Publications emerging from these studies

Crispin P, Forwood K. Near infrared spectroscopy in anaemia detection and management: A systematic review. Trans Med Rev 2020 (Online ahead of print). doi.org/10.1016/j.tmrv.2020.07.003

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Crispin P. Comment on Ferritin as a functional biomarker of iron in children and young adults. Br J Haematol 2019 187(2): 261-263 doi: 1111/bjh.16148 (letter)

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Abbreviations

6MWT – Six minute walk test

A5 - Amplitude at 5 minutes on thromboelastogram

A10 - Amplitude at 10 minutes on thromboelastogram

A20 - Amplitude at 20 minutes on thromboelastogram

AABB – American Association of Blood Banks

ACT – Australian Capital Territory

APTT - activated partial thromboplastin time

AUC – Area under the curve

CCI: Corrected (platelet) count increment

CLI 30 - Clot lysis at 30 minutes on thromboelastogram

CPP - Cryopreserved platelets

CT - Clotting time on thromboelastogram

DMSO - Dimethyl sulfoxide

FACIT – Functional Assessment of Chronic Illness Therapy scale

FACT – Functional Assessment of Cancer Therapy scale

FACT-An – Functional Assessment of Cancer Therapy Anaemia subscale scale

FACT-BMT – Functional Assessment of Cancer Therapy Bone Marrow Transplant subscale

FBC – Full Blood Count

FITC - Fluorescein isothiocyanate

FSC - Forward scatter

FTOE – Fractional tissue oxygen extraction

FVII - Factor 7

FVIII - Factor 8

FX - Factor 10

FXa – Activated FX

FXIII - Factor 13

Hb – Haemoglobin

HbA – haemoglobin A

HbF – Foetal haemoglobin

HBV – Hepatitis B virus

HCV – Hepatitis C virus

HIV – Human immunodeficiency virus

HREC – Human Research Ethics Committee

IRF – Immature reticulocyte fraction

LHD – Low haemoglobin density

LI30 - Lysis at 30 minutes (the clot strength 30 minutes after the MCF is reached, as a proportion of the MCF)

LI60 - Lysis at 60 minutes on thromboelastogram (the clot strength 60 minutes after the MCF is reached, as a proportion of the MCF)

LOESS – Locally estimated scatterplot smoothing

MAF – microcytic anaemia factor

MCF: Maximum clot firmness on thromboelastogram

MCH – Mean cell haemoglobin

MCHC – Mean cell haemoglobin concentration

MCV – Mean cell volume

ML: Maximum lysis on thromboelastogram

MRV – Mean reticulocyte volume

NBA – National Blood Authority (of Australia)

NIR - Near infrared

NIRS – Near infrared spectroscopy

NR - Normal range

OR - Odds ratio

PBM – Patient blood management

PDMS - Polydimethylsiloxane

PE - Phycoerythrin

PFA - Platelet function analyser

PICO – Population; Intervention; Comparison; Outcomes

PRP: Platelet rich plasma

PT: Prothrombin time

RCT – Randomised controlled trial

RSF – Red cell size factor

ROC – Receiver operator characteristics

SABM – Society for the Advancement of Blood Management

SCOR – Splanchnic to cerebral oxygen saturation ratio

SPSS – Statistical Package for the Social Sciences

SSC: Side scatter

SmO_2 – Muscle oxygen saturation

StO2 - Tissue oxygen saturation

TACO – Transfusion associated circulatory overload

tHb – Total haemoglobin

TRALI – Transfusion related acute lung injury

Tsats – Transferrin saturation (expressed as a percentage)

VWF: von Willebrand factor

Xa: activated factor ten (X)

Chapter 1. Introduction to Patient Blood Management and the problems in measurement

The modern view of patient blood management (PBM) has arisen paradoxically from transfusion medicine. Successively, schools of thought focusing on transfusion alternatives and PBM emerged. "Transfusion alternatives" implies that blood product transfusion is the standard of care. PBM is best considered as a holistic approach to patient care where the blood is carefully considered and managed as an organ, but there remains confusion in the literature with some still maintaining a blood product focus when discussing the term. In this chapter I will show that the culture in medicine has seen blood as dispensable since the availability of transfusion and that transfusion practices have been uncritically maintained despite threats posed to and by the blood supply. While recent decades have seen change due to evidence refuting ingrained practices from randomised controlled trials, there remains considerable scope to improve the way we measure triggers for intervention in PBM. I propose that advancement of PBM requires a critical look at the way we measure intervention triggers and their outcomes. This requires exploration of new tools because some of our current approaches poorly predict therapeutic efficacy. Without new ways to measure therapeutic outcomes, there will be limited capacity for transfusion science to focus on what is clinically meaningful. It may also mean considering new products that best fulfil clinical needs. This will form the basis for exploring, in a holistic sense to mirror the ethos of PBM, how changing the way we measure the potential effects of blood transfusion may change how we prevent and manage deficiencies in the blood.

A brief history of transfusion

The first transfusion of blood has been attributed to Richard Lower in 1665 who transfused blood between dogs in England.[2] Thereafter, the mystique around transfusion began. Jean-Baptiste Denis in France within the next few years transfused lamb's blood, apparently in an effort to imbue the calm spirit of the lamb into the troubled recipient. One recipient, a mentally ill homeless man, died. A reaction following the transfusion included chills and black urine – the first (heterologous) transfusions were complicated by acute haemolytic transfusion reaction. Meanwhile, Lower and Edmund King in

England had also transfused lamb's blood to improve demeanour in 1666. Baptiste went to court, successfully defending a charge of murder. The practice of transfusion was however banned by Parliament in 1670 and there was a long hiatus before these would again be tried.

It should be noted that although there has been much written about transfusion in England and France, Marinozzi and colleagues have reported on transfusions around the same period in Italy.[3] They document transfusions, again from sheep, to humans from 1667-8. Although there are no confirmed homologous transfusion cases, techniques for it were described.

It was physician James Blundell in England who is attributed with conducting the first homologous human blood transfusions beginning in 1818. Dismayed at seeing young women succumb to post-partum haemorrhage, he had a period developing the technique in dogs.[4] While it may seem obvious today, transfusing to restore lost blood, rather than to transfer the personality attributes of the donor to the recipient was step forward on the earlier attempts at transfusion as it targeted a correction based on the anatomy and physiology rather than belief. When he transfused people he enlisted their relatives, colleagues or husbands as donors, cannulated vein to vein and reported some excellent success. He eventually reported on ten cases, five successful, with various donors. He never reported a syndrome of acute haemolytic transfusion reaction in any of his cases, although two cases died before the transfusions were complete. He did however have a clear recognition of the need to closely observe patients during transfusion, and to stop if they deteriorated. Other reports of serious transfusions reactions did come to light and Blundell suggested compiling these cases and reporting them when a substantial body of evidence had been collected.[5] Unfortunately, no such publication eventuated. By 1852 Soden was able to collate 37 reports of transfusion for obstetric haemorrhage.[6] There were two cases where the physician thought that a reaction to the transfusion may have contributed to the patient's demise.

Landsteiner published on blood groups in humans in 1901, detected by agglutination when red cells and serum are mixed between some individuals and not others.[7] He suggested these may be

accounting for adverse reactions to blood, a fact later confirmed, and the work was later lauded as a major advance in medicine and awarded a Nobel prize. Based on the known frequency of ABO antigens, more than one third of random human-human transfusions would have major ABO compatibility. Given the severity of many of these reactions it can be reasonably concluded that Soden's report is subject to selection bias, and it seems likely from Blundell's own instructions on what to expect and how to observe patients during transfusion, that he also encountered severe reactions.[5] From the earliest history of transfusion, success has been celebrated and a blind eye turned to adverse events.

There were several obstacles to transfusion in the first half of the twentieth century. Compatibility was a critical issue for blood bankers. ABO incompatibility is easy to detect with saline agglutination tests, but for most other antibodies the indirect agglutination test is required to exclude them. Its introduction in 1945 enabled the detection of IgG antibodies acquired from previous red cell exposure more reliably and there was a steady stream of new blood group systems identified during the late twentieth century, albeit with less frequent or important clinical impact.[8]

A second issue for blood bankers was storage. Defibrination to stop clotting, or citrate anticoagulant, were initially tried and both could enable the storage of refrigerated blood, both stopping the need for donor-recipient connections and enabling the rapid deployment of blood to where it was needed. Citrate prevailed and enabled the later separation of blood components.

A third major issue was technique, with this issue in the domain of clinicians. Cannulation was often not easy and used metal needles. For this reason, anaesthetists were often required as part of, or even to manage, transfusion services.[9] The availability of stored blood rather than direct donor to recipient transfusions enabled more standardisation in this process. Barriers to transfusion fell away and blood transfusion became routine. Expertise in blood transfusion was thereafter vested in laboratories and blood donor services with the importance of ensuring that blood was available and safely cross-matched for the donor.

Blood transfusion use increased. There was a common perception of the need for blood when the haematocrit dropped below 30%, or haemoglobin concentration less than 100g/L (10g/dL) – the so-called "10/30 rule." This has been attributed to Adams and Lundy's 1942 treatise on the anaesthetic management of the patient with poor surgical risk.[10] This paper has only very limited reference to transfusion in patients with concurrent respiratory disease:

> "This condition [anaemia], owing to the lowered oxygen carrying capacity of the blood, interferes with adequate transportation of oxygen to the tissues. When concentration of haemoglobin is less than 8 to 10 grams per cubic centimetres of whole blood it is wise to give a blood transfusion before operation."

The only other discussion is on the use of transfusion to improve haemorrhagic shock. What arose as an opinion about a subgroup of higher risk patients appeared to be extrapolated to the entire population. Transfusions to top up haemoglobin became commonplace, even when there were reversible causes of anaemia to be attended to. Transfusion became normal and easy, and there was a common blindness to its limitations and risks. As Crosby noted in 1958:[11]

> "[Citing legal opinion] 'A person would be liable for damages following and caused by a transfusion that was not indicated by the facts of the case. Even if no negligence could be proved **. . .** an action for damages would lie if the plaintiff could prove that the transfusion was not indicated.'
>
> "Transfusing to a haemoglobin number is a "flagrant examples of what might be called the secretarial practice of medicine. In such a practice a stenographer types out the laboratory request, and when the patient flunks her hemoglobin test the stenographer types out a transfusion request.
>
> "Much of a person's 15 grams [/L] of hemoglobin is a reserve against strenuous exertion. Where there is no requirement for exertion, an individual can well tolerate a lower level of

> hemoglobin. For a sedentary life, 10 grams is often sufficient, and most bed-fast patients are comfortable with as little as *5* or 6 grams. Many patients can learn to live with a chronic anemia, and a little quiet encouragement is often as helpful as repeated blood transfusions."

Crosby concluded with a further challenge to the professionalism of his colleagues, "*What do you treat? The patient, or his lab report, or your own anxiety?"* That the appeal failed is evidenced by the fact that over 40 years later randomised trials in surgical patients used a haemoglobin threshold of 100g/L as the control – the presumed standard of care.[12]

Just as it was when heterologous transfusion began, there remains a lack of recognition of the potential harms of blood transfusion from clinicians at the bedside. The potential for transfusion to precipitate fatal pulmonary oedema due to transfusion associated circulatory overload (TACO), has long been recognised,[13] yet it does not feature prominently in modern haemovigilance reports. This may be because of better management of the complication, diuretics used to prevent it in at risk patients, or perhaps that it is "expected" that a degree of fluid overload might accompany a transfusion and it is not considered worthy of reporting. There has been a notable wide variation in the reported incidence of transfusion related acute lung injury (TRALI). One could argue that documenting each reaction is not critical if the benefit outweighs the risk. But that can't be said for the vast majority of transfusions. Alter and Klein note the prevalence of hepatitis in blood transfusion recipients in the United States prior to 1970 was as high as 33%.[14] Despite our inability to test for hepatitis C virus (HCV- then known as non-A, non-B), rates of transfusion continued to rise.

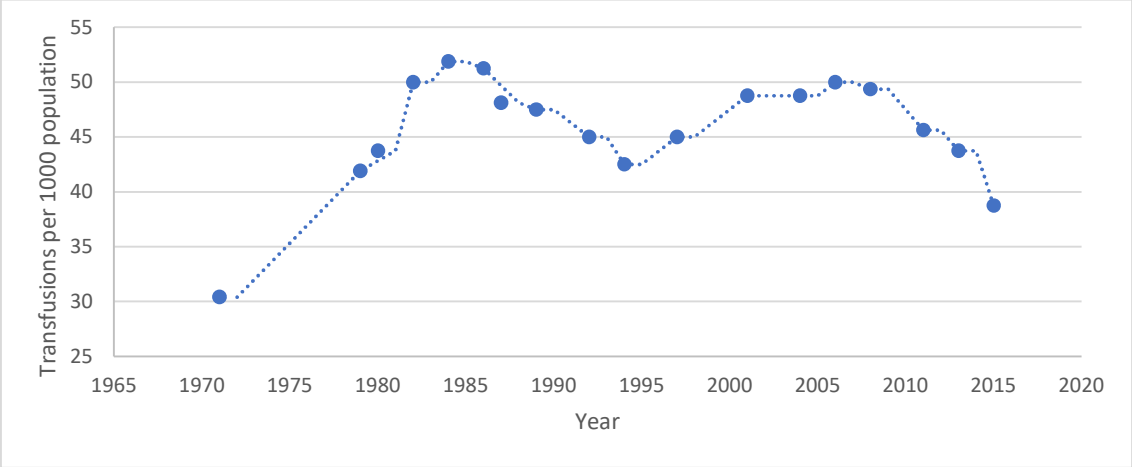
It has been suggested that there was a turning point in considering the risk of transfusion in the early 1980s in response to the human immunodeficiency virus (HIV) epidemic. Certainly, the slow pace at which hepatitis was being addressed was eclipsed by the rapid pace required after the discovery of an immunodeficiency illness primarily in men who had sex with men in the United States in 1981, then cases in haemophilia patients in receipt of clotting factors and other transfusions within the next two years.[14] There was no direct way to screen for it until anti-HIV serology could be developed and

implemented in 1985. Even now, in the United Kingdom, questions are being asked about the slowness of blood banks to respond to HIV. But how did clinicians respond at the bedside?

Surgenor and colleagues proclaimed a downturn in red cell transfusions in the United States in response to HIV in an article published in the New England Journal of Medicine in 1990.[15] They surveyed blood collection and use between 1982 and 1988. The *rate of growth* of transfusions declined between 1982 and 1986, but it was not until the 1987 survey that an actual *per capita* fall in transfusions was recorded. Growth in autologous donations was noted, although this finding rests only on changes between 1986 and 1987 as prior data were not available. The authors rightly reported that the study did not investigate the cause for these changes, but did note the synchrony with the HIV epidemic and an awareness of blood transmission in contributing to transmission. However, a fall in rates of transfusion did not occur until after implementation of HIV serological screening. The authors of this paper noted that the ability to reduce transfusions suggests an element of "latitude in the decisions to administer transfusions." This argument was supported by large regional variation in red cell transfusion rates.

Examination of the continuing reports of red cell transfusion rates before and since the HIV epidemic in the United States using the same methodology are summarised in Figure 1.1. There was a decline in the rates of transfusion during the mid-1990s, only to see a rise again after 1994. Transfusion rates again started to decline after 2005. These data do not show, or even investigate, causality. There was a recognition that HIV was transfusion transmissible by 1983, serological testing implemented in 1985 and hepatitis C serology in 1990.[14] Perhaps transfusion was perceived as "safe" again. The first major randomised controlled trial of liberal versus restrictive transfusion strategies was published in 1999, with more to follow.[16] The subsequent plateau and decline in transfusions rates suggests that clinicians may have responded less to the perceived risks than the reports emerging of lack of benefit. It suggests that Crosby's call almost a quarter of a century earlier against unnecessary transfusions was not heeded, even with emerging awareness of infectious threats.

Figure 1.1 Estimated red cell transfusions in the United States of America by year. Data collated from sequential studies. [15,17-25]



Another important outcome in the blood banking sector following HIV was the adoption of the "precautionary principle." This prioritises public health over economic gain and recognises uncertainty when considering the appropriate "level of evidence" required for intervention or maintaining the status quo.[26] Where the risk is high, only a low level of confidence in the evidence may be required to support a particular approach.

This risk-based approach is likely to be intuitive to many clinicians. Indeed, "*primum non-nocere*" (first do not harm) is often enshrined in codes of ethics as an expression of non-maleficence. However, in transfusion medicine its application appears to have been predominantly left to the blood banking sector. It is apparent from the history of transfusion that clinicians interpret data within a professional cultural context that overlooks harm while perceptions of benefits, often passed between generations of teachers to students, are held as truth in the absence of evidence for or against and the risk-benefit ratio not reconsidered. It appears that practice did not undergo sustained change despite the emergence of increasing risk and the precautionary principle not applied to clinical transfusion practice. The academic paradigm has become one based on large population randomised studies,

where these and meta-analyses are graded as high level evidence and a failure to demonstrate such evidence may be interpreted as a reason to maintain the *status quo*.

Inappropriately applied, this "evidence-based" approach may enshrine current practice despite emerging risks. While new evidence was eventually forthcoming, a reappraisal of practice based on the available evidence should have been the predominant response, adopting a precautionary principle. To look at risk we have to look beyond randomised trials and include cohort studies.[27] Where would we be if blood banks had waited for a randomised trial before implementing risk management strategies with HIV? It seems inconceivable to contemplate such an approach, yet this appears to have been the response at the bedside in transfusion medicine. Liberal transfusion strategies persisted despite evidence of new risks waiting for randomised trials to disprove efficacy where practice had emerged in an evidence vacuum. This also despite evidence from Jehovah's Witnesses without cardiovascular disease who have refused blood transfusion that mortality is not increased compared with those that have transfusion, even at haemoglobin levels significantly lower than those used in restrictive arms of clinical trials.[28] Transfusion was a belief-based practice.

The early history of transfusion has taught us that we need to get the indication for transfusion right. In the early decisions to transfuse we can see evidence of the culture of medicine at the time impacting on individuals. The middle of the twentieth century saw the expansion of transfusion to apply a "rule" that had no basis in evidence and likely did not even represent the opinion of the authors to whom it is most frequently attributed. It is evidence of the powerful effect of the culture in medicine. The latter part of the twentieth century saw the dogged persistence with liberal transfusion and further highlights the importance of cultural norms rather than evidence in directing practice. How is it that clinicians failing to adjust practice to an emerging threat are spared the scrutiny applied to blood banks who clearly responded quicker?

Patient Blood Management (PBM)

The phrase "patient blood management" to the best of my knowledge, first appeared in an opinion paper by Isbister in 2005.[29] The first Medline reference to it is in 2007 by the same author (Pubmed search October 10, 2019).[30] It refers to the practice of optimising the blood of a patient as an organ in its own right, aiming to optimise red cell mass prior to expected losses, prevent excessive bleeding by medical and surgical means and to consider the tolerance for anaemia before replacing blood. It is not simply transfusion avoidance, and although it may have modern roots within transfusion circles, it is best seen as a reclamation of what has been lost. What was lost was not knowledge, although arguably lessons learned in history may be buried from modern clinical decision-makers, but a cultural approach involving attention to haematological system integrity in favour of an easy and acceptable replacement therapy in the form of transfusion.

Patient blood management is distinguished from blood management, the latter term having been applied frequently in blood banks in reference to issues like stock holdings and rotations. These are important issues in maintaining the blood supply and to ensure availability to patients when they need it, but are different to managing a patient. Cardiac management would be seen as optimising a person's heart function rather than the movement of donated organs for transplant, but with blood this would create ambiguity.

There is inconsistency in the perception of PBM. It is difficult to detach from a product-focused paradigm with many proponents coming from the blood banking sector. By way of example, Sekhar and colleagues reported on a PBM program for platelets where the objectives were to improve appropriate use of platelets, reduce wastage and reduce costs.[31] These are all worthy aims, but it focused on the use of product rather than patients' needs. The report included no assessment of patients with thrombocytopenia who may have potentially benefitted from platelet transfusion, no assessment of efficacy in achieving haemostasis or measures that were conducted in addition to platelet transfusion to maintain or achieve haemostasis. The reported outcomes were consistent with

the aims and worthwhile. But they were product rather than patient focused and therefore, at least in my view, should not be considered PBM.

This ambiguity is reflected in a variety of definitions for PBM (see Table 1.1), some notably limiting the scope to include only patients who might otherwise need a transfusion. Others have a broader scope and while recognising potential harms of transfusion, also recognise potential harms of anaemia or uncontrolled bleeding in their own right. Some organisations have provided different definitions in varying circumstances. Notably, while Australia's National Blood Authority (NBA) has coordinated the development of national PBM guidelines that have been lauded, they have two definitions that are significantly different. In the companion documents to the Guidelines, the NBA cites a definition from the Society for the Advancement of Blood Management.[32,33] This definition includes no reference to blood products, focusing instead on a goal to improve patient outcomes by actively considering how best to optimise the components of blood, preventing bleeding. By contrast, the definition in the Guidelines states the aim of PBM is to avoid blood product transfusion.[34]

Table 1.1: Variations in definitions of Patient Blood Management

Organisation	**Definition**
AABB (American Association of Blood Banks)[35]	An evidence-based approach to optimizing the care of patients who might need transfusion

Australian Commission for Safety and Quality in Health Care[36]	Patient Blood Management (PBM) takes an individualised, multidisciplinary approach to the management of a patient's blood, through assessment and the development of a management plan to: • optimise a patient's own blood (identify and address the health conditions that might lead to a blood transfusion such as anaemia or iron deficiency) • minimise blood loss (such as surgical techniques that reduce blood loss) • optimise tolerance of anaemia (with appropriate management, the body may tolerate anaemia without resorting to blood transfusion). PBM should be the standard of care applied by all clinicians for patients facing a medical or surgical intervention who are at high risk of significant blood loss.
European Union[37]	Adopted WHO definition
International Foundation for Patient Blood Management[38]	An evidence-based bundle of care to optimise medical and surgical patient outcomes by clinically managing and preserving a patient's blood
National Blood Authority (NBA), Australia	Endorse SABM definition[32] Patient blood management aims to improve clinical outcomes by avoiding unnecessary exposure to blood components. It includes the three pillars of: • optimisation of blood volume and red cell mass • minimisation of blood loss • optimisation of the patient's tolerance of anaemia. Patient blood management optimises the use of donor blood and reduces transfusion-associated risk.[34]

Society for the Advancement of Blood Management (SABM)[33]	**Professional Definition:** Patient Blood Management (PBM) is the timely application of evidence-based medical and surgical concepts designed to maintain haemoglobin concentration, optimize haemostasis and minimize blood loss in an effort to improve patient outcome. **Public Description:** Patient Blood Management (PBM) is the scientific use of safe and effective medical and surgical techniques designed to prevent anaemia and decrease bleeding in an effort to improve patient outcome.
World Health Organisation[39]	Patient blood management (PBM) is a patient-focused, evidence-based and systematic approach to optimize the management of patient and transfusion of blood products for quality and effective patient care. It is designed to improve patient outcomes through the safe and rational use of blood and blood products and by minimizing unnecessary exposure to blood products. Essential elements of patient blood management include: the prevention of conditions that might otherwise result in the need for transfusion (through health promotion and screening for early detection), appropriate diagnosis and optimal treatment, including the use of alternatives to transfusion, good surgical and anaesthetic techniques, the use of alternatives to blood transfusion and blood conservation.

PBM has been characterised by "three pillars." These are based on a perioperative model. Gombotz et al studied transfusion variation in orthopaedic surgery in Austria.[40] Pre-operative anaemia was found to be a risk factor for transfusion, as was surgical duration and technique impacting on surgical blood loss. Finally, post operatively the willingness to give transfusions also varied, and application of the evidence on the safety of restrictive transfusion thresholds varied. Pre-operative optimisation, surgical technique to prevent blood loss and considering tolerance for anaemia have been outlined as the pillars.[41]

Current evidence suggests that transfusion avoidance and improved patient outcomes are likely to frequently align, so the distinction between the definitions may seem academic. It is not. The stated goals of these definitions are to either reduce transfusion, or to improve patient outcomes by a holistic approach to the blood as an organ system. One definition does not consider the patient within scope

if they are not likely to need a transfusion, the other allows that there may still be benefits and has the patient outcomes as the goal. While reducing transfusion was an outcome of a local obstetric PBM program, I will argue through data presented in this thesis that significant benefits have accrued to women who would not have approached transfusion thresholds.[42] The definitions also matter, especially as programs to implement PBM emerge, because resources will be directed to meet the perceived objective. If the goal is to reduce transfusion, then resources may be limited to achieve that goal, whereas if we choose to improve patient outcomes by managing the blood, then the outcome is measured not just by units saved, but by improvements in our patients. In this thesis I have adopted the SABM definition as it describes precisely the broad scope of work that is needed to maximise the blood for the benefit of patients, of which transfusion management is a subset.

The evidence behind PBM has been reviewed recently in the perioperative context and concern expressed over a lack of evidence for PBM programs.[43] While this statement reflected a consensus group interpretation of the literature, there is also no consensus on what a PBM is program is. In most cases a program is seen as a systematic approach to implementing strategies to manage patients' blood, in particular optimising red cell mass prior to impending losses, and preventing unnecessary losses. In the consensus statement, Mueller and colleagues defined it as having interventions from at least two of the three PBM "pillars."[43] This is somewhat arbitrary and it could be argued that the simplest program conceptually (although not necessarily in practice) could be to feedback data to clinicians on the transfusion rates and indications from themselves and peers. In this model, once appraised of the potential benefits, clinicians may learn from their peers and so although the program itself targets one of the PBM pillars individually it may reach them all.

Caution needs to be exercised when insisting on randomised clinical trials to assess the efficacy of multimodal interventions. Randomised controlled trials are specifically designed to establish whether a particular intervention is efficacious, acknowledging that effectiveness may depend on factors not controlled within the study. Multimodal interventions may incorporate practices with no benefit or

even harm, when other interventions have an overriding benefit. In sepsis, transfusion to achieve a haematocrit of more than 30% was incorporated into an early intensive intervention strategy which significantly improved survival.[44] A meta-analysis of randomised trials comparing only transfusion at higher (90-100g/L) or lower (70g/l) haemoglobin thresholds has by contrast shown no difference in mortality between the groups.[45] The first study would have a high transfusion threshold adopted based likely on the efficacy of concurrently implemented interventions. Randomised trials can work well to demonstrate efficacy of single interventions, but when used with multimodal interventions do not indicate which of them is working, or indeed whether some components of the intervention could be causing harm.

There are adequate data to support many PBM interventions, such as perioperative iron replacement. It would be ethical to compare outcomes from hospitals or surgeons that did and did not implement this practice. However, if an intervention is known to help it would not be ethical to randomise an institution not to treat. Programs are, by most definitions, multifaceted, drawing on the evidence shown to effect good outcomes. Implementation science is about showing how best to introduce new processes or procedures. While randomised trials may help to support implementation, a lack of evidence on how to implement should not prevent institutions undertaking sensible or trial proven practices, as individual processes or part of a wider strategy.

The Western Australian Government implemented a perioperative PBM program in 2009[46] and were recognised as world leaders.[47] Already with low rates of per capita transfusion, the focus was not on benchmarking to other jurisdictions, although it did allow clinicians to compare transfusion rates and haemoglobin triggers as a part of the implementation strategy,[48] but to focus on what more could be done. Australia, through the NBA, published the first of six PBM Guidelines in 2011.[49] There was a thorough review of the literature, engagement with experts throughout the process and expert opinion provided within the guidelines. Gaps in evidence were also highlighted and were considerable. These first guidelines covered critical bleeding and were only able to make one firm

evidence-based recommendation: that institutions should have a common agreed plan to manage critical bleeding and massive transfusion. The evidence for what should be in it was too poor to make firm recommendations on the content, but the value of everyone involved in critical bleeding united in one agreed plan was recognised.

Transfusion within a culture of PBM

If the principles of PBM are adopted, transfusion is not to be avoided, but to be used when it is the most appropriate therapy. For red cell transfusions, there remains a dearth of randomised controlled data in support of when to transfuse, and plenty of data to suggest when it is safe not to.[50] Randomised trials have been done, mostly comparing liberal vs restrictive red cell transfusion strategies, with few studies favouring liberal transfusion.[51] As shown by figure 1.1, we are in an era of declining red cell transfusions and are likely still in an era of over-transfusion. Under-transfusion has yet to be defined, but that is not to say that it does not exist. Ultimately, the demonstration of clinically meaningful outcomes requires clinical trials, but that leaves an open question on who to include in such trials and what they should compare.

All trials in red cell transfusion to date have used the haemoglobin concentration. While this correlates with red cell mass, the latter may be more relevant. The former is affected by fluid status as well as red cell mass. Patients may have different needs. Cyanotic comorbidities affect the amount of oxygen that can be carried on red cells; vascular and cardiac comorbidities may affect perfusion of red cells carrying oxygen to the tissues. The amount of activity alters tissue demands, as do hypermetabolic states. While some trials have examined transfusion in subgroups with particular comorbidities, thus stratifying for some of these factors, clinical trials have generally not accounted for this individual variability. This has sparked debate on the applicability of our clinical trials in certain populations, such as the elderly or frail.[52] Perhaps more significantly though, it highlights the lack of good measures to indicate the impact of anaemia in patients with varying needs. The haemoglobin concentration is being used as a trigger for transfusion, despite its failings, due to a lack of alternatives.

If the evidence of benefit from red cell transfusion is scarce, there is even less for other fresh blood products. With the exception of thrombotic thrombocytopenic purpura, the evidence for plasma transfusion for improving outcomes based on randomised trials, is minimal.[53] There is only a little more for platelet transfusions. It is harder to conduct placebo-controlled efficacy trials without transfusions when they have become the standard of care. Plasma trials have used standard coagulation tests or viscoelastic tests, acknowledging that the way we measure the effect of transfusion may be important. There are also more options for treating coagulation problems, with fresh frozen plasma, cryoprecipitate or fractionated plasma factor concentrates. Likewise, the role of platelet transfusions varies in practice due to the uncertainty about when they may be of value.

Measuring to improve

It is often said in the field of quality improvement that you cannot improve what you cannot measure. Whether it is attributed to W. Edwards Deming or Peter Druker, it is an over-simplification of their initial statements. It is however true that if we don't measure in some form we cannot establish whether a change is an improvement. Both measurement and the ability to change a process are required to consciously undertake improvement, and the outcomes measured are the most likely to be the outcomes improved. Other outcomes may also improve, often labelled as the Hawthorne effect, but it is also possible that other outcomes not targeted may get worse. It follows that choosing the outcomes to measure is critical to good quality improvement.

The process of measuring and feedback can, but does not necessarily, change outcomes. This technique was used by the Western Australian Patient Blood Management Program to successfully reduce transfusions.[46,48] Providing information to compare transfusion practice on a "dashboard" so that clinicians could easily manipulate the data for comparison was effective, without having to specifically devise individual programs in different centres or departments. It was combined with information about situations when red cell transfusion lacked proven benefit, enabling clinicians to reflect on their own practice and compare against their peers, procedure by procedure. It enabled

visibility of transfusions where the triggers were likely to be inappropriate to prevent future episodes. However, there are still few data on when transfusions should be given.

Looking at both sides

Measurement is also essential to understand the risks and benefits. While the benefits of early transfusions were promulgated, the need for a systematic approach to measuring harm was acknowledged, but not implemented. Without a systematic approach to measuring benefits and harms, the perceived lifesaving effect of blood transfusion in critical bleeding could be extrapolated to less emergent areas, a process termed "indication creep" and still prevalent today.[54] During the 1990s the need for greater understanding of adverse transfusion events was recognised internationally and processes developed to enable systematic reporting, a field termed haemovigilance.[55,56] There are problems with haemovigilance even when programs are in place, principally because it relies usually on both recognition and reporting of adverse transfusion events by clinicians.

In my own institution, adverse transfusion reactions are actively followed up by the transfusion clinical nurse consultant and haematologist overseeing transfusion. Clinicians recognise potential immediate transfusion reactions at the bedside. Blood is often stopped and returned to the transfusion laboratory. Reporting of adverse events through the hospital risk management system is usually undertaken by the clinical laboratory rather than clinicians. Reactions where the transfusion is not ceased are infrequently reported.

We studied adverse events associated with lower doses of intravenous immunoglobulin at the transition between 6% and 10% intravenous immunoglobulin products.[57] With different products and concentrations of immunoglobulin available, our hospital protocol for intravenous immunoglobulin infusion had uniform infusion rates by volume. The transition to a higher concentration for a large number of people presented the opportunity to examine how higher immunoglobulin infusion rates

due to the more concentrated product would affect reactions to the products. Patients were recruited prospectively and asked to report any adverse events during or after infusion.

The study showed that the higher infusion rate had acceptable toxicity. There was a higher rate of reactions after the switch to a new product, as has been seen when patients move from one product to the next in previous studies.[58] Although 49% of patients in the study reported at least one adverse reaction during or after intravenous immunoglobulin infusion, none were reported through the usual hospital reporting mechanisms. The study showed a weakness in reporting adverse transfusion events. Although these were all mild, a failure to understand the impact of transfusion practices, including adverse events, skews our decision making. Trials of intravenous immunoglobulin for the prevention of infection in haematological malignancies have shown benefit in reducing infection risk.[59-61] Cost-effectiveness has been questioned. When overall quality of life was considered in one study there was no net benefit with intravenous immunoglobulin.[62] The primary outcomes of reduced infection rates is relevant to determine efficacy of immunoglobulin therapy, but to demonstrate effectiveness needs to consider the overall impact on quality of life. To do this, adverse events need to be measured, and ideally overall quality of life.

Adverse event monitoring therefore takes on additional importance when studies have examined only benefit as the primary outcome. Aware of these data, clinicians may offer treatment in circumstances where the benefit is minimal, or perhaps there is no benefit when the intervention is the subject of "indication creep." This is even more likely when lack of measurement induces blindness to the impact of transfusion on quality of life. It highlights the importance of haemovigilance, and as electronic medical records become more widely used at the bedside, more active surveillance may help clinicians appreciate the impact these are having on our patients. This thesis will not expand further upon the role of haemovigilance, but it is worthwhile acknowledging this as a manifestation of a common problem in transfusion medicine – measuring only one part of a system.

Measuring only one side of a clinically relevant system is also seen in the field of coagulation. The commonly used tests of coagulation, prothrombin time and activated partial thromboplastin time, have been developed to evaluate the effects of anticoagulants, warfarin and heparin in particular. They have found other uses. Prothrombin time is sensitive to factor V and factor VII deficiency and so is a prognostic marker in liver disease.[63] It is appropriately used to measure coagulopathy of liver disease for this reason. However, this is often extrapolated to guide the prescription of plasma to prevent bleeding. The result we measure is abnormal, indicating a deficiency of clotting proteins, so these are replaced. The liver makes many proteins, and in haemostasis it manufactures natural anticoagulants as well as clotting factors, which are both depleted in liver disease. This results in a risk of bleeding and clot formation in liver disease. Prophylactically trying to correct coagulopathy has not been shown to reduce bleeding and is not recommended prior to procedures in current guidelines.[64,65] Notwithstanding the fact that replacement therapy for labile factors like factor V is extremely short-lived and therefore unlikely to be effective in prophylactic situations, the approach itself is based on the assumption that we have measured an imbalance – that a prolonged prothrombin time indicates an imbalance in haemostasis, without considering the impact of reduced anticoagulants. Here, at least theoretically, assays based on whole blood have merit.[64] A larger discussion on whole blood coagulation assays will follow in chapter 4. It is worthwhile mentioning coagulation here as an example of how transfusion therapy has probably been frequently misdirected by incompletely measuring a system and using the tools we have at hand for whatever purpose arises, rather than seeking better tools.

Quality of life

Measuring quality of life requires quite a different set of tools. This is important, particularly in transfusion where many transfusions are given to relieve symptoms. Quality of life has been the primary outcome in many studies evaluating the efficacy of erythropoietin,[66] but is less commonly used in transfusion.[67,68] Staibano and colleagues reviewed the use of patient centred outcome tools

for anaemia in 2018.[69] Of the 130 studies included, only 5 were for the assessment of transfusion as an intervention, with erythropoiesis stimulating agents and iron being most commonly studied. Oncology and renal settings were most commonly evaluated and the Functional Assessment of Cancer Therapy (FACT)[70] / Functional Assessment of Chronic Illness (FACIT) scales being the most commonly used tools.

The ability to measure outcomes relevant to patients is critical when discussing transfusion. Randomised studies in acute illness have largely used mortality as the primary outcome. While this is highly relevant in this setting, in the chronic anaemia setting, transfusion aims to improve quality of life. Studies with erythropoiesis stimulating agents have set higher haemoglobin targets up to 110-120g/L and show improved quality of life. Some have argued that liberal transfusion strategies targeting similar levels in myelodysplastic syndrome and other chronic anaemias may improve quality of life.[71] To date, data supporting one strategy over another are limited. A systematic review in 2015 found only a single small randomised study in myelodysplastic syndrome, closed due to poor recruitment.[72] It is not easy to translate the outcomes from studies evaluating erythropoiesis stimulating agents to transfusion as the former requires only infrequent self-administered subcutaneous injections, whereas there is a significant cost, as perceived by patients on their quality of life, with transfusion dependency.[73]

Development and validation of such tools requires input from consumers and clinicians, usually beginning with qualitative research to determine domains to be considered and how relevant they are to a particular group.[74,75] Triangulation with data external to the instrument itself, such as measures of functional capacity, the levels of physiological variables, such as haemoglobin concentration, or clinical assessments, such as clinically diagnosed depression. Due to these difficulties, studies may use more than one outcome assessment score, or include other similar tools, such as visual analogue scales or assessments of physical function. The latter have included measures of exercise capacity,

such as 6 minute walk tests and, more recently, measures of activity, such as activity tracking with step counters.[68,76,77]

There are multiple quality of life scoring questionnaires available, usually scoring systems with different domains. While these aim to bring objectivity to quality of life assessment, there are inherent potential problems, such as what aspects of quality of life to measure and how they should be weighted. General instruments may be supplemented by additional scales with questions that relate to particular conditions or circumstances, as the general instruments may be insensitive to particular issues under question. There is an element of subjectivity in that the instrument chosen is weighted towards issues that investigators think are most relevant to the particular population in question.

Choosing the right instrument does matter. In a recent randomised study of transfusion at higher or lower haemoglobin thresholds in haemopoietic stem cell transplantation, the FACT-BMT (Bone marrow transplant subscale) was the primary outcome.[78] While the outcome is entirely consistent with other data in the acute setting showing a lack of difference between higher and lower haemoglobin thresholds, and the choice of the FACT-BMT appears entirely appropriate for the setting, the score itself has questions related to engagement and trust with their medical and nursing carers and its sensitivity symptoms of anaemia could be questioned. The study cited also used FACT-An (Anaemia), which has been well-validated for symptoms of anaemia. Overall, there was no convincing difference between the liberal and restrictive transfusion groups, although only just over one third of participants completed the FACT-An assessments, so the power to detect a difference is uncertain. Despite this, there was a significant difference between the adjusted FACT-An scores at day 7 favouring the restrictive transfusion group. It highlights the importance of selecting a questionnaire that is appropriate for both the study setting, and the study outcomes in question.

The Law of the Instrument

It is very easy to measure haemoglobin concentrations and platelet counts. Modern automated analysers do so reliably and there is no doubt that they correlate with outcomes. They have significant limitations. Platelet transfusions do not differentiate well between haematological malignancy patients who will bleed and those who will not.[79] In measuring the haemoglobin concentration we fail to see the expanded red cell mass of pregnancy and it pays no heed to a person's ability to tolerate anaemia. But these are the tools we have.

Kaplan noted in *The Conduct of Inquiry* in 1964 the propensity to design research and measure outcomes with the methodological tools we have at hand.[80] He called it the "law of the instrument" and provided the now often quoted analogy, "Give a small boy a hammer, and he will find that everything he encounters needs pounding." While speaking of the behavioural sciences, this is also true in medical science. We measure with the tools we have, not necessarily with those that are most ideal. For transfusion, the impact of anaemia on oxygen delivery and functional outcomes; the impact of platelet or plasma transfusion on whole blood haemostasis and bleeding; for immunoglobulins the effect on infection rates or improvement in the underlying immune condition.

What should we make of Kaplan's boy? From the boy's frame of reference, the hammer may be his only tool as he seeks to understand the world. It will work well nailing timber together, and he'll learn quickly that it is effective at opening windows, though the unintended consequences may be severe. Understanding is absolutely required, as from the boy's viewpoint "*everything* he encounters *needs* pounding." There is no option, for he seeks understanding and he has only one device. As with the scientist, when he tries his tool, he will learn when it works and when it falters.

As a child myself, a hammer was a useful tool to crack open macadamias. With their round shell an off-centre hit could see the nut fly across the yard and be lost. Too hard a hit could see the nut and shell pulverised onto the rock or timber on which it was set. A straight tap we learned, was required, but hard enough to crack the shells. These are tough and require significant force beyond the standard

kitchen nutcracker, but respond well to additional force applied using a threaded cracker. The hammer was not the best tool, but it was the one we had. We learned how to use it as best we could. Of course, we couldn't use a tool we didn't have, but we need to acknowledge the flaws in our approach while exploring new avenues.

In patient blood management there are useful tools that we don't yet adequately apply or even know how to use. Measuring iron deficiency is one such circumstance. There is a good test in the serum ferritin, but still questions about its application. Population normal ranges don't necessarily correlate well to treatment thresholds when iron deficiency anaemia is prevalent. There is still work to be done to understand treatment thresholds. There may also be better ways to test or screen by measuring the impact of iron deficiency on erythropoiesis.

There are potential options to measure the impact of red cell transfusion on physiological parameters. There is acceptance that tolerance of anaemia varies widely, but without systematic ways to measure this it is hard to study. The haemoglobin concentration is very much like our hammer for nut cracking. It sometimes works well, but sometimes the results are well off the mark. The solution needs a different way of thinking.

Our ideal approach to the boy with a hammer would be threefold:

1. Learn when to use the hammer, and when not to;
2. Learn the best way to use the hammer, when it is required;
3. Acquire or develop more appropriate tools. The hammer can't do everything.

An additional problem with transfusion trials is selection bias. The strong belief that transfusion works may limit recruitment to trials for those considered most at risk from not having transfusion. This was recently demonstrated (even if somewhat staged) in a randomised controlled trial of parachutes.[81] The trial showed parachutes were no more effective than backpacks when jumping off planes. While extreme, the study clearly demonstrated the impact of selection bias on outcomes, with people

recruited only when they were approached while sitting on a small stationary plane parked at an airport, not when approached to leap from a commercial plane in flight. The same bias has the potential to confound studies in transfusion where there have been now generations of medical practitioners trained in the "10/30" rule or some other, more recent arbitrary transfusion trigger. The same biases could equally apply to maintaining haemostasis. One way around this selection bias is to completely change our frame of reference. New tests, linked to physiological outcomes, could serve to detach us from old ways of thinking. As I shall discuss later when reviewing near infrared spectroscopy (NIRS), failure to blind clinicians used to prescribing according to the current paradigm may be a potential source of significant error hampering the evaluation of new techniques.

To be accepted as potential replacement strategies to transfusion decisions, new tests need to have face validity. They need to have a pathophysiological rationale for claiming superiority. This then needs to be supported by evidence of clinical utility and ultimately clinical trials comparing different approaches. The endpoints of studies need to be diverse yet appropriate to established benefits and risks and early studies may need to examine surrogate markers. It is a big task that needs to start small and progress to larger more definitive studies once testing approaches and their rationale have been elucidated.

In the remainder of this thesis I will identify areas where understanding the ways we measure in patient blood management could be improved and investigate ways this may be achieved. Patient blood management is multidimensional, from identifying and actively managing those at risk of bleeding or anaemia, to applying appropriate selection of patients for transfusion and considering pharmacological, surgical, anaesthetic and system factors that could be enhanced. So too, is this body of work. Focusing on only one facet of patient blood management would belie what it is.

This work shall also aim to address research questions at various stages of maturity. It shall explore the potential for new tests, implementing old tests in new settings, and refining the understanding of well-established tests so that they can be used more effectively in patient management. It is a

deliberate approach to adopt a multi-faceted research toolkit as this is required in order to advance patient blood management.

I shall explore better ways to detect iron deficiency in chapter 2. Ferritin has known limitations, but remains the best test in most circumstances. I shall explore those limitations and argue that current interpretations based on population norms do not meet the needs of patient blood management programs, where correlation with functional outcomes and benefit from therapeutic interventions is required. There are gaps in our understanding of how ferritin may be used and I shall investigate pregnancy, one of these known gaps. This has been done by retrospectively analysing data before and after implementation of patient blood management in obstetrics.[1] I shall also explore the potential for red cell and reticulocyte indices to be used as screening tests for iron deficiency, as these are produced without additional samples by current automated analysers and have the advantage of being able to screen for patients at risk from the laboratory without additional tests being ordered.[82,83] This has been done by a prospective observational study in antenatal clinics, correlating novel red cell and reticulocyte indices to ferritin.

In Chapter 3 I shall explore some of the limitations of using haemoglobin concentration as a transfusion trigger and seek to determine whether measuring oxygen saturation directly in tissue with near infrared spectroscopy may be an alternative, more physiologically sound basis upon which to base red cell transfusion decisions. There have been numerous data published on NIRS and I shall bring these together in a systematic review aiming to clarify the role of NIRS in transfusion decision making. Understanding that the impact of anaemia may only become apparent under additional physiological stress, this shall be further explored through a prospective observational study evaluating the impact of anaemia on tissue oxygenation during exercise, both aerobic and short isometric, and seeing how this correlates with functional performance as measured in the six minute walk test (6MWT).[84]

Chapter 4 will explore haemostasis, both how we measure it and how we respond to those abnormalities. I have replicated and extended the work of others examining the potential for

additional fibrinogen replacement to compensate for thrombocytopenia. In doing so, blood constituents were evaluated to determine in vitro which could be used to enhance haemostasis, as measured by rotational thromboelastometry. A single arm phase 2 interventional study with cryoprecipitate was then conducted to determine whether it may improve haemostasis. During this, I sought to develop a new way of measuring the difference in haemostasis at very low platelet counts under high shear stress with a whole blood coagulation assay. This study also examined changes in conventional coagulation assays, particularly clot formation curves, during the interventions.

There remains ample scope to refine patient blood management practice. While there is room to improve implementation of what we know, understanding how better to measure the impact of anaemia, haemostasis and transfusion therapies may be the best way to advance the field.

Chapter 2. Detecting and managing iron deficiency

Iron deficiency is the most common cause for anaemia worldwide.[85,86] While it impacts developing populations more than the developed world, it remains an issue regardless of the economic status of countries and individuals. Iron absorption is limited and impacted by the source of iron; its ionic state and concurrent food intake, such as tannins in food, may inhibit absorption. Changes to stomach pH may influence the ionic state of iron and impact absorption, an under-recognised issue with long term proton pump inhibitor use.[87] As such, the iron content of food does not necessarily translate into improved absorption.

The best sources of iron are haem-based, meaning the intake of meat is associated most clearly with iron stores across populations.[88] Iron demands during the growth and development years is high and at these times iron stores are generally poor. The same is true in women until menopause, with menstruation and pregnancy increasing iron loss. Physiologically then, reduced or absent marrow iron stores are common. Dietary depletion or pathological blood loss, particularly from heavy menstrual or gastrointestinal bleeding, are commonly seen in addition to low levels of absorption. Tipping from the common state of depleted iron stores to iron deficiency anaemia is relatively frequent.

Perhaps because it is common, iron deficiency may be frequently overlooked. A PBM approach would encourage us to look not just at the anaemia, but to pre-emptively address non-anaemic iron deficiency in situations where bleeding is anticipated, like surgery with a high risk of bleeding. Despite this many people reach elective operating theatres without attention having been paid to their anaemia or iron deficiency.[89,90] In most cases the failure is a lack of implementation into practice of what we know rather than knowledge deficits, but there are still many areas where uncertainty exists and these should be addressed by further research.

Despite the high prevalence of iron deficiency in the community, there remains no consensus on how to detect and manage it. Detection requires simple, readily available tests that are sensitive to iron deficiency, low cost and accessible, given the frequency of iron deficiency in the community. Ferritin is the most commonly used for iron deficiency and haemoglobin is used for anaemia. Both have their benefits and pitfalls and while the assays for both are widely available and standardised, both are subject to areas of uncertainty in their use and interpretation.

Perhaps the biggest controversy with measuring markers of iron stores, is the importance, or otherwise, of non-anaemic iron deficiency. Just as there has been indication creep in the application of transfusion, the treatment of iron depletion without anaemia due to the false attribution of symptoms to it, may lead to excessive use and exposure of large numbers of people to therapy from which they will not benefit. By contrast, if iron depletion without anaemia does cause symptoms or places people at risk, then the high prevalence means a high potential to improve the health of the population through identification and intervention.

Non-anaemic iron deficiency is largely beyond the scope of my research, except where it identifies people at risk of being unable to adequately respond to anticipated blood loss. However, when it comes to making decisions about screening and therapy, an understanding of the current literature in the field is relevant and shall be briefly addressed here.

It is well-established that depletion of iron stores occurs prior to the onset of anaemia. Iron depleted erythropoiesis can be identified by reduced haemoglobin and red cell size, and these may be identified in reticulocytes prior to the effect being measurable on the long-lived total red cell population.[91] The physiological impact occurs prior to the ability to detect it in standard red cell parameters. Red cells are not the only site where iron is required. Myoglobin is also iron rich, but every cell uses iron in electron transport processes. Population based studies have suggested that iron depletion may impact on fatigue, cognition and exercise performance in the absence of anaemia.[92] Iron is essential to human development and in utero, infant or early childhood, iron deficiency has been correlated with adverse

behavioural outcomes in later life.[93,94] Prospectively, iron therapy has been shown to improve fatigue in adolescent females with iron deficiency with no or mild anaemia.[95] Oral iron therapy has also been shown to improve exercise performance in young iron depleted non-anaemic women, including during pregnancy, and reduces later behavioural issues in low birth weight infants[96-99] The benefits of iron therapy in pregnancy are accepted and the WHO recommends replacement therapy in all pregnant women, with the dose dependent on population risk for anaemia.[100] There remains controversy about the best way to screen and replace iron in pregnancy in Australia, which is best solved by further research.[101,102]

There are various definitions of iron deficiency based on ferritin. Thresholds range from 10 to 30µg/L and may be higher with concurrent conditions.[103,104] The basis for these thresholds vary. Based on a normal population (using non-parametric statistics as ferritin does not have a Gaussian distribution), women may be considered normal with a ferritin more than 8µg/L,[105] but iron stores are generally depleted from the marrow when ferritin is less than 30µg/L.[106] Given that human development and evolution has occurred in the context of much of the population having marginal iron stores at best, it could be argued that the presence of marrow storage iron is not necessarily a prerequisite for normal functional iron use. Ferritin is also an acute phase reactant. It also increases in liver disease, and in particular, increases in non-alcoholic steatohepatitis. The increasing body mass of the population therefore impacts on ferritin.[105,107] The effect of this on the diagnostic utility of ferritin at the lower end of the range for the detection of iron deficiency remains unknown. The 5th centile for ferritin is higher in overweight women, but whether this is due to increased body mass affecting ferritin production or improved iron stores due to their dietary intake is unknown.[107]

An alternative approach to a statistical population-based approach to establishing a reference range is to determine the functional impact. Below what ferritin level can a functional impairment be measured? This is difficult as the effect of iron deficiency other than on erythropoiesis is difficult to assess and may be long term. An alternative approach, asking below what ferritin level iron

supplementation improves outcomes, may be valid in some circumstances, but is confounded by the potential for iron (particularly intravenous iron) to improve performance in functional iron deficiency. This is well established in renal failure and more recently in cardiac failure.[108-110] The level at which a therapeutic intervention leads to improvement is problematic for defining a "normal" range, but from a PBM perspective defining at what point intervention should occur is ideal.

The ferritin at present remains the best measure of iron stores, but there is a lack of consensus about its use. Ideally, for the purposes of PBM, a level at which replacement therapy impacts haemoglobin levels should be identified. Having established anaemia in pregnancy as a risk factor for transfusion,[42] and both under diagnosis and under treatment of iron deficiency, we undertook a practice improvement project to improve outcomes in pregnant women, specifically aiming to reduce the incidence of anaemia at delivery.[111] As a quality improvement activity, the project's approach was based on what was considered the best available evidence. This included using a ferritin threshold of 30µg/L to define iron deficiency.[103] It provided an opportunity to explore the impact of ferritin levels during pregnancy and by comparing the rate of anaemia at delivery, verify or refute the value of this a priori threshold.

The lack of consistency around the lower ferritin reference range and the general lack of recognition of iron deficiency as a clinical issue, means that iron studies are often performed relatively late. In pregnancy, some guidelines only suggest screening with the haemoglobin and without a ferritin level, non-anaemic iron deficiency won't be detected.[112] It would be useful to have a screening test based on commonly performed parameters, such as the red cell indices, in order to identify people with early iron deficiency. Such screening would be useful in the setting where bleeding may be anticipated and the marrow need to respond by increasing erythropoiesis, such as pre-surgical assessments and during pregnancy. Automated blood count analysers have the potential to screen in this way.

The capability for analysers to look at red cell indices varies. Analysers that examine individual red cells can look at the amount of haemoglobin in individual reticulocytes. The percentage of

hypochromic reticulocytes has been used to identify iron deficiency and functional iron deficiency.[113] A similar approach has been advocated with analysers that measure haemoglobin in red cell lysate, but as individual red cell haemoglobin concentrations are not measured, different parameters need to be used.[114] In Beckman Coulter counters, the low haemoglobin density (LHD) is one such parameter previously used to detect iron depletion. LHD is a sigmoidal transformation of the mean cell haemoglobin concentration (MCHC), defined by the equation:

$$LHD\% = 100\sqrt{1 - \left[\frac{1}{1 + e^{1.8(30 - MCHC)}}\right]}$$

I have previously examined the potential for low haemoglobin density (LHD) to identify iron deficiency.[82] LHD was compared with mean cell volume (MCV), mean cell haemoglobin (MCH) and haemoglobin for the detection of iron deficiency. Initially, the performance of LHD was verified using criteria from Urrechaga et al:[115] Normal as ferritin >200µg/L and MCV >85fL; Iron deficiency as adults with anaemia (haemoglobin <100g/L), ferritin <50 µg/L with serum iron <7.5 µmol/L and Tsats <20%. With 1080 iron deficient samples and 8119 meeting the criteria for normal, an LHD of 5.9% provided a sensitivity of 92% and specificity of 63% for iron deficiency.

The study population omitted large numbers of people with non-anaemic iron deficiency and ferritin values closer to the cut-points. Therefore, the analysis was repeated comparing those who were iron deficient, now defined by reference ranges derived from the (non-parametric) 2.5 to 97.5 centiles (ferritin <20µg/L for women over 50 years and men, ferritin <10µg/L for women 18-50 years old and ferritin <6µg/L for children under 12 years old). This found 2239 (7.4%) of 30276 adults were iron deficient. In this setting, LHD cut-off of 5.9% had a sensitivity of 74% and specificity of 60%, with the receiver-operator characteristics (ROC) curve having an area under the curve (AUC) of 0.74 (95%CI: 0.73-0.75). By contrast, the routinely performed MCV had an AUC of 0.81 (0.80-0.82) with a sensitivity of 77% and specificity of 69% at the ROC derived cut-point of 88fL. Further results of the study are shown in table 2.1.

The study showed LHD and MCV performed similarly in children, but the LHD and MCV had lower discrimination (AUC of 0.66 (0.65-0.66) and 0.62 (0.61-0.63)) for iron deficiency in pregnancy. The latter was postulated to be due to the rapid changes in iron stores due to the expansion of red cell numbers during pregnancy. If iron depletion occurs rapidly, this may not be detected across red cell parameters that measure the entire red cell population, with a life span of approximately 120 days.

In this chapter I explore these issues further. Using data before and after implementation of a quality improvement intervention to increase the rate of measurement of ferritin and treatment of iron deficiency in pregnancy,[111] I examined the predictive value of ferritin in early and mid-pregnancy for anaemia at delivery and the impact of a systemic approach to iron management. With a population of iron replete women, haemoglobin ranges were re-evaluated. Furthermore, due to the poor performance of MCV and LHD at detecting iron deficiency in pregnancy, in a separate study I evaluated the performance of reticulocyte-based indices for the detection of iron depletion.[83] Finally in this chapter I explore some ways in which changes in haemoglobin and red cell indices across a population may be used to verify ferritin levels.

Table 2.1: Patient characteristics and results. Reproduced from Crispin et al, reproduced under licence.[82]

Group	Total number	Female (%)	Age in years (SD)	Positive for condition *n* (%)	LHD% AUC (95%CI)	Suggested cut off value (%)	Sensitivity (%)	Specificity (%)	MCV AUC (95% CI)	MCV suggested cut off value (fL)	Sensitivity (%)	Specificity (%)
Adults[1] (very normal v iron deficient)	9199	42.3	64.2 (17.6)	1080 (11.7)	0.9 (0.89-0.91)	5.9	92	63	0.94 (0.93-0.95)	86	87	91
Adults - iron deficiency[2]	30276	61.5	55.4 (20.2)	2239 (7.4)	0.75 (0.73-0.75)	5.9	74	60	0.81 (0.80-0.82)	88	77	69
Adults - functional iron deficiency[3]	30276	61.5	55.4 (20.2)	6092 (20.1)	0.66 (0.65-0.66)	na	na	na	0.62 (0.61-0.63)	na	na	Na
Antenatal - iron deficiency[2]	545	100	31.3 (6.2)	143 (26.2)	0.60 (0.54-0.65)	5.9	75	50	0.67 (0.62-0.72)	89	75	50
Antenatal with ferritin <30	545	100	31.3 (6.2)	389 (71)	0.48 (0.44-0.54)	na	na	na	0.56 (0.51-0.61)	na	na	na
Paediatric	2263	43.2	5.1 (3.4)	76 (3.4)	0.79 (0.73-0.85)	5.9	75	68	0.85 (0.81-0.90)	79	88	68

Very normal: ferritin > 200µg/L and MCV >85fL, iron deficient: ferritin < 50 µg/L, serum iron < 7.5 µmol/L, transferrin saturation < 20% and haemoglobin <110g/L

Ferritin <20 for females >50yo and males; <10 for females 18-50yo

Ferritin <300µg/L and transferrin saturation <20% with a haemoglobin of <135g/L and not meeting the criteria for absolute iron deficiency

Revisiting the ferritin range: A retrospective study on the impact of ferritin during pregnancy on anaemia at delivery

Objectives:

- To identify the optimum approach and timing to screen with ferritin for iron deficiency in pregnancy.

Aims:

- To determine the value of ferritin <30µg/L as a screening tool in the first and second trimester of pregnancy for the detection of anaemia prior to delivery, and at which point, or points, screening should be undertaken;
- To determine whether ferritin is a preferable screening tool for pre-delivery anaemia than haemoglobin in early pregnancy;
- To determine the value of transferrin saturation <20% as a screening tool in the first and second trimester of pregnancy for the detection of anaemia prior to delivery;
- To determine the optimum cut-point of ferritin as a screening tool for iron deficiency in pregnancy

Hypotheses:

- That ferritin <30µg/L has a high sensitivity for anaemia at delivery when measured in first and second trimesters;
- That ferritin <30µg/L is more sensitive and less specific for anaemia prior to delivery when measured in first and second trimesters than ferritin <10µg/L;
- That the sensitivity of ferritin <30µg/L is better than anaemia (as defined in each trimester) in first and second trimesters for the detection of anaemia at delivery;

- That transferrin saturation <20% is not superior to ferritin <30µg/L in first and second trimesters for the detection of anaemia prior to delivery.

Methods

Setting

The study was performed at the Centenary Hospital for Women and Children and ACT Pathology in Canberra. The Centenary Hospital is a tertiary referral centre including neonatal intensive care and high risk materno-fetal medicine unit, but also having low risk deliveries undertaken within midwife led and general practitioner shared care pathways. Hence, there are multiple pathways monitoring for women during pregnancy. The service delivers approximately 3600 babies each year. The immediate catchment area is metropolitan with a high socio-economic demographic, but the extended catchment for tertiary care includes surrounding rural areas and regional towns.

Prior to the implementation of a quality improvement intervention there was no standardised approach to the detection and management of iron deficiency. The laboratory reference range for ferritin for women reported the lower normal limit as 10µg/L based on an approximation of the 2.5-97.5 population centiles. As such, it was felt that treatment of women with ferritins above this level was unlikely. This was the impression of obstetric co-investigators and was also suggested during an audit of post-partum haemorrhage management.[42] This audit identified unmanaged iron deficiency as a potential area for improvement.

Population

The initial cohort included in this study was selected to include women immediately preceding and during the intervention phase of a quality improvement project. The study has been described elsewhere.[111] In brief, collaborators from obstetrics, the ACT Blood Counts Program (a clinical transfusion and PBM program) and the Red Cross Blood Service (now Lifeblood) identified barriers to appropriate anaemia prevention, identification and management through the multiple antenatal care pathways. Ferritin screening was conducted during pregnancy in conjunction with routine antenatal

testing (at booking and at 26-28 weeks gestation) and at 32-36 weeks gestation to determine the response to iron supplementation if required. Treatment was offered with oral iron if the ferritin was <30µg/L, a threshold based on Royal College of Pathologists of Australasia guidelines for ferritin reporting.[103] Intravenous iron was offered to non-responders. The project also included education for medical staff and midwives, both on iron management and the indications for transfusion in the peripartum period, promotion of iron therapy rather than transfusion for minimally symptomatic women and promotion of active management of the third stage of labour. The project tools have refined through other institutions, but remain the mainstay of tools available as the Maternity Patient Blood Management Toolkit (available at Transfusion.com.au).

Women were included in the study if they delivered at the Centenary Hospital for Women and Children between January 1996 and October 2015 and were divided into two cohorts: an initial cohort between July 2014 and October 2015, immediately before and during the quality improvement intervention, and; a second cohort up to June 2014 to test the hypotheses generated from the first cohort.

Data analysis

Information was extracted from the ACT Pathology laboratory information system on women who had iron studies and / or haemoglobin values measured during the study period. Using medical record numbers, data were linked for the initial cohort (around the time of the quality improvement project) with the maternity care database, which included major aspects of antenatal care, confinement and early neonatal outcomes. Iron therapy was not consistently recorded in this database. For the second cohort, pregnancy and the expected date of confinement were as provided by the requesting clinician and recorded in the pathology information system. Data were de-identified prior to analysis. The study was approved by the ACT Health Low Risk Ethics Committee and conducted in accordance with relevant local privacy regulations and the Declaration of Helsinki.

The primary outcome of the study was the ability of first and second trimester ferritin (<30µg/L) to predict anaemia prior to delivery prior to the quality improvement intervention. It was expected that the quality improvement intervention would prevent the development of anaemia subsequent to its implementation, so results were compared immediately prior to and subsequent to implementation (based on implementation impacting outcomes from 1 November 2015). Pre-delivery haemoglobin was defined as the first measured haemoglobin between 36-42 weeks gestation. Gestational age was defined by treating clinicians in accordance with best clinical practice. The analysis was repeated for ferritin <10µg/L, anaemia and for transferrin saturation <20%. The proportion of women developing anaemia with low ferritin in each of the two trimesters was compared with Chi-squared or Fisher exact tests (based on expected frequencies from the data), as were the frequencies before and after the intervention. ROC curves were constructed for the evaluated tests. Analyses were carried out in Excel (Microsoft, CA. USA) and SPSS v23 (IBM, NY, USA). Values were considered significant with 2-sided $p<0.05$.

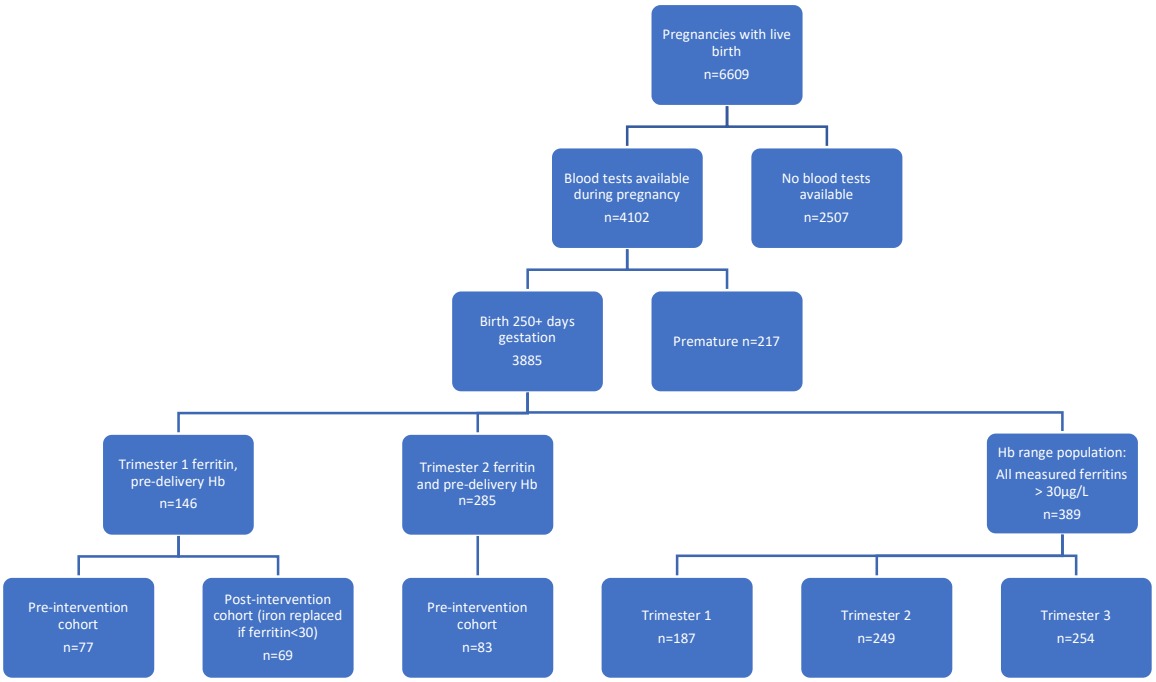
Anaemia was defined as a haemoglobin of less than 105g/L in the first and third trimesters and less than 110g/L in second trimester. In order to examine the validity of these ranges, the haemoglobin 95% range was determined for women who had ferritins above 30µg/L at each time point they had measured during pregnancy, defining an iron replete population.

Results

During the initial cohort period, 4102 pregnant women had haemoglobin or iron measurements. They had a mean age of 29.7 years (95% range 19.7-39.8) and median gestational age of 276 days (range 141-299) at the time of delivery. Women who delivered prior to 36 weeks gestation were excluded. The flow chart for the selection of women for the initial cohort is shown in figure 2.1. Only 146 (3.56%) women had a ferritin measured in first trimester and subsequent haemoglobin measured pre-delivery, with 77 (53%) in the pre-intervention period and 69 (47%) in the post intervention period. Anaemia

was seen in 5 of 24 women (20.8%) with a low ferritin compared with 2 of 53 (3.8%) with a normal ferritin (p=0.027, Fisher exact test) in the pre intervention period. During the intervention 19 women had a low ferritin and only one was anaemic at the time of delivery. This compares with one woman being anaemic from the 50 women who had higher ferritin levels (p=0.48). Thus, ferritin in first trimester appears to predict women at risk of anaemia at delivery, but not when iron therapy is implemented.

Figure 2.1: Flow chart for the selection of women in the initial cohort. Reproduced from Crispin et al 2019,[1] used under licence.



Repeating the analysis for second trimester showed that the ferritin did not correlate with anaemia at delivery. There were 285 women with ferritins during second trimester and pre-delivery haemoglobins available, 83 (29.1%) in the pre intervention period and 202 post intervention. The increased number post intervention reflecting one of the intervention parameters (increased screening). Pre-intervention low ferritin was more frequent, seen in 59 (71.1%) of women and of these 8 had later anaemia prior to delivery. A similar proportion of women were anaemic at delivery (3/24, 12.5%, p=1

by Fisher exact test) in the cohort who had ferritin more than 30µg/L. The results of the initial analysis are shown in table 2.2.

The findings suggest that a ferritin of under 30µg/L identifies women at higher risk for anaemia at delivery, but that this is not true in second trimester, where the prevalence of iron deficiency (defined as ferritin <30µg/L) is higher.

Transferrin saturation has been suggested as an alternative to ferritin for screening for iron deficiency in pregnancy. Using the same methodology, women with transferrin saturation levels were identified and the incidence of anaemia at delivery compared for levels above and below 20%. There were only 32 women in the pre-intervention period with transferrin saturations in first trimester and haemoglobins determined prior to delivery and 32 in second trimester. Low transferrin saturation was associated with later anaemia however in first trimester, with 4 of 7 developing anaemia compared with 1 when the saturation was normal (p=0.004, Fisher exact test). As with ferritin, transferrin saturation lost its predictive value in second trimester with anaemia being seen in 4 of 16 women with low saturations and 2 of 15 with normal saturations (p=0.65).

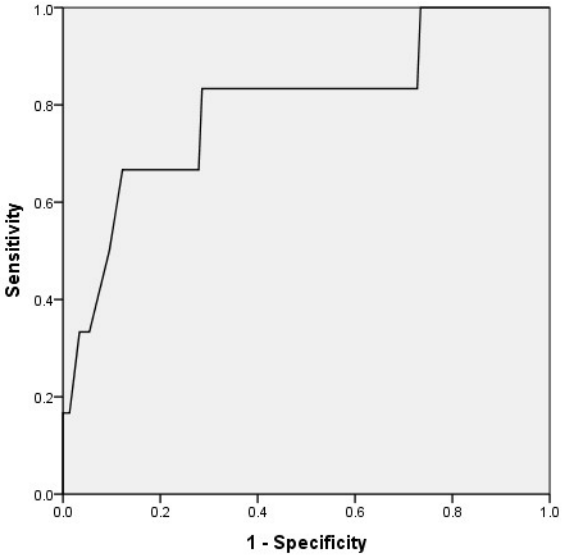
The haemoglobin in first trimester was also evaluated in the sample prior to the intervention. Of 270 women with haemoglobin measurements in first trimester and pre-delivery, 11 had anaemia in first trimester, with 5 having anaemia pre-delivery. A normal haemoglobin was seen in first trimester in 259 (95.9%) women of whom 22 (8.5%) went on to have anaemia at the time of delivery making a low haemoglobin in trimester 1 predictive of later anaemia (p=0.002, Chi-squared).

All three tests were predictive of anaemia prior to delivery. The sensitivity and specificity of each was calculated. The ferritin in trimester one had a sensitivity of 83% and a specificity of 67% for anaemia prior to delivery. The transferrin saturation had similar results at 75% and 72%, respectively, but the haemoglobin had a poor sensitivity of 23% while retaining a similar specificity of 69%. A ROC (figure 2.2) curve for ferritin was constructed and had an area under the curve of 0.80 (95%CI 0.59-1).

Table 2.2: Results for the initial cohort of women, before and during the intervention to improve the detection of iron deficiency and its management. Reproduced from Crispin et al 2019,[1] used under licence.

	Trimester 1			**Trimester 2**		
	Pre-delivery anaemia	**Normal Hb at delivery**		**Pre-delivery anaemia**	**Normal Hb at delivery**	
Pre-intervention						
Ferritin <30µgL^{-1}	5 (6.5%)	19 (24.7%)	N= 77	8 (9.6%)	51 (61.4%)	N=83
Ferritin ≥30 µgL^{-1}	2 (2.6%)	51 (66.2%)	P=0.027	3 (3.6%)	21 (25.3%)	P=1.0
Transferrin saturation <20%	4 (12.5%)	3 (9.4%)	N=32	4 (12.9%)	12 (38.7%)	N=31
Transferrin saturation ≥20%	1 (3.1%)	24 (24%)	P=0.004	2 (6.5%)	13 (41.9%)	P=0.65
Anaemia (Trimester 1 <110gL^{-1}; Trimester 2 <105gL^{-1})	5 (1.9%)	6 (2.2%)	N=270	12 (1.8%)	22 (3.4%)	N=653
Normal haemoglobin	22 (8.1%)	237 (88.8%)	P=0.002	44 (6.8%)	575 (88.1%)	P<0.001
Post-intervention						
Ferritin <30µgL^{-1}	1 (1.4%)	18 (26.1%)	N=69	6 (3.0%)	125 (61.9%)	N=202
Ferritin ≥30 µgL^{-1}	1 (1.4%)	49 (71.0%)	P=0.48	5 (2.5%)	66 (32.7%)	P=0.52
Validation cohort						
Ferritin <30µgL^{-1}	6 (14.3%)	7(16.7%)	N=42	67 (14.0%)	277 (57.7%)	N=480
Ferritin ≥30 µgL^{-1}	4 (9.5%)	25 (59.5%)	P=0.046	21 (4.4%)	115 (24.0%)	P=0.36

Figure 2.2 ROC curve for ferritin in first trimester to detect anaemia pre-delivery



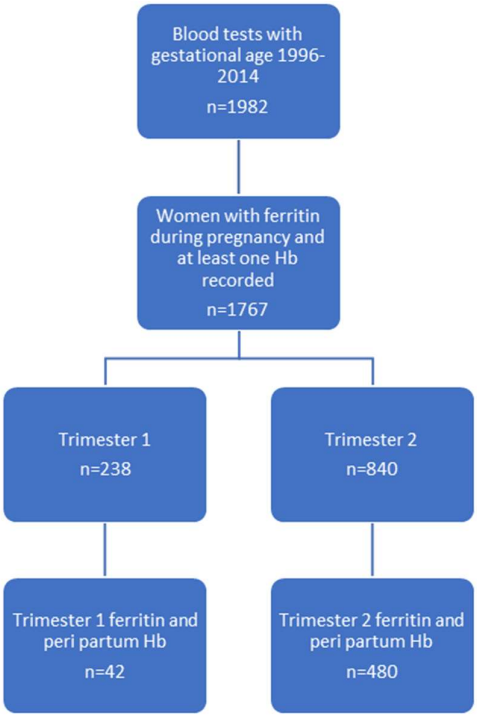


Figure 2.3: Flow chart for the selection of women in the validation study. Reproduced from Crispin et al 2019,[1] used under licence

In order to confirm the finding that ferritin in first, but not second, trimester identifies women at risk of anaemia prior to delivery, a second sample was taken from the laboratory information system for the period from 1996 until the start of the initial cohort. Pregnancy dates were taken as provided on pathology requests as the time as much of this period preceded the implementation of the antenatal database. Using the data from the initial cohort and an α of 0.05, a minimum of 61 women were required to have ferritin in first trimester and haemoglobin pre-delivery to confirm the predictive value of ferritin with 90% power. Despite the long timeframe, only 1767 women had a ferritin measured and gestational age simultaneously recorded. The flow chart for inclusion in shown in figure 2.2. Of these, only 42 had a ferritin in first trimester and haemoglobin pre-delivery, and 480 in second trimester. In trimester one, 6 of 13 (46%) had later anaemia prior to delivery and 4 of 29 did so when the ferritin was >30gμ/L. Although the numbers were small, this was statistically significant ($p<0.05$, Chi-squared test). In second trimester, 67 of 344 (19.5%) women with a low ferritin had anaemia at delivery, compared with 21 of 136 (15.4%) with normal ferritins ($p=0.36$). This second cohort confirmed the predictive value of ferritin in first trimester. It also confirmed the high frequency of low ferritin levels in second trimester (71.6%) and their lack of correlation with anaemia at delivery.

Haemoglobin reference ranges

Women with at least one ferritin performed during pregnancy and no low ferritin readings were selected. Haemoglobin levels were available for 187 in first trimester, 249 in second trimester and 254 women in third trimester. Determining a parametric 95% reference range gave estimates for non-iron deficiency ranges of 109-150g/L in first trimester, 99-139g/L in second trimester and 102-144g/L in third trimester.

Pregnancy outcomes were assessed from the initial cohort, as listed in table 2.3. No association was found between iron deficiency or anaemia and birth weights or gestational age at birth. The amount of perinatal bleeding was not significantly different between women with and without iron deficiency in early pregnancy.

Table 2.3 Complications of pregnancy with and without iron deficiency or anaemia. Reproduced from Crispin et al 2019,[1] used under licence.

Iron depletion or anaemia	**With condition**		**Without**		
	Median	**Range**	**Median**	**Range**	
Anaemia					
Estimated blood loss (mL)					
Trimester 1	300	100-1500	350	50-3200	P=0.438
Trimester 2	416	50-3000	400	100-2700	P=0.21
Gestational age at delivery(days)					
Trimester 1	278	216-293	277	145-296	P=0.57
Trimester 2	274	206-296	274	216-293	P=0.61
Birth weight (g)					
Trimester 1	3290	890-4230	3380	360-5450	P=0.06
Trimester 2	3325	940-5320	3200	1400-4918	P=0.16
Iron depletion					
Estimated blood loss (mL)					
Trimester 1	350	100-2000	300	100-3000	P=0.13
Trimester 2	350	100-2700	400	50-3000	P=0.21

Reticulocyte indices in pregnancy

Background:

We have previously shown that LHD was poor at differentiating iron deficient from non-iron deficient women in pregnancy.[82] From the previous study in this chapter we know that iron deficiency is common in this cohort. There would be significant benefits in being able to detect iron deficiency using the routinely performed full blood count (FBC) rather than having to perform additional ferritin tests in all women.

Red cell size factor (RSF) has been used to detect iron deficiency.[110,116] As it uses reticulocytes it may be better at detecting changes in red cell production during pregnancy when iron stores are rapidly declining.

Aims:

- To improve antenatal screening for iron deficiency by using red cell indices, which are routinely recommended in pregnancy, rather than ferritin.

Objectives:

- To determine whether red cell / reticulocyte indices from the Beckman Coulter DXH 800 correlate with serum ferritin in pregnant women and to determine their utility as a screening tool for iron deficiency in this population. There are a number of experimental indices produced by the instruments to be examined, however RSF has correlated best in non-pregnant population.

Hypotheses:

- That RSF will identify iron deficiency in pregnancy (as determined by the serum ferritin) with a sensitivity and specificity of more than 80%.

Methods:

Setting and participants

This study was conducted at the Centenary Hospital for Women and Children in Canberra, recruiting women from the antenatal clinic. It began following the previously described quality improvement project, which implemented widespread ferritin screening.[111] Women who were to have blood counts and ferritin performed as part of standard screening procedures were recruited by their treating obstetrician (Dr Farah Sethna). Women who were being treated for known iron deficiency or with known thalassaemia were excluded. Samples were drawn as per standard practice for full blood count and ferritin, with the additional test of reticulocytes added for the purpose of this study. This did not require additional blood draws or volume. Women included in this prospective study provided written informed consent. The study was approved by the ACT Health Low Risk Ethics Committee (ETHLR.16.198) and was conducted in accordance with the Declaration of Helsinki and all relevant institutional and jurisdictional privacy regulations.

Data on novel indices were not uploaded to the laboratory information system from analysers. A process for regular data extractions was put in place. While this was effective initially, there were errors in data extraction and transmission during the course of the project.

The planned recruitment was of 150 women. Based on the previously reported study, 26% of women presenting for antenatal care with had ferritin levels below the laboratory normal range (10ug/L), with a total of 71% having levels where treatment is currently recommended (<30ug/L).[82]

Recruitment was intermittent and times of peak recruitment correlated with data loss associated with data extraction difficulties from the analyser, leading to a significant number of tests not having data recorded on RSF. Examination of the population in November 2018 found a lower rate of iron deficiency than expected, probably due to the widespread use of iron screening both in the clinic and by referrers, resulting in these women being treated before arrival. It was calculated that with data loss issues and change in iron deficiency prevalence in clinic that the study was not going to have

enough women to answer the question using the pre-specified power calculations. Therefore, a retrospective component was added.

Reticulocyte parameters and ferritin are routinely measured when haemoglobin electrophoresis is performed for suspected thalassaemia or haemoglobinopathy. Data on women who had these performed during the timeframe of the study had been captured, but not extracted. A retrospective study to examine these results was therefore undertaken, following approval the ACT Health Low Risk Ethics Committee. As haemoglobin electrophoresis is often performed for undiagnosed microcytosis, this population was predicted to be enriched for iron deficiency. Informed consent was waived by the Ethics Committee due to the use of de-identified data only, however privacy and data integrity considerations remained the same as for the prospective study.

Laboratory assays

FBC and reticulocyte counts were performed the Beckman Coulter DXH 800 analysers. Ferritin was measured on the Architect (Abbott Diagnostics, IL, USA). Both tests were conducted in accordance with the manufacturer's recommendations and laboratory standard operating procedures including maintenance of controls and external quality assurance. Experimental red cell indices data were stored on the analysers and periodically uploaded by laboratory staff. All other data were extracted from the laboratory information system.

Established iron deficiency was defined as a ferritin of <10µg/L, based on the population 95% reference range.

Statistical analysis

Based on a prior study showing a sensitivity of 98.8% and specificity of 89.6% for RSF in detecting iron deficiency[117] it was estimated that a minimum of 117 patients were required to have an 80% chance of demonstrating that RSF is effective in pregnant women, with a significance level of 5%. In order to account for a potential lower sensitivity in pregnancy (as the efficacy is unknown in this setting), a target of 150 women was decided upon as the recruitment target.

ROC curves were used to determine the cut-off values for the experimental indices in detecting iron deficiency (ferritin<10ug/L). The primary outcome was the area under the curve (AUC) of the ROC curve. Sensitivity and specificity were determined for the cut-off value of 87.7fL for RSF, which was derived from a non-pregnant population, and from a value derived from the ROC curve in this study including only pregnant patients. ROC curves were also developed for other red parameters including MCV, MCH, the microcytic anaemia factor (MAF) and the mean reticulocyte volume (MRV). Similar analysis was conducted for the immature reticulocyte fraction (IRF), although as a measure of the proportion of younger reticulocytes, this would be expected to correlate with response to treatment rather than iron deficiency. The optimal cut off values were determined by Youden's index.[118] Comparisons between patient groups were performed using independent t tests, or Chi squared tests for dichotomous variables. Stepwise linear regression was performed to assess for independence of variables associated with iron deficiency. Statistical analysis was performed in SPSS version 24 (IBM, NY, USA), except for comparison of AUC, which were in R[119] version 3.5.2 with the pROC package.[120] A $p<0.05$ was considered significant.

Results

There were 134 women with data extracted for the study, of which 13 were excluded due to thalassaemia and one excluded post hoc due to active acute leukaemia, which had not been anticipated in the study plan. These were from prospective (n=70) and retrospective (n=50) cohorts. The mean age was 29.9 years at the time of blood sampling. Results were from first trimester in 12 (10%), second trimester in 50 (42%) and third trimester in 58 (48%), leaving first trimester pregnancies, where ferritin screening is most effective, under-represented.

Despite the addition of retrospective cohort where iron deficiency was expected to be more common, only 15 (12.5%) of women were iron deficient with a ferritin of <10µg/L. Using the pre-determined cut-off of 87.7fL for RSF, women with levels above this had a mean ferritin of 36.6 µg/L, while below this it was 19.4 µg/L (P=0.02). This cut-point gave a sensitivity of 47% and specificity of 77%.

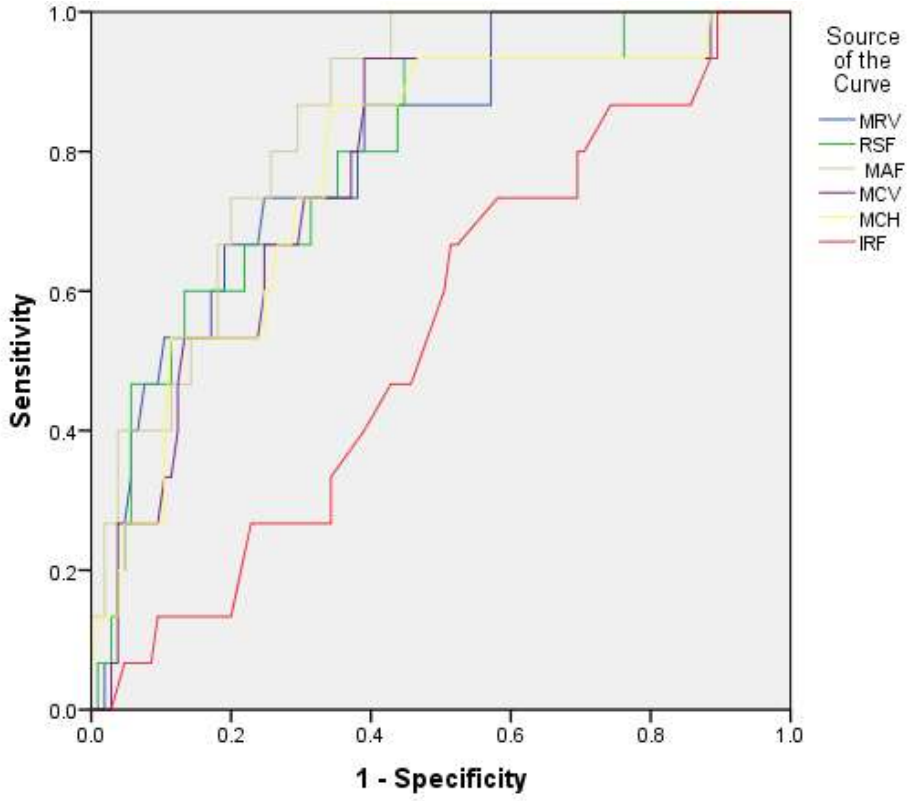


Figure 2.4: Red cell and reticulocyte parameters ROC curves for the detection of iron deficiency (ferritin<10µg/L). IRF was not discriminatory and was significantly inferior to all the other red cell parameters. Excepting IRF, there was no significant difference in the AUCs between red cell parameters for the detection of iron deficiency (P=0.1). MRV: Mean reticulocyte volume; RSF: Red cell size factor; MAF: Microcytic anaemia factor; MCV: Mean cell volume; MCH: mean cell volume; IRF: Immature reticulocyte fraction. From Crispin et al, 2019, used under licence.

A ROC curve was constructed (figure 2.4) and the optimal threshold for RSF was determined as 94.4fL. This improved sensitivity to 93% with a specificity of 55%. The AUC for RSF ROC curve was 0.80 (0.68-0.91).

The MRV uses only reticulocyte parameters rather than the combination of reticulocyte and entire red cell population parameters used in the RSF, so it was postulated that it may be more sensitive to emerging iron deficiency during pregnancy. The ROC curve also had an AUC of 0.80 (0.70-0.91), no different from the RSF (p=0.25). The MAF had the highest AUC of any red cell parameters tested. The AUC of 0.85 (0.77-0.93) and at a threshold of 103.5 had a sensitivity of 93% and specificity of 65%. The IRF did not discriminate between iron deficient and non-iron deficient women. Tested red cell parameters are shown in table 2.4.

Table 2.4: Red cell and reticulocyte parameters for the detection of iron deficiency.
Sensitivity and specificity were not calculated where the ROC was not significantly different from the line of unity (AUC=0.5). Cut off values were determined by Youden's index.[118] (AUC: Area under the curve; CI: Confidence interval; NC: Not calculated; MRV: Mean reticulocyte volume; RSF: Red cell size factor; MAF: Microcytic anaemia factor; MCV: Mean cell volume; MCH: mean cell volume; IRF: Immature reticulocyte fraction). From Crispin et al 2019, used under licence.[83]

Parameter	**Iron deficiency (Ferritin<10µg/L)**					**Early iron deficiency (Ferritin<30µg/L)**				
	AUC	**95% CI**	**Cut off**	**Sensitivity**	**Specificity**	**AUC**	**95% CI**	**Cut off**	**Sensitivity**	**Specificity**
RSF	0.80	0.68-0.91	94.4fL	93%	55%	0.62	0.52-0.72	95.7fL	68%	56%
MRV	0.80	0.70-0.91	102.9fL	87%	61%	0.56	0.46-0.67	NC	NC	NC
MAF	0.85	0.77-0.93	103.5	93%	65%	0.66	0.56-0.76	109.5	60%	60%
MCV	0.77	0.65-0.89	86.6fL	93%	61%	0.65	0.55-0.75	86.6fL	60%	73%
MCH	0.78	0.66-0.90	28.65pg	87%	66%	0.65	0.55-0.75	28.7pg	56%	79%
IRF	0.54	0.40-0.68	NC	NC	NC	0.52	0.41-0.62	NC	NC	NC

Currently available red cell parameters MCV and MCH were also compared. The MCV had an AUC of 0.77 (0.65-0.89). The optimum threshold to detect iron deficiency was 86.6fL, giving a sensitivity of 93% and a specificity 61%. The laboratory lower limit of normal (80fL) gave a sensitivity of 83% with a

specificity of 87%. The MCH had a similar sensitivity of 87%. As multiple red cell indices were associated with iron deficiency, step-wise linear regression was undertaken to determine collinearity and whether a score with multiple indices may improve the test characteristics. In this model, only the MAF, with the highest AUC, was independently correlated with a ferritin <10μg/L, indicating collinearity of all parameters and no additional value in combining them to screen for iron depletion.

Comparison between the red cell and reticulocyte parameter ROC curves was undertaken. IRF was significantly inferior to all other parameters and did not detect iron deficiency. With IRF excluded, all other parameters showed no significant difference between the AUCs (p=0.1).

Early iron deficiency was defined as a ferritin <30μg/L, as it is known that marrow iron stores are depleted below this level. A majority of women (n=68, 57%) met this criterion. The AUC for ROC curves are shown in table 2.4. RSV, MCV, MCH and MAF were all statistically different from AUC of 0.5, indicating a capacity to distinguish between iron depleted and non-iron depleted women, however the sensitivity and specificity were low. This is shown in figure 2.5. An alternative definition of iron deficiency, transferrin saturation <11% (n=11), did not show improved discriminatory capacity.

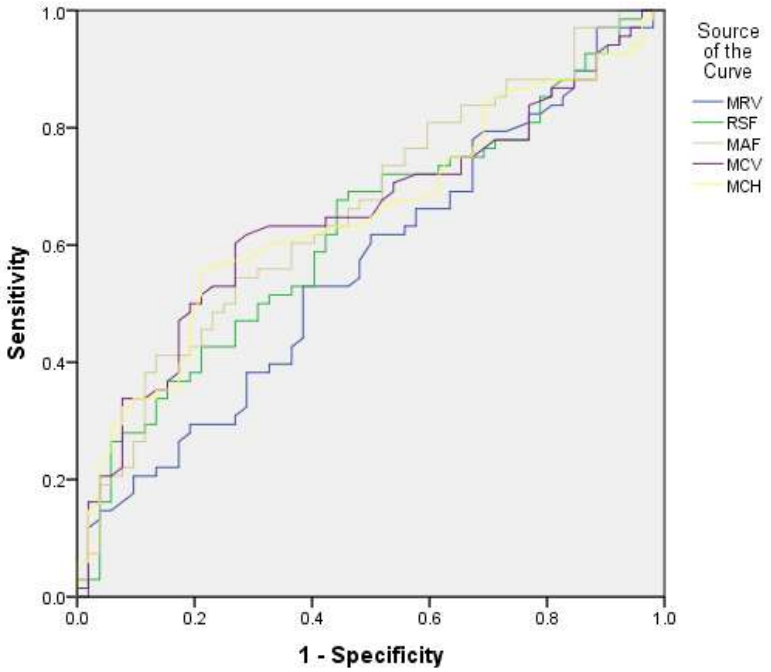


Figure 2.5: Red cell and reticulocyte parameters ROC curves for the detection of iron depletion (ferritin<30µg/L). MRV was not discriminatory, while other values were all significantly different from AUC of 0.5. MRV: Mean reticulocyte volume; RSF: Red cell size factor; MAF: Microcytic anaemia factor; MCV: Mean cell volume; MCH: mean cell volume.

There were 13 women with haemoglobinopathy in the retrospective database excluded from the initial analysis. These included 5 with β thalassaemia minor, 4 with α thalassaemia trait, 1 with HbH disease, 1 with HbA/E, 1 with HbS/C and 1 with sickle cell disease. Combining this group showed a lower MCV (72 v 81fL, p=0.02) and MCH (23.5 v 26.6pg, p=0.03) than the iron deficiency group. The RSF showed no significant difference in RSF (p=0.27) or MRV (p=0.5). The low numbers and heterogenous nature of the haemoglobinopathy group precludes a definitive conclusion on the efficacy of the latter tests, and the ability to differentiate may depend on the degree of iron deficiency and anaemia.

Revisiting the Ferritin Range: Impact on haemoglobin

The studies above have included variations in the ferritin range used to define a normal ferritin. In the first study, I sought to validate the range chosen for use in the quality improvement program, based on RCPA guidelines.[103] A ferritin of <30µg/L was predictive of anaemia at delivery when tested in the first trimester, indicating that iron stores were not adequate in some women to accommodate the loss of iron to the fetus and expansion of the red cell mass during pregnancy. However, in second trimester, ferritin<30µg/L was not associated with pre-delivery iron deficiency. As noted in the introduction to this chapter, there is variability in the accepted iron range. Population data collected here may be able to indicate at what level of ferritin the haemoglobin begins to fall across the population.

Aim:

- To improve the definition of iron deficiency by determining its impact on haemoglobin across a population and to evaluate the effect of patient selection on ferritin ranges determined from large retrospective pathology cohorts.

Objectives:

- To determine the ferritin level in a population of women undergoing screening at which haemoglobin levels fall.
- To compare ranges so obtained by using women selected from the history as being normal, compared with an unselected cohort.

Hypothesis:

- That haemoglobin levels in women selected for screening will fall as haemoglobin falls from a ferritin level of 30µg/L.
- That using changes in haemoglobin concentration across a population to determine the serum ferritin lower range in unselected patient retrospective patients leads to an artificially

elevated value than when compared with a level based on patients positively selected due to referral for routine screening.

Methods:

Ferritin, haemoglobin levels and red cell indices were extracted from the laboratory information system for the studies noted above and deidentified for the period July 2014 to June 2016. The study was exempted from ACT Health Human Research Ethics Committee review as there was no risk to the participants and it was aimed at verifying pathology laboratory reference ranges. Data were collected and maintained in accordance with local privacy regulations and the study conducted in accordance with the Declaration of Helsinki and the NHMRC National Statement on Ethical Conduct of Human Research (updated 2015).

Samples were excluded with a ferritin of 600µg/L or more in order to minimise the effect of inflammation on haemoglobin and red cell parameters. A subgroup of samples from outpatient collections referred from general practice was selected to exclude people with illness requiring specialist attendance. Samples were then positively selected to define a screened population to represent a relatively normal community-based population. The history as transcribed from the laboratory request into the laboratory information system was used with the selected population containing the “check,” or “screen,” but not modifiers, such as “follow up,” “pregnant” or “diabetes”. The intention was to create a population of people referred for screening, so where there was doubt about the indication for the test (including no history), these were not included in the normal group.

Analysis

The data were plotted to examine the relationship between ferritin and haemoglobin and described, including descriptive statistics. Local regression (Locally estimated scatterplot smoothing, LOESS) was used to assist descriptive analysis and determine trends. The plateau haemoglobin and the ferritin level at which plateau is reached were determined. The subgroup selected on history was compared with the unselected patients. This process was repeated for the MCV. Medians were compared with

the Mann-Whitney test. Plateau levels were based on inspection of trends, and best fit curves. Analyses were performed in Excel (Microsoft, CA. USA), LOESS in SPSS v24 (IBM, NY, USA) and exponential curve fitting in Prism (Graphpad, CA, USA).

Results

There were 46498 samples from adults during the data extraction period. Excluding results with a ferritin >600µg/L left 13332 males and 26084 females. Of these only 347 (2.6%) of males and 676 (2.6%) of females met the criteria for screening samples. Following inspection of the data, an exponential – plateau distribution was considered the best fit. This was defined by:

$$Y = YM - (YM - Y0)xe^{-kxFerritin}$$

Where Y is the haemoglobin or MCV in the respective analysis, YM is the level of plateau, Y0 is the value of Y at ferritin of 0 and k is the constant derived from curve fitting, by least squares. These are plotted for the selected and unselected cohorts in figure 2.6. The values from the regression models are shown in table 2.5.

All populations showed a fall in haemoglobin and MCV with low ferritins. The unselected populations had a lower asymptote haemoglobin in both males and females than the group who had been referred for screening. The results of MCVs were less consistent, with both having a slightly higher asymptote MCV value in the unselected population than in the screening population. However, the lowest MCV values in the regression models were lower in selected females and higher in selected males than in their unselected counterparts. There are also anomalies with these models. Figures 2.6A and 2.6E show the unselected populations by sex. While one model has been fitted, the higher ferritin values appear bimodal, perhaps indicative of people with concurrent inflammation having a higher ferritin relative to their haemoglobin, although this cannot be proven from the data available. This pattern is not apparent in the screening-selected population.

These graphs point to a fall in haemoglobin and MCV at levels above 30 µg/L, although they make the assumption of one distribution across the analysed ferritin range. Local regression (LOESS) was performed in a subpopulation of women to explore the difference in haemoglobin and red cell indices responses to ferritin as the ferritin increases. For unselected women, the LOESS, using 15% of values for local analysis showed a rise with increasing ferritin until 75-100µg/L, followed by a gradual fall in haemoglobin (figure 2.7A).

Restricting the analysis to ferritin <55 µg/L in order to examine the lower limit only, ferritin values were rounded to the nearest integer and medians for each value obtained and plotted. LOESS using 60% of observed values was performed on both selected and unselected cohorts. While the unselected cohort showed a continuing rise throughout this range, the selected cohort showed a peak at approximately 27 µg/L (figure 2.7B). There was a significant difference in the haemoglobins with the same ferritin values within this range ($p<0.001$), between the selected and unselected groups, indicating the impact of comorbidities when using unselected laboratory referred samples to determine reference ranges for ferritin, likely due to the effect of inflammation on the ferritin level. Based on the selected cohorts, using the regression models shown in figure 2.6, estimated ferritin values at which haemoglobin and MCV begin to fall were 27 µg/L and 29 µg/L, respectively for females and 30 µg/L and 32 µg/L for males. While the models are not conclusive, they are supported by the alternative LOESS model and are consistent with a ferritin level of approximately 30µg/L before haematological effects are observed across otherwise reportedly healthy populations.

Figure 2.6: Haemoglobin and MCV in relation to ferritin levels. Populations are restricted to adults and have ferritins under 600µg/L. Unselected females (A,B) are compared with those selected based on a referral for screening (C,D). The same approach is shown for males (E-H).

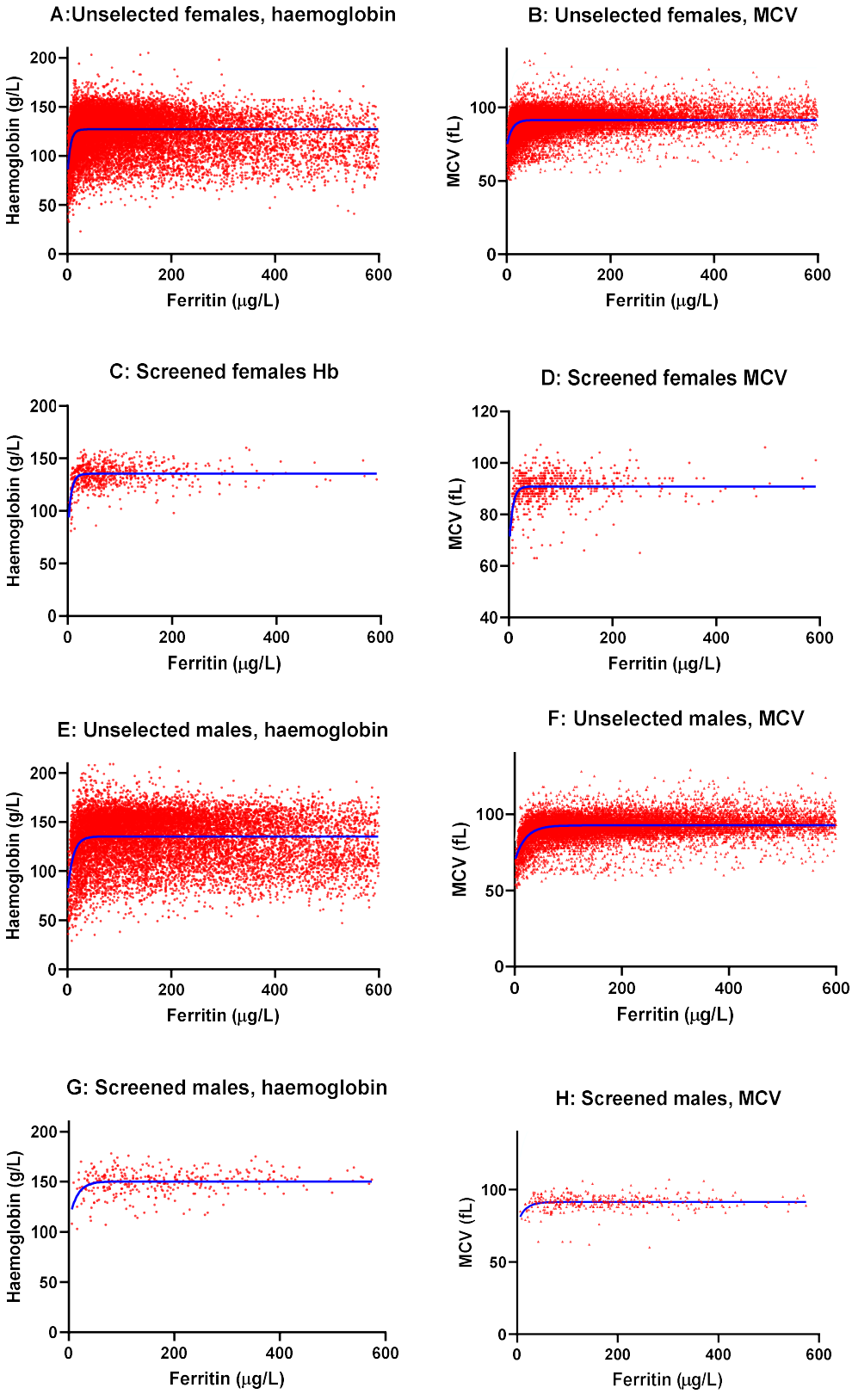
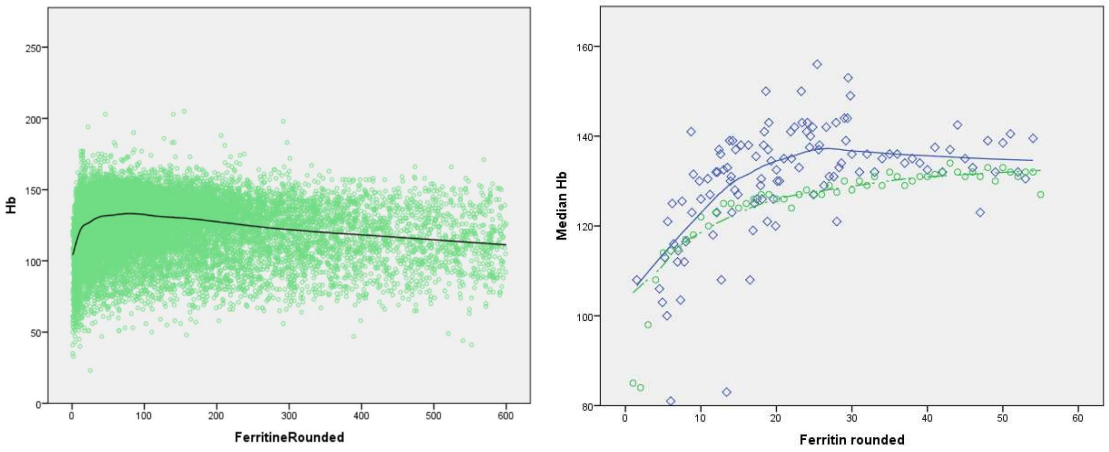


Table 2.5: Parameters of the exponential-plateau best fit curve models

	Haemoglobin			MCV		
	Level at plateau (YM, g/L)	**Level at ferritin of 0µg/L (Y0, g/L)**	**Constant (k)**	**Level at plateau (YM)**	**Level at ferritin of 0µg/L (Y0, g/L)**	**Constant (k)**
Unselected females	127.1	78.57	0.173	91.6	73.5	0.101
Selected females	135.4	78.3	0.161	90.8	64.6	0.152
Unselected males	135.4	75.75	0.095	92.7	69.5	0.050
Selected males	150.3	108.7	0.067	91.45	76.2	0.067

Figure 2.7: Local regression analysis (locally estimated scatterplot smoothing, LOESS) of haemoglobin levels by ferritin in adult females. The effect of inflammation at higher ferritin levels, reducing haemoglobins correspondingly is shown (A). The difference between haemoglobin levels in those undertaking screening tests (blue line), and unselected (green line) are shown in B.



Discussion

The PBM "first pillar"

The research presented in this chapter has addressed issues related to the first pillar of PBM, the prevention and treatment of anaemia and optimisation of the red cell mass. The studies have explored the potential to improve the detection of iron deficiency, for which, despite its frequency, there is still no consensus. They have demonstrated that ferritin screening has utility in pregnancy, however, have suggested that screening is best conducted in first trimester. Ferritin screening loses its value after this point. There remains a reluctance to screen with ferritin, and so these studies explored alternatives that could be performed automatically with the full blood count. While these were effective, they were no better than currently used red cell indices. These studies indicate that currently used red cell indices, particularly the MCV, could be reinterpreted to optimally screen for iron depletion and contribute to improved patient blood management.

In the three pillar model of PBM, the first pillar has been defined as the detection and treatment of anaemia,[41] [121] optimisation of the patient's red cell mass,[32] or more simply, optimisation of the patient's own blood.[36] The latter definition is preferable in the setting where an impending challenge to the integrity of the circulation is foreseen, as the appropriate response is to maximise the patient's ability to manage. This means more than detecting anaemia. If someone is iron depleted their haemoglobin might be within the normal range yet impair the individual's capacity to make red cells in response to blood loss. This can be improved by adequate and timely correction.[122] The first definition follows an illness model, whereas the second adopts a "wellness" approach. These studies adopted an approach that in the face of expected blood loss we should optimise the patient's own blood.

What's a range for?

In determining the appropriate range for iron stores we need first to understand our purpose for testing. A "normal" population-based range is helpful for determining whether an explanation should

be sought for a particular aberrant parameter. Levels falling outside this range do not necessarily indicate pathology and the normal range should not be seen as a surrogate treatment threshold. Cholesterol is an example of an analyte with a normal range that differs between populations and has longitudinal variation over time.[123] As a treatable risk factor for cardiac disease though, the decision to treat is based on levels as well as patient risk factors – the *range* and *treatment thresholds* are not the same. Ferritin is similar. Population levels have changed over time[107] and although there is uncertainty as to the level at which iron replacement should be added, it is also dependent on patient circumstances. As noted previously, there is debate about the impact of non-anaemic iron deficiency.[96,98,99,124] In the circumstance of impending potential blood loss though, our goal should be to have adequate iron stores to optimise the red cell mass and enable the marrow to respond to blood loss. These studies have specifically targeted that goal while pursuing streamlined screening methods.

Reinterpretation of MCV rather than novel indices for detection of iron depletion

The aim of the first series of studies was to determine whether novel red cell indices, which are routinely produced, but not reported, could be used rather than ferritin to screen for the need for iron supplementation pre-operatively and antenatally. The study used a broader population and identified a lack of screening in these settings. While LHD had previously been shown to be useful,[115,125-127] this study showed a lower utility when the iron deficient was less severe, as is seen across a normal population. The MCV had a higher AUC than the LHD and the LHD performed poorly in pregnancy, one of the populations of interest.

It was then hypothesised that the red cell indices including reticulocyte parameters may be able to detect the early changes of iron deficiency during pregnancy, when iron stores are rapidly changing. Red cells survive for over 100 days. During pregnancy erythropoiesis affected by iron deficiency well within this time frame resulting in an affected group of reticulocytes prior to changes being seen across the whole red cell population. For this reason, indices based on reticulocytes were examined. However, the RSF and the MRV were also not significantly better than the MCV.

Performing the ROC curves in both of these studies led to very similar results for the MCV. Critically, when using the ROC curve to derive the optimal cut-off value for the detection of iron deficiency, levels of 86-88fL were found irrespective of the population. Rather than a new test, these results suggest we need to consider the way we use the MCV. For thalassaemia detection, values lower than 80fL are better cut-off values.[128] When screening for iron depletion in a PBM setting, an MCV of 87 had the best performance characteristics. An approach where the MCV is used to screen and ferritin added for those with values below 87fL could reduce the cost of testing, while still improving detection rates.

Ferritin ranges

There were different definitions of iron deficiency used in my prior LHD study.[82] The first definition was based on a prior publication suggesting that LHD was superior to other red cell indices for iron deficiency detection, but which excluded "grey-zone" cases such as non-anaemic iron deficiency.[115] The differences were demonstrably more pronounced when this approach was used rather than more typical definitions across a whole population. It does raise the question of how best to define iron depletion.

While traditional methods for defining reference ranges are based on population frequency, the high prevalence of iron depletion is a factor if a normal range is set by statistical means alone.[105,129,130] An alternative approach is to examine at what point low iron contributes to functional impairment.[131] In these studies I have examined this in two ways. The first was to retrospectively review the impact of ferritin measured during pregnancy on the incidence of anaemia prior to delivery. The second was to determine below what level of ferritin does the haemoglobin or MCV begin to fall.

Across a population iron sufficiency could be described as the point where further increases in iron stores lead to no further improvement in red cell parameters. It is accepted that this accounts only for the effect of iron on erythropoiesis, and not in the multitude of other functions in which it plays a role.[132,133] At present, the postulated effect on non-erythropoietic roles, such as exercise performance

or cognition, can only be measured by functional outcomes. The effect of erythropoiesis could be seen as a surrogate for cellular iron depletion more generally though.

Using this approach, I have demonstrated that below ferritin levels approximating 25-35µg/L both the MCV and the haemoglobin fall, both in men and women. This approach has been tried in adolescents and children using all samples in a pathology laboratory information system.[131] While it may be argued that across a large community laboratory that reference ranges so obtained may approximate a normal population, I have demonstrated that significant differences exist between those referred for screening and unselected samples. The difficulty in establishing a population reference range, especially in children, is well known, with ideally large community sampling required. While there is an appeal in being able to analyse data for this secondary purpose, it must be acknowledged that even the samples nominally sent for screening may not be representative of the population. The approach nevertheless has merit, as it does define a functional limit above which raising ferritin shows no apparent improvement in haematological parameters. The approach would be an ideal adjunct to population based normal range studies.[130]

The level obtained was consistent with the 30µg/L level chosen for use in a quality improvement program in obstetrics. Retrospective analysis showed that anaemia was increased with ferritin levels below this at delivery unless iron therapy was instituted. These data showed a benefit for ferritin screening in first trimester of pregnancy, but not in second trimester. A low transferrin saturation was similarly effective. While a low haemoglobin in first trimester was predictive of anaemia at the time of delivery, it was insensitive, and a normal haemoglobin could not be relied upon to exclude anaemia at the time of delivery. Ferritin screening therefore is better at identifying women who may benefit from treatment.

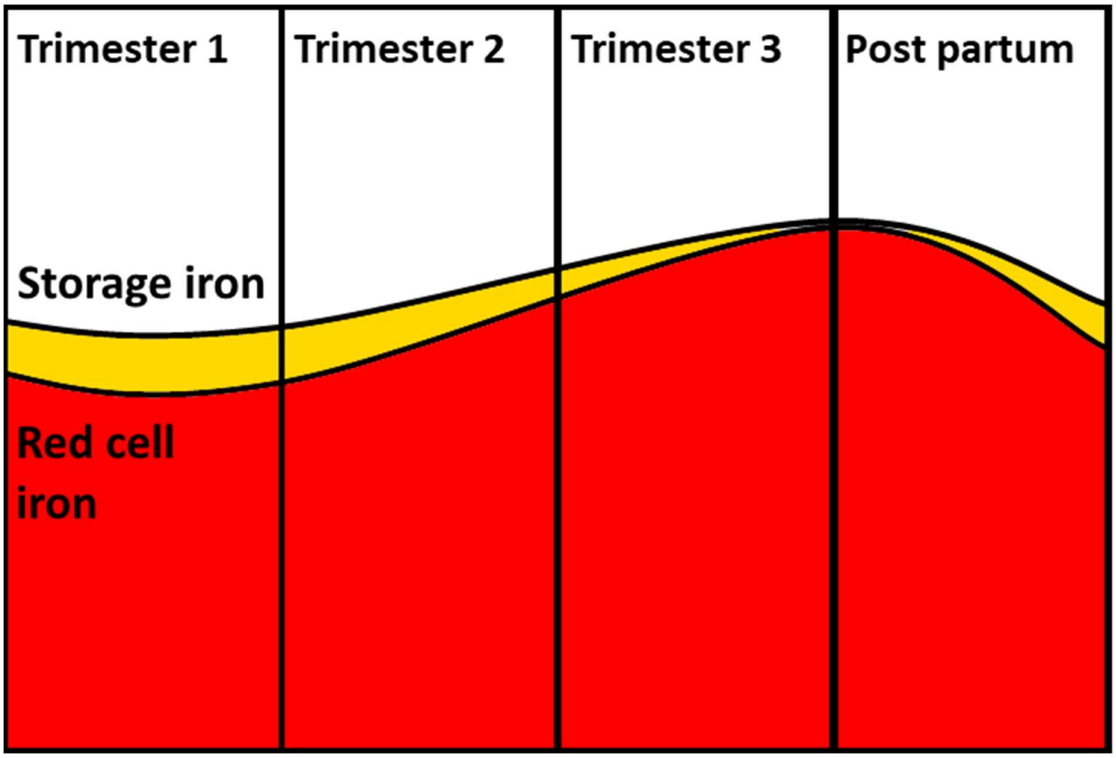
Ferritin screening in the second trimester was unexpectedly found to be not predictive. A repeat analysis on a preceding retrospective cohort confirmed the predictive value of ferritin in trimester one and not trimester two. While this is a new finding, Milman showed a lack of correlation between the

haemoglobin and measures of iron stores from early second trimester as pregnancy continued.[134] The difference in performance of the iron studies compared with the haemoglobin is likely due to the expansion of red cell mass in pregnancy.

Pregnancy is associated with an expansion of both the plasma and red cell volume, with the former exceeding the latter.[135,136] This leads to a fall in haemoglobin concentration, as shown again here, in the second and third trimesters. Women who are anaemic at the start of pregnancy, from whatever reason, are likely to remain anaemic unless it is specifically treated. Women with borderline iron stores may begin with normal haemoglobin, but fail to expand the mass of red cells during pregnancy. Indeed, competing demands from the growing fetus may reduce iron stores and actual red cell mass may decrease in pregnancy. The increased demands during pregnancy mean that the majority of women have depleted marrow iron stores beyond the first trimester, as shown in figure 2.8. As the ferritin is measured following a substantial expansion in the red cells, the shift from storage iron to erythrocyte iron may have already occurred. This study has underlined the importance of measuring ferritin prior to the expansion of red cell mass, if it is to be used to screen for later anaemia.

It may be inferred that a low ferritin towards the end of pregnancy is likely to be less critical for most women, provided the haemoglobin is adequate. Without bleeding, iron scavenged from senescent red cells will be returned to marrow stores as the expanded red cell mass returns to normal post-delivery. In women who have significant bleeding, the lack of storage iron may inhibit their capacity to regenerate new red cells. A PBM approach to surgery would be to ensure that storage iron is adequate to cope with these demands. Aiming for a normal ferritin at the end of pregnancy may lead to a significant expansion in total body iron, as the ferritin does not account for the iron present in the erythroid compartment. There is currently a lack of long-term data to compare replacing to a normal ferritin or to normal haemoglobin levels.

Figure 2.8: Changes in iron distribution during pregnancy. In young women iron stores make up a small component of body iron. Iron is transferred to red cells and to the developing fetus. Iron absorption is increased. Ferritin changes with storage iron, not the iron included in red cells. Reproduced from Crispin et al 2019,[1] used under licence.



Based on work conducted in large part by Stephens in our group, a normal haemoglobin will help to prevent transfusion in the post-partum period.[42] There are concerns about increasing iron stores. Studies across populations have shown association between higher haemoglobin concentrations and higher ferritins and adverse pregnancy outcomes.[137-139] These should not be seen as reasons not to act on iron deficiency as potential risks, even to the fetus, remain.[133] Ferritin is an acute phase reactant. The haemoglobin concentration will reflect the red cell volume as well as the plasma volume. The failure to expand plasma volumes has been associated with preeclampsia and placental disorders.[139] It is likely that the association between higher ferritin or haemoglobin with adverse pregnancy outcomes is due to conditions that impact on inflammatory markers and plasma volumes rather than cause and effect. More research into the association is required.

The appropriate guidance for managing non-anaemic iron deficiency however remains an area of controversy. Treatment of women has been shown to improve haemoglobin levels following iron infusion, and as a PBM strategy to optimise the blood, has merit.[140] Whether this effect is observed in all trimesters is unknown. Larger studies with longer follow up to observe potential adverse effects are likely to be needed to overcome these concerns.

It could also be argued that replacing iron stores to normal marrow levels is unnecessary, as recent data has shown the ability for perioperative iron infusions, even intra-operatively, to decrease the need for transfusion. A similar approach, identifying women with post-partum haemorrhage and giving iron early, may be effective and better targeted. Iron infusion in pregnancy can be carried out safely[140-142] however it remains uncertain as to whether a pre-emptive approach during pregnancy or rapid iron supplementation in the event of bleeding is the better strategy for women with non-anaemic iron deficiency.

Limitations

There are limitations to the studies presented here. Retrospective studies are prone to biases due to the selection of people for testing being unaccounted for. In these studies, I have endeavoured to allow for these biases. When examining ferritin's effect on haemoglobin across a population I have shown the effect that referral bias has. When using all pathology samples in adults, the association between haemoglobin and ferritin is affected by the illnesses in the referred population, with very different, more plausible outcomes when the cohort is selected based on a referral for "screening."[130] Although this is a preferred approach, it is still only one of a number of ways in which the effective lower reference limit, where intervention may help, could be determined and it is important to triangulate the results from different methods, potentially including different ways to look at the data statistically. Although this has been done with LOESS and exponential plateau curves, confirming a result with different statistical methods may give confidence in the analysis, but does not overcome biases that may be present in the original data.

There were a number of assumptions inherent in the retrospective studies. These included an assumption that iron deficiency (defined as a ferritin <30µg/L) was infrequently treated in obstetrics prior to the implementation of our quality improvement strategy. Based on the quality improvement team's knowledge of practice in the clinical setting, this was considered likely to be true, although the timeframe for the confirmatory study was long and knowledge of prior practice likely to be incomplete. The fact that the results were reported using population derived values (ferritin <10µg/L) and there were no local guidelines suggesting screening or intervention at higher levels, also support this assumption.

Statistically, the power of a study is determined by the number of events in question rather than the overall number of samples included. Retrospectively, there were few events. As the focus on first rather than second trimester ferritin screening in pregnancy emerged as a hypothesis from the data, a confirmatory study was performed. This also had limited numbers of events, and while it statistically proved the hypothesis as stated, greater confidence could be achieved with a larger data source. Unfortunately, this is not easy to find, since it requires an untreated population where assumptions can be safely made, or the actual treatment known.

The low number of women presenting with anaemia at delivery is also noted. It draws into question the cost effectiveness of screening. Cost effectiveness analyses have not been performed for any of these strategies and they depend on a number of assumptions, including how high throughput pathology tests, with significant overheads and staffing costs required, but low marginal costs once established, are accounted for in such studies. The use of MCV as a screening tool to decide on who needs ferritin tests done could potentially reduce costs in the pre-admission and pregnancy settings, but if recollected samples are required, any cost savings may be lost and to the patient this would add an additional small burden.

Recommendations

These studies support a ferritin level of 30µg/L as a reasonable threshold above which, in the absence of significant concurrent illness, intervention for iron deficiency is unlikely to be required. Specific studies in renal and cardiac failure provide better data for the use of iron in the presence of concurrent inflammation.[108,109,143] Where ferritin thresholds are implemented in pregnancy, measurement in the first trimester is preferred to identify women most at risk of anaemia.[1]

There are numerous guidelines for the detection and management of iron deficiency in pregnancy. Universal supplementation for all women has been advocated by the WHO,[100,144] with the degree of iron replacement determined by whether the woman is from a high or low risk population for anaemia. Guidelines in some developed countries recommend targeted screening for high risk women using the haemoglobin.[112,145,146] A significant difficulty with this approach is how to identify women who are "high risk" when the frequency of iron depletion, as in this study from a high socioeconomic locality, is around 30%. These women are at increased risk of anaemia in later pregnancy and ideally should be treated. Ferritin is the best way to determine who is at risk, both from our data and from prior studies.[147] In view of the prevalence of iron deficiency, screening with first trimester ferritin is best considered as a way to identify the over 60% of women who do not require the internationally recommended iron supplementation and can be spared the potential side effects during pregnancy.

Where screening with ferritin is not specifically adopted, these results suggest that in high prevalence populations, such as young women, in pregnancy and pre-operatively, that re-interpretation of red cell indices could improve detection. Specifically, an MCV threshold of under 87fL based on these data and derived ROC curves, will optimise the sensitivity and specificity of the test for iron deficiency. In doing so we must be careful not to consider this a redefining of the normal range or remove other thresholds in use for the identification of thalassaemia. This approach is preferable to using novel red cell indices from Beckman Coulter instruments, which I have shown do not add significantly to the

detection of iron deficiency. The MCV, being commonly measured, could be applied to all analysers currently available.

Chapter 3. Tissue Oxygen Saturation. A better transfusion trigger?

Background

Although national guidelines recommend that transfusion be considered based on patient symptoms and comorbidities within the haemoglobin range of 70-100g/L, they also acknowledge that lower haemoglobin levels may be well tolerated in some people and provide little advice on how the decision to transfuse red cells could be made within this range.[34,146,148] While clinical signs and symptoms may be considered well established, there are problems with this assertion. Firstly, as noted in Chapter 1, the history of transfusion has been marked by over-valuation of red cell transfusions. As one potentially treatable cause for fatigue, transfusion for anaemia may be seen as an easy solution, even when the benefit is small. The benefit in post partum anaemia is in many cases minor and short-lived over iron replacement,[149] yet over-transfusion in this context remains an ongoing issue.[42] Secondly, the signs, symptoms and impact of anaemia are not universally agreed and applied.

Recent clinical trials exemplify the lack of agreement on signs and symptoms of anaemia. While normovolaemic haemodilution is associated with cardiovascular compensation, avoiding hypotension until very low haemoglobin concentrations,[150] hypotension has been used as an indication for transfusion in stable post-operative patients in clinical trials.[12] Finally, if there is to be an evidence-based approach to transfusion then researchers need measurable indications at which to consider transfusion. The clinical Gestalt view is hard to measure, but with all the variabilities and deficiencies that it entails, arguably remains the standard of care.

The haemoglobin concentration has traditionally been used in clinical trials, applied as either higher or lower haemoglobin triggers across broad indications, somewhat detached from specific physiologic patient needs.[12,16,45,149,151] This may give impression that transfusion is indicated when the haemoglobin is less than 70g/L in intensive care settings,[16] or <80g/L in major orthopaedic surgery[12], whereas studies have actually not yet defined populations that benefit. They have taken a population-

based approach, using haemoglobin, a known adverse prognostic factor in many conditions where it is a *marker* of morbidity rather than its cause. It is not surprising that transfusion threshold studies have shown no improvement in mortality.

The use of haemoglobin concentration as a trigger within these studies may be a problem. Red cell mass is associated with performance in athletes. In training, this may be underestimated due to expanded plasma volume. Physiologically, this is also seen in pregnancy. Iatrogenic haemodilution may precipitate transfusion when the decision to transfuse is based on haemoglobin concentration thresholds rather than total haemoglobin mass (which is not easy to measure). In such circumstances, having a restrictive intravenous fluid protocol, reducing crystalloids and colloids rather than red cell transfusions, actually also reduces red cell transfusion.[152] This may be counterintuitive since an expanded intravascular volume should be able to maintain microvascular capillary density and therefore tissue oxygenation better than a contracted one, and suggests that by using the haemoglobin concentration as a trigger we are likely to be frequently over-transfusing. Using haemoglobin concentration does not take into account an individual's ability to compensate for anaemia by cardiovascular adaptation, their metabolic demands or indeed their functional needs. Ideally, a transfusion trigger should measure the physiological impact of anaemia and this should translate to improved measurable outcomes, such as symptoms and functional performance before any effect on mortality is detectable. At present, such a measure does not exist.

There are a variety of potential ways to measure the adequacy of oxygen delivery by red cells. Lactate accumulation is used in clinical practice as a measure of anaerobic metabolism in sepsis and trauma, indicating poor tissue perfusion. As a systemic measure from mixed venous or arterial blood, it may not indicate regional hypoperfusion. It is also known to be poorly responsive to anaemia.[150]

Another potential way to measure the impact of anaemia and transfusions is to measure tissue oxygen saturation (StO_2), which may be non-invasively measured using near infrared spectroscopy (NIRS). This technique uses the difference in light scatter between oxygenated haemoglobin (or myoglobin)

within the near infrared range to detect the proportion of each in the tissues (Figure 3.1). Light will either travel through tissues or be absorbed or reflected by tissue chromophores. Light within the NIRS range of wavelengths is able to penetrate through tissue, being reflected along the way and absorbed by haem. Absorption is complete through large vessels, so NIRS measures microvascular oxygenation.[153] As light travels through tissues it reflects at different depths and angles, which can be described by mathematical models and the amount reflected is quantified by the Beer-Lambert law.[153]

As the amount of light reflected and absorbed depends on the path length through the tissues, a smaller amount of light is expected from deeper tissues. In order to measure oxygenation in deeper tissues, there needs to be greater distance between the light optode from the receiving optode. Two optodes can enable the subtraction of superficial from deeper pathways, optimising detection for NIRS signal to deeper tissues (Figure 3.2).[153] A larger fat layer will therefore make it more difficult to target deeper tissues due to the greater depth required. NIRS in muscle will also be affected by myoglobin as an alternative chromophore to haemoglobin.

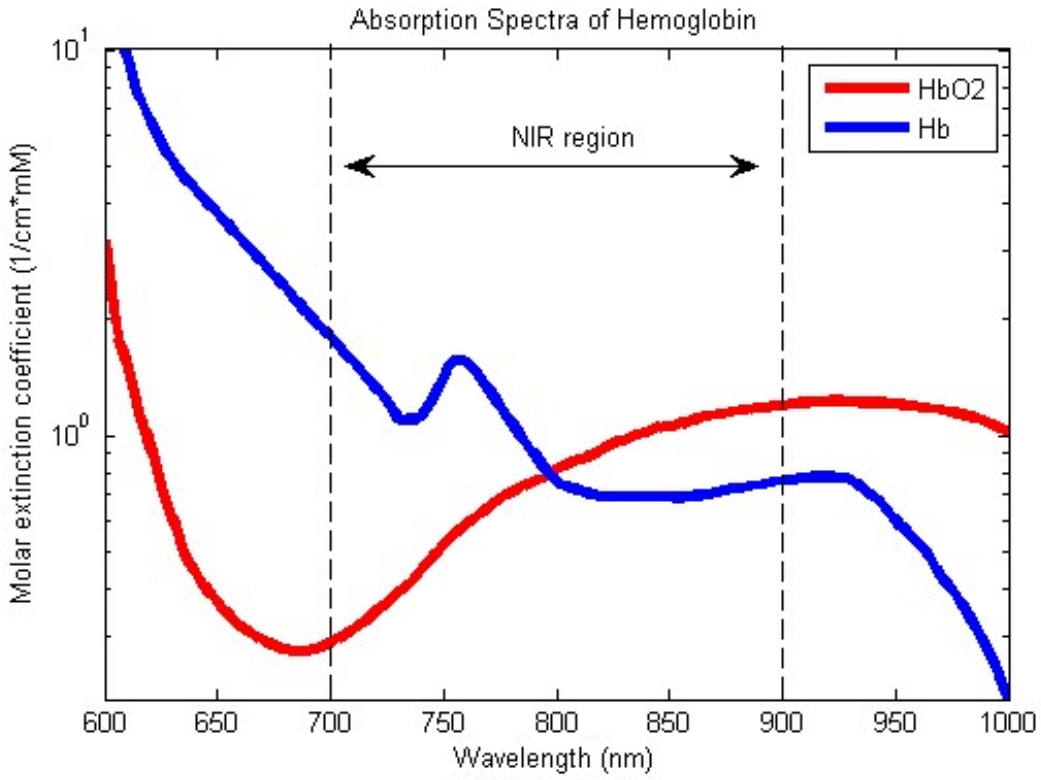


Figure 3.1: Absorption of light by haemoglobin and deoxyhaemoglobin with the near infrared range. (Adrian Curtin, https://www.wikiwand.com/en/Functional_near-infrared_spectroscopy, Creative Commons Attribution Share-alike 3.0)

The degree to which the NIRS signal through muscle represents haemoglobin or myoglobin is uncertain. They both have similar absorption spectra and so the calculation of saturation will be the same, no matter which is used. As the myoglobin and haemoglobin oxygen dissociation curves are very different, the different readings will reflect different oxygen tensions. While NIRS readings are usually attributed to haemoglobin in the tissues, a recent nuclear magnetic resonance imaging study has suggested that the majority of the signal is attributable to myoglobin.[154] If the amount of haemoglobin and myoglobin in the tissues is constant, then for the purposes of detection of regional oxygenation, the distinction is not required since the saturation will reflect the relative molar concentrations of oxygenated haem. The uncertainty that exists however should lead to caution when interpreting StO_2 results from different sites, such as brain and muscle.

While the principle is the same as that used in pulse oximetry, there are differences. Pulse oximetry measures multiple times each second, so can measure rapid changes in light absorption. Frequent continuous readings create the typical sine waved plethysmograph, and by subtracting maximum from minimum absorption levels, and background light measured during a phase where neither light optode is active, to measure specifically pulsatile arterial oxygen saturation. They typically use light at 660nm and 940nm, in the infrared range, and while light absorption is usually measured through thin tissues, light reflectance may also be used. NIRS devices do not aim to measure pulsatile waveforms, so do not select for arterial blood and NIRS signal is therefore representative of local tissue saturation.

As NIRS measures oxygenated and deoxygenated haem, devices frequently report the sum "total haemoglobin." The term is somewhat misleading, as the devices never measure absolute concentrations. Without knowing the exact path length, this is not possible. A closer measure to

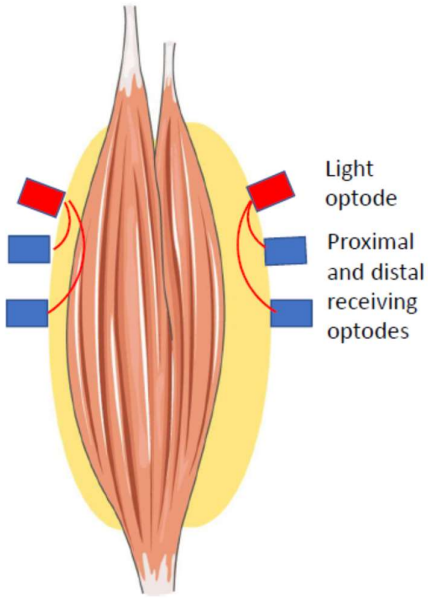


Figure 3.2: The effect of inter-optode distance on NIRS measurements. Light emitted from the light optodes (red) is reflected through tissues and detected by receiving optodes (blue). Reflected light paths are represented by arcs (red). Only a small fraction of emitted light reaches each receiving optode. The depth of the path is proportional to the inter-optode distance. Absorption in deeper tissues can be measured by subtracting absorption from the shorter superficial paths through subcutaneous tissue (left). If subcutaneous tissue is too deep for inter-optode distance, the measurements will not indicate muscle oxygen saturation (right). Muscle image from Servier Medical Art (smart.servier.com), Creative Commons Licence.

haemoglobin concentration in the tissues may be obtained using spatially resolved spectroscopy. In this technique receiving optodes at differing distances are used, and if the absorption is assumed to be uniform, the absolute haemoglobin can be better estimated by using the change in absorption between the different path lengths.[155] However, an increase in "total haemoglobin" is not necessarily proportional to an increase in oxygen delivery (even assuming arterial oxygen saturation remains

constant), since the haemoglobin within the light path will not necessarily increase with increased rate of blood flow.

While the proportion of oxygenated haemoglobin in the tissues is measurable with NIRS, it is difficult to validate the results obtained in absolute terms. Devices use different wavelengths, inter-optode distances and algorithms. While devices may have linearity, the absolute values are not necessarily comparable between devices, with differences shown between commercial devices when compared against a phantom.[156]

Despite these limitations, NIRS has the capacity to measure oxygenation in the tissues and has been used clinically and in the research setting for this purpose. Changes in StO_2 may be due to changes in blood flow, both systemically and regionally, or in the oxygen carrying capacity of the blood, which is largely reflected in the haemoglobin concentration. NIRS has been used to measure StO_2 in sickle cell disease and peripheral vascular disease where macrovascular and microvascular insufficiency is expected, in trauma and cardiac surgery as a measure of both poor tissue perfusion and anaemia, and in neonates.[155]

There have been many studies evaluating NIRS for the detection of anaemia or changes in haemoglobin and they are used in a variety of clinical settings. Yet, evidence of clinical benefit remains elusive or contradictory and measurement of StO_2 has not found its way into evidence based clinical practice guidelines. There are several potential reasons for this. Too much heterogeneity within and between studies makes setting clinical intervention thresholds difficult. There may be too few intervention trials or the results inconsistent. Finally, studies may show minimal to no effect if the threshold for transfusion is still largely based on haemoglobin and hypoxia is not present prior to transfusion. A benefit from transfusion will not be seen if the transfusion is given at a level of haemoglobin not associated with reduced tissue oxygenation.

As discussed in Chapter 1, transfusion based on haemoglobin concentration may not be ideal, but is generally practiced and has largely been the measure upon which clinical trials have been based. If

we are to compare StO_2 and haemoglobin concentration, there needs to be an identifiable measurable endpoint that indicates a positive response to transfusion and potentially adverse outcomes related to non-transfusion. Few studies have examined clinically relevant endpoints. The clinical setting may confound this. Intensive care (ICU), neonatal intensive care (NICU), neurosurgery and cardiac surgery, where NIRS are often used, are circumstances where comorbid conditions are frequent, and anaemia may be only one factor impacting on regional StO_2. Showing a benefit from an alternative transfusion trigger based on StO_2 would require large clinical trials comparing adverse event rates, hospital utilisation and functional recovery rates with enough power to overcome these confounders. As yet, it remains unclear where a threshold for transfusion based on StO_2 would be set.

One way to establish potential transfusion threshold levels for StO_2 is to examine functional and quality of life outcomes in a less critically ill cohort. Ambulant patients are able to report on symptom changes, functional outcomes, such as ability to conduct daily activities, and exercise performance testing are feasible. Demonstrating the impact of transfusion on these outcomes would give an indicator of when anaemia has become clinically meaningful and when transfusion has resulted in clinical improvement.

The best conditions for measuring StO_2 are also uncertain. Resting levels are most commonly used. Others have used dynamic approaches to measure microvascular flow and tissue oxygenation.[157-159] Venous occlusion allows the change in haemoglobin (noting the limitations above) to be measured as a way of determining venous flow. Complete vascular occlusion creates hypoxia and following release there is a reactive hyperaemia during which changes in oxygenation may be measured. Vascular occlusion requires a high pressure tourniquet for prolonged periods (3-6 minutes), an uncomfortable procedure for most, particularly in unwell patients.

A potential alternative to vascular occlusion, which reduces oxygen supply, is exercise, which increases oxygen consumption. In an isometrically contracted muscle, the ability of red cells to access active muscle tissue and transfer oxygen may also be impaired by compression of the small vessels. Isometric

contraction may be an alternative approach to measure the microvasculature response to hypoxia and is likely to be more comfortable for patients than a sustained tourniquet eliciting compression well above systolic blood pressure.

There have been no studies looking at the impact of anaemia on StO_2 during exercise, but the concept arises readily from the bedside. Patients at rest can often tolerate very low haemoglobin levels. Many will report difficulty with exercise capacity within the range of daily activities. We know that exercise performance at maximum capacity is associated with total red cell mass, but if someone is functioning well below maximum performance do they function at a capacity proportionately less because of anaemia, or does anaemia induce a threshold limiting maximum performance, but below which activity is normal? This can only be determined by measuring performance in submaximal exercise.

Measuring StO_2 during exercise also offers the opportunity to measure functional capacity. There are a variety of techniques that may be used. Incremental stress tests are used most commonly and are specifically designed to induce cardiac stress in order to identify electrocardiographic evidence of ischaemia. While this may be an outcome associated with anaemia, it is likely to be more reflective of coronary arterial insufficiency. Ergometric testing may be used in athletes to measure VO_{2max} and could be used, however this correlates with peak performance. The association between peak performance and red cell volume is established, as noted in Chapter 1, and for most patients peak level exercise performance is not a part of daily activities, or even a goal. The 6 minute walk test (6MWT) is a standardised test that correlates well with functional performance in a variety of settings.[160] Normative values have been obtained.[161,162] It has been used in the assessment of the impact of anaemia,[12,163] although prior to this study the effect of transfusion on 6MWT distances had not been well studied. Improvement in 6MWT distances with transfusion in chronic anaemia was however documented by others during the course of this study.[164]

These studies sought to understand the potential for StO_2 to detect clinically significant hypoxia and therefore act as a transfusion trigger. In order to achieve this, two projects were undertaken:

1. A systematic review of the literature evaluating the impact of haemoglobin or anaemia on StO_2 and transfusion. There have been previous systematic reviews assessing the impact of NIRS or StO_2, each generally confined to a particular clinical situation. In this review I planned to amalgamate data from different clinical settings in order to establish the utility of NIRS for the detection of clinically significant anaemia and its use as a transfusion trigger.
2. A clinical observational study, measuring the StO_2 in exercising muscles during aerobic (6MWT) and isometric muscle contraction in people with and without anaemia and correlating this with submaximal exercise capacity (as measured by 6MWT distance) and haemoglobin concentration. Where there is a significant change in haemoglobin concentration, generally due to transfusion, repeated assessment to measure the impact of the change was undertaken.

Near infrared spectroscopy in the detection and management of anaemia – A systematic review:

Research Question:

What is the role of near infrared spectroscopy in the detection of anaemia (or the impact of anaemia) and as a trigger for transfusion?

PICO statement:

Population

Human studies, including an anaemic population or with a change in haemoglobin. Excluding studies specifically targeting sickle cell and/or HB S/C disease.

Intervention

1. Changes in NIRS with change in haemoglobin or anaemia
2. Use of NIRS to direct red cell transfusion

Exclude studies where NIRS used as endpoint in multiple interventions (eg. Algorithms that specify inotropes and transfusion)

Comparison

Any control, including subjects as own control (pre & post an intervention). The study type was not specified. Studies containing primary data (not reviews) were included.

Outcomes

1. Detection or quantification of the effect of anaemia using a NIRS derived threshold;
2. Correlation of NIRS-derived parameters with haemoglobin;
3. The effect of NIRS based approaches to transfusion on the rate of red cell transfusion (proportion with any transfusion and the number of transfusions per individual)

4. The effect of NIRS based transfusion strategies on patient outcomes including cardiac events, mortality, hospital and ICU length or stay and other outcomes considered by the authors to be related to transfusion or red cell mass.

Methods

Searches were undertaken in the following databases using the search strategies:

- National Library of Medicine (Pubmed; All fields): ["Anaemia" OR "Transfusion"] AND ["Near Infrared (Infra Red) Spectroscopy" OR "Tissue oxygen saturation"].
- EMBASE: ["Anaemia" (expanded term) OR "Transfusion" (keyword)] AND ["Infrared spectroscopy" OR "Near Infrared spectroscopy" OR "Infrared spectrophotometry"]
- Web of Science: Topic search on ["NIRS" or "Near infrared spectroscopy"] AND ["Transfusion" OR "Anaemia"]
- In addition, active protocols were searched for in Prospero for "Oxygen saturation" and "Infrared spectroscopy." Potential similar protocols were examined. Clinicaltrials.gov was searched using the search terms "Anaemia" (condition) AND "infrared".
- Opengrey.eu was searched with the terms "Infrared spectroscopy" AND ["anaemia" OR "Transfusion" OR "Saturation" OR "Oxygen"]]

Where necessary, search terms were repeated with United States spelling ("anemia").

Only articles in English were included. Duplicates were removed. Titles and abstracts were reviewed by two authors (Philip Crispin and Kathryn Forwood) for inclusion or exclusion against the PICO criteria. Where there were discrepancies, these were resolved by consensus agreement.

Articles were retrieved and reviewed against PICO criteria in full. Further duplicates were removed. Data were extracted into an Excel spreadsheet (2019 Microsoft Redmond, WA) and, where applicable, into RevMan (v5.3 The Nordic Cochrane Centre, Copenhagen).

Qualitative descriptions of studies were undertaken. Studies were reviewed for risk of bias using QUADAS-2 guidelines.[165]

Meta-analysis was planned where there were multiple studies addressed similar or identical clinical questions with comparable quantifiable reported outcomes, sub-grouped by patient population. Quantitative data were pooled for similar effect using studies with similar methodological quality (with case-control, individual as own control, randomised studies evaluated separately). Funnel plots were constructed to consider publication bias where data were suitable and pooled for analysis. Heterogeneity was assessed by visual inspection of forest plots and I^2 with studies excluded only on the basis of study features (such as a unique population, device or setting) if heterogeneous. Outliers were able to be excluded for sensitivity analysis or examined to determine potential unrecognised confounders, however where pooled meta analysis was performed, the primary results were reported without the post hoc exclusion of outliers. Random effects model was planned. Results were reported in line with PRISMA guidelines.[166]

Results

The search was conducted on 10 May 2019.

There were 500 abstracts obtained from Pubmed, with an additional 756 from EMBASE and 47 from Web of Science. A total of 1303 abstracts were reviewed, with 93 classified as possibly meeting the research question and complete articles retrieved. A further 4 articles were obtained on review of the references of included articles, making a total of 97 full text articles reviewed.

The search of Clinicaltrials.gov found one ongoing trial comparing transfusion based on haemoglobin or NIRS-based criteria in the NICU and a further observational study ongoing examining NIRS variables in relation to the time between red cell irradiation and transfusion and necrotising enterocolitis. Two further observational studies reporting on NIRS variables before and after transfusion were found and remain unpublished (in ICU and NICU).

Following review of the 97 full text articles, 69 studies remained for inclusion in this study. Excluded papers included redundant data (14), testing by means other than NIRS (n=6), meaningful data not presented or able to be abstracted (7) and one article had been retracted. The selection diagram is shown in figure 3.3.

The studies were categorised by the population and / or effect being assessed, with neonatal, paediatric, critical bleeding in trauma and other settings grouped together. In addition, studies that evaluated NIRS as a potential trigger for transfusion were grouped to describe the efficacy of NIRS in this setting.

Assessment of study quality

Studies were generally of low concern with respect to applicability, in part due to the broad nature of the review inclusion criteria. The risk of bias was assessed as low for 14 studies, with most having a high or uncertain risk of bias, as shown in table 3.1.

Figure 3.3: Flow diagram of study selection

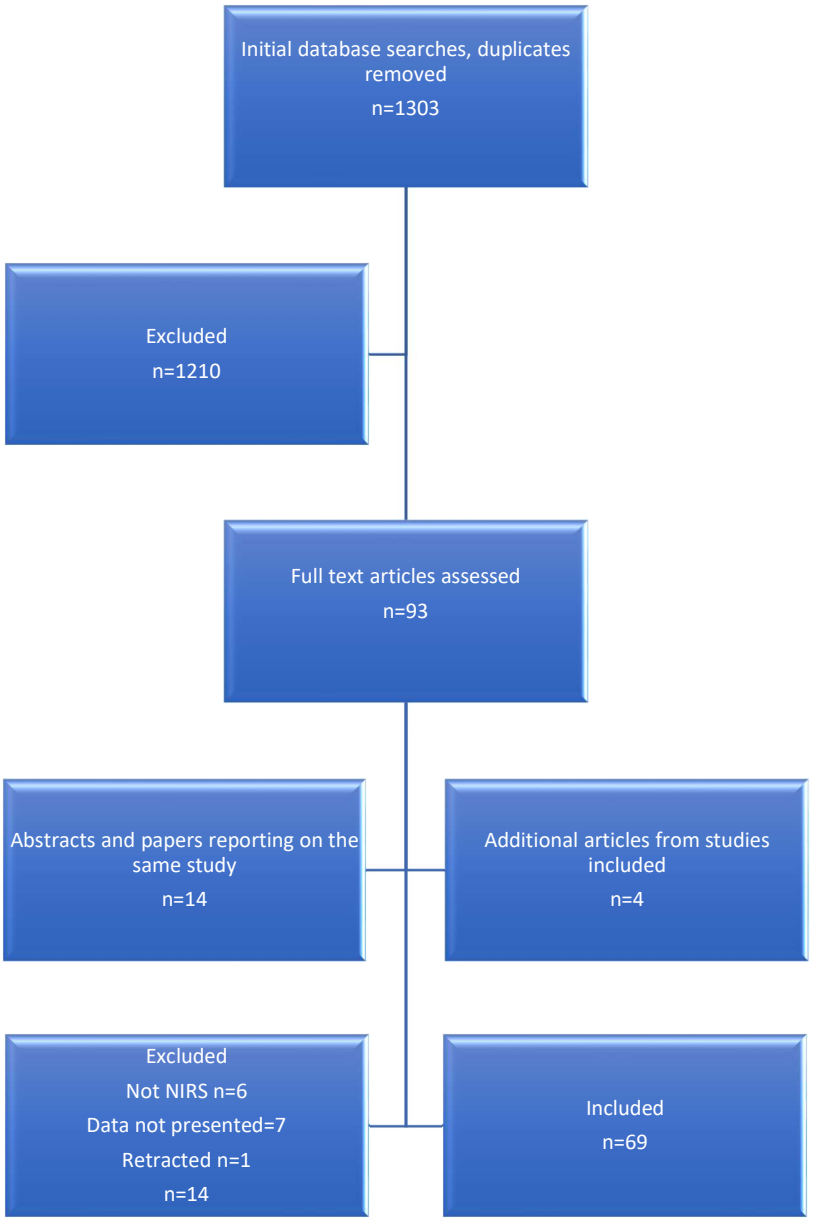


Table 3.1: Bias and applicability assessment for included studies.

Study ID	**Could the selection of patients have introduced bias?**	**Are there concerns that the included patients and setting do not match the review question?**	**Could the conduct or interpretation of the index test have introduced bias?**	**Are there concerns that the index test, its conduct, or interpretation differ from the review question?**	**Could the reference standard, its conduct, or its interpretation have introduced bias?**	**Are there concerns that the target condition as defined by the reference standard does not match the question?**	**Could the patient flow have introduced bias?**
Aktas 2016[167,168]	High risk	Low concern	High risk	Low concern	High risk	Low concern	Unclear risk
Ameloot 2015[169]	Low risk	Low concern	High risk	Low concern	High risk	Low concern	Low risk
Andersen 2015[170]	Low risk	Low concern	High risk	Low concern	Low risk	Low concern	Low risk
Baenziger 2009[171]	High risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	High risk
Bailey 2012[172]	Low risk	Low concern	Low risk	Low concern	Unclear risk	Low concern	Low risk

Banerjee 2016[173]	Unclear risk	Low concern	Low risk	Low concern	Unclear risk	Low concern	Unclear risk
Beekley 2010[174]	Low risk	Low concern	Unclear risk	Low concern	Low risk	Unclear concern	Low risk
Carlile 2015[175]	Low risk	Low concern	High risk	Low concern	High risk	Low concern	Low risk
Cem 2014[176]	High risk	low concern	Low risk	Low concern	Low risk	Low concern	High risk
Cerussi 2005[177]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Low risk
Creteur 2009[157]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Damiani 2015[178]	High risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk
Dani 2002[179]	Unclear risk	Low concern	Low risk	Low concern	Low risk	Low concern	High risk
Dani 2010[180]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Low risk
Dhabangi 2016[181]	Unclear risk	Low concern	Unclear risk	Low concern	Low risk	Low concern	Low risk
Donati 2014[182]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk

El-Dib 2016[183]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Low risk
Fiser 2014[184]	Low risk	Low concern	Low risk	Low concern	High risk	Low concern	Low risk
Green 2007[185]	Low risk	Low concern	High risk	Low concern	High risk	Low concern	Unclear risk
Hakim 2016[186]	High risk	Low concern	High risk	Low concern	High risk	Low concern	Unclear risk
Ito 2017[187]	Low risk	Low concern	Low risk	Low concern	Unclear risk	Low concern	Low risk
Jani 2019[188]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk
Khasawneh 2014[189]	Low risk	Low concern	High risk	Low concern	High risk	Low concern	High risk
Kiraly 2009[190]	High risk	Low concern	High risk	High concern	High risk	High concern	Unclear risk
Kobayashi 2017[191]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Kowalsky 2011[192]	Low risk	Low concern	Low risk	Low concern	Low risk	Unclear concern	Low risk
Kuo 2011[193]	Low risk	Low concern	Unclear risk	Low concern	Low risk	Low concern	Low risk

Leal-Noval 2017[194]	High risk	Low concern	Low risk	Low concern	Low risk	Low concern	Unclear risk
Lejus 2015[195]	High risk	Low concern	High risk	High concern	High risk	High concern	High risk
Liem 1997[196]	Unclear risk	Low concern	Low risk	Unclear concern	Low risk	Unclear concern	Low risk
McCredie 2017[197]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Memtsoudis 2015[198]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Menke 2004[199]	Unclear risk	Low concern	Low risk	High concern	Low risk	Low concern	Low risk
Meznar 2009[200]	Unclear risk	Low concern	High risk	Unclear concern	Unclear risk	Low concern	Unclear risk
Miller 2017[201]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk
Mintzer 2014[202]	Unclear risk	Low concern	Low risk	Low concern	Unclear risk	Low concern	Unclear risk
Moore 2008[203]	Low risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Low risk
Muthuchellappan 2018[204]	Unclear risk	Low concern	High risk	Low concern	Unclear risk	Low concern	Unclear risk

Naidech 2008[205]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk
Neunhoeffer 2018[206]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Podbregar 2015[158]	Unclear risk	Low concern	Low risk	Low concern	Low risk	Low concern	Unclear risk
Razlevice 2016[207]	Unclear risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Reisner 2016[208]	Unclear risk	Low concern	Unclear risk	Low concern	Low risk	Low concern	Unclear risk
Roberson 2012[209]	Low risk	Low concern	High risk	High concern	Low risk	High concern	Low risk
Rogers 2017[210]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Sadaka 2011[211]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk
Saito-Benz 2019[212]	Unclear risk	Low concern	High risk	Low concern	High risk	Low concern	High risk
Sandal 2014[213]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk
Schenkman 2017[214]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk

Seidel 2013[215]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk
Smith 2008[216]	Unclear risk	Low concern	Low risk	Low concern	Low risk	Low concern	Unclear risk
Sood 2014[217]	Low risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk
Stowell 2017[218]	Unclear risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Sung 2011[219]	Low risk	Low concern	Low risk	Low concern	Low risk	High concern	Unclear risk
Tobias 2011[220]	Unclear risk	Low concern	Low risk	Low concern	Low risk	High concern	Unclear risk
Torella 2002[221]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Torella 2002b[222]	Unclear risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Torella 2003[223]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Torella 2004[224]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk
Van Hoften 2010[225]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk

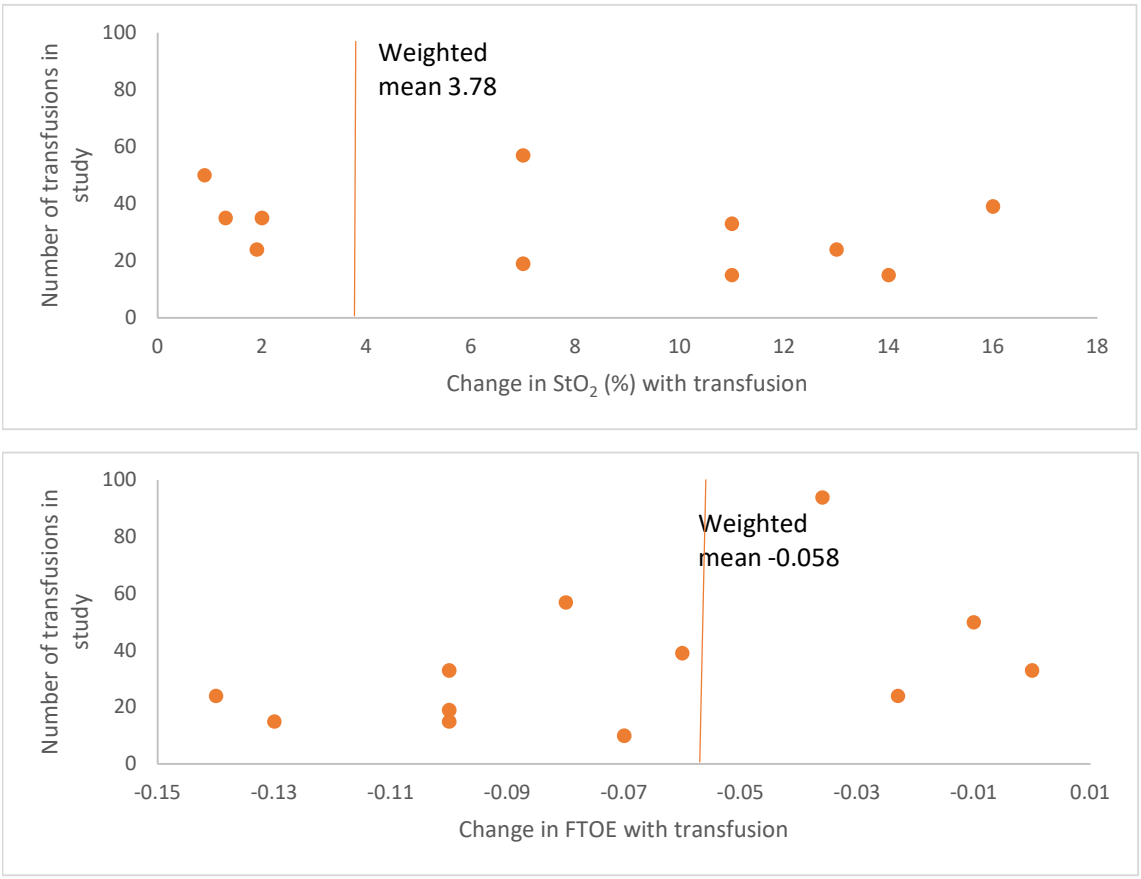
Vora 2017[226]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Low risk
Vretzakis 2013[227]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Walz 2017[228]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Wardle 1998[229]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Wardle 2000[230]	Unclear risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk
Wardle 2002[231]	Low risk	Low concern	Low risk	Low concern	High risk	Low concern	Unclear risk
White 2015[232]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	High risk
Yoshitani 2005[233]	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk	Low concern	Unclear risk
Yuruk 2012[234]	Low risk	Low concern	Low risk	Low concern	Low risk	Low concern	Low risk

Neonatal

There were 26 included studies using NIRS in neonates, which are summarised in table 3.2. A majority of these (20) were prospective observational studies, two were retrospective and there were three case-control studies. There was a single randomised controlled trial. These studies included observations related to 1178 transfusions in infants. NIRS probes were used to measure at cerebral (17 studies), splanchnic (9), muscle (5) and renal (4) sites using 13 different devices. Some papers used more than one site. There were 18 studies reporting on StO_2 and 17 studies reporting on FTOE before and after transfusion and three studies reported correlations between haemoglobin or haematocrit and tissue oxygenation. One study reported a change in total haemoglobin in cerebral tissue with transfusion of anaemic, or venesection of polycythaemic, neonates,[196] however total haemoglobin values were not reported by most studies as the values are arbitrary. In order to evaluate potential bias, funnel plots for StO_2 and FTOE, using cerebral NIRS (as this was the most commonly reported site) were constructed (Figure 3.4). These suggest a potential bias towards studies with a positive result.

Of the studies that reported on changes with transfusion only three didn't show an increase in StO_2 or decrease in FTOE. These were at splanchnic (2)[226,232] and cerebral (1)[188] sites. A further four studies showed no significant difference before and after transfusion in subgroups.[193,215,229,231] Asymptomatic neonates demonstrated no reduction in forearm muscle FTOE in one study, whereas symptomatic neonates did.[229] This is in contrast to a later study that also used forearm FTOE where neonates transfused based on physician discretion within a randomised trial for symptoms did not show a benefit.[231] Another showed improvement in transfusion for neonates with cyanotic heart disease in the lower two tertiles of haemoglobin concentrations, but not when the haemoglobin was above 133g/L.[193] While this may lead us to speculate that a lack of benefit from transfusion in infants with higher haemoglobin values is reflected in the lack of response in regional oxygen saturation, there were not enough data from other studies to confirm this.

Figure 3.4: Funnel plots of StO_2 (a) and FTOE (b) in studies reporting NIRS values before and after transfusion.



There were three studies reporting a correlation with the pre-transfusion haemoglobin or haematocrit and measures of tissue oxygenation, with two showing a higher StO_2[225] or reduced FTOE[170,225] with higher haemoglobin and the other no significant relationship.[215] Due to heterogeneity these could not be combined. A single study derived a receiver operator characteristic curve that suggested the splanchnic to cerebral oxygen ratio would predict the need for transfusion, however this was derived post hoc and requires prospective validation.[172]

While the majority of studies showed an increase in tissue oxygenation or a reduction in tissue oxygen extraction, the effect size was small. Owing to the lack of standardisation of instrumentation, the absolute values measured are not necessarily comparable.

Table 3.2: Studies using NIRS in neonatal setting

Study	N	Number of transfusions	Study type	Intervention	Device	Site	Measurement	Change	Correlation	p	Comments
Aktas 2016[167,168]	35	35	Observational	Transfusion	Sensmart X-100	Cerebral	StO_2	1.3		0.01	
						Splanchnic	StO_2	12		0.03	
						Renal	StO_2	2		0.01	
Andersen 2015[170]	24	24	Observational	Correlation	NIRO-200	Cerebral	FTOE		-0.47	0.04	
Baeziger 2009[171]	10	10	Observational	Transfusion	Cerebral Redox Monitor 2001	Muscle - gastrocnemius	StO_2	3.33		0.001	
							FTOE	-0.07		0.005	
Bailey 2012[172]	52	52	Observational	Transfusion	INVOS 5100C	Cerebral and Splanchnic	SCOR				Post hoc benefit from transfusion ROC
Banerjee 2016 group 1[173]	17	17	Observational	Transfusion	NIRO 300	Splanchnic	StO_2	15.4		0.01	
							FTOE	-16.3		0.004	
Banerjee 2016 group 2[173]	20	20	Observational	Transfusion	NIRO 300	Splanchnic	StO_2	13		0.01	
							FTOE	-14.4		0.005	
Banerjee 2016 group 3[173]	15	15	Observational	Transfusion	NIRO 300	Splanchnic	StO_2	12.5		0.01	
							FTOE	-12.9		4E-04	
Cerusi 2005[177]	17	10	Case control	Transfusion	OptiplexTS	Muscle - interscapular	StO_2	14		0.004	
Dani 2002[179]	14	14	Observational	Transfusion	N-300	Muscle - right hand	StO_2	11.3		1E-04	
Dani 2010[180]	15	15	Observational	Transfusion	INVOS 5100C	Cerebral	StO_2	14		1E-04	
						Splanchnic	StO_2	20		1E-04	
						Renal	StO_2	20		1E-04	
						Cerebral	FTOE	-0.13		1E-04	
						Splanchnic	FTOE	-0.19		1E-04	

						Renal	FTOE	-0.2		1E-04	
El-Dib 2016[183]	72	72	Observational		FORE-SIGHT	Cerebral	FTOE	-0.34		0.001	
Jani 2019[188]	50	50	Observational	Transfusion	NIRO-200	Cerebral	StO_2	0.9		0.9	
							FTOE	-0.01		0.8	
Kuo 2011 high[193]	61	61	Retrospective	Transfusion	Not stated	Cerebral	StO_2			0.28	
Kuo 2011 low[193]	61	61	Retrospective	Transfusion	Not stated	Cerebral	StO_2			0.01	
Kuo 2011 medium Hb[193]	61	61	Retrospective	Transfusion	Not stated	Cerebral	StO_2			0.03	
Liem 1997[196] transfused	13	13	Observational	Transfusion	In house	Cerebral	tHb	1.79			
Liem 1997 [196]venesected		0	Observational	Venesection	In house	Cerebral	tHb	-1.86			
Miller 2017[201]	30	30	Observational	Transfusion	INVOS 5100C	Splanchnic	StO_2	-5.41		0.001	
Mintzer 2014[202]	10	10	Observational	Transfusion	INVOS 5100C	Cerebral	FTOE	-0.07		0.05	
						Renal	FTOE	-0.18		0.05	
						Splanchnic	FTOE	-0.05		0.05	
Neunhoeffer 2018[206] biventricular cardiac	24	24	Observational	Transfusion	O2C	Cerebral	FTOE	-0.14		0.001	
							StO_2	13		0.001	
Neunhoeffer 2018[206] general	15	15	Observational	Transfusion	O2C	Cerebral	FTOE	-0.1		0.002	Post hoc, cFTOE<0.4 suggested benefit from transfusion
							StO_2	11		0.001	
Neunhoeffer 2018[206] univentricular cardiac	19	19	Observational	Transfusion	O2C	Cerebral	FTOE	-0.1		0.017	

Neunhoeffer 2018[206] univentricular cardiac	19	19	Observational	Transfusion	O2C	Cerebral	StO_2	7		0.007	
Razelevice[207] 2016	44	44	Observational	Surgery	INVOS	Cerebral	StO_2			0.001	<50% or >20% fall associated with transfusion
Saito-Benz 2019[212]	24	24	Observational	Transfusion	Sensmart X-100	Cerebral	StO_2	1.9		0.007	
							FTOE	-0.02		0.01	
Sandal 2014[213]	39	23	Case control	Transfusion	INVOS 5100C	Cerebral	StO_2	16		0.002	
							FTOE	-0.06		0.014	
						Splanchnic	StO_2	24		1E-04	
							FTOE	-0.13		0.007	
Seidel 2013[215]	76	76	Observational	Transfusion	INVOS 5100C	Cerebral	StO_2			0.05	
				Transfusion		Renal	StO_2			0.05	
				Correlation		Cerebral	StO_2		0.09	0.45	Correlation with Hb
				Correlation		Renal	StO_2		-1.14	0.22	Correlation with Hb
Sood 2014[217]	57	147	Observational	Transfusion	INVOS 5100C	Cerebral	StO_2	7		1E-04	Correlation with Hb
							FTOE	-0.08		1E-04	
						Splanchnic	StO_2	4.6		1E-04	
							FTOE	-0.05		1E-04	
van Hoften 2010[225]	33	47	Observational	Transfusion	INVOS 4100	Cerebral	StO_2	11		0.001	
							FTOE	-0.1		0.001	
							StO_2		0.41[#]	0.001	
							FTOE		-0.46[#]	0.001	Spearman correlation with Hb

Vora 2017[226]	13	13	Observational	Transfusion	INVOS 5100C	Splanchnic	StO_2	0		1	Spearman correlation with Hb
Wardle 1998[229] asymptomatic	94	24	Case control	Transfusion	NIRO 500	Muscle - forearm	FTOE	0		0.74	
		18						-0.06		0.001	
Wardle 2000[230]	94	46	Observational	Transfusion	NIRO 500	Cerebral	FTOE	-0.04		0.001	
Wardle 2002[231]	74		RCT	Transfusion	Not stated	Muscle - forearm					
Wardle 2002 Conventional - low Hb[231]		8	RCT	Transfusion	Not stated	Muscle - forearm	FTOE	-0.05		0.01	Transfused in NIRS = 56, v 84 in conventional p=0.64
Wardle 2002 Conventional - symptoms[231]		8	RCT	Transfusion	Not stated	Muscle - forearm	FTOE	-0.08		0.02	
Wardle 2002 NIRS high FOE[231]		18	RCT	Transfusion	Not stated	Muscle - forearm	FTOE	-0.18		0.001	
Wardle 2002 NIRS low Hb and high FiO_2[231]		8	RCT	Transfusion	Not stated	Muscle - forearm	FTOE	-0.05		0.005	
Wardle 2002 NIRS symptoms[231]		23	RCT	Transfusion	Not stated	Muscle - forearm	FTOE	-0.03		0.07	
White 2015[232]	23	23	Observational	Transfusion	FORE-SIGHT	Splanchnic	StO_2			0.86	
							FTOE			0.74	

Abbreviations: FTOE – Fractional tissue oxygen extraction; RCT – Randomised controlled trial; SCOR – Splanchnic to cerebral oxygen ratio; StO_2 – Tissue oxygen saturation; tHb – Total haemoglobin (units are arbitrary) # - Spearman correlation; * - Pearson correlation

The single randomised trial in neonates established transfusion thresholds based on NIRS (FTOE>0.47 using a partial venous occlusion technique) and haemoglobin, compared with haemoglobin and clinical features alone.[231] There was no significant difference in the number of children transfused or the number of transfusions per child between the groups. However, adherence to the protocol was poor with a large number of children transfused due to clinician concern in the NIRS group. The authors indicated a failure of the NIRS-based approach to detect neonates in need of transfusion according to the clinicians' views. It is noted that the study was not blinded and that there was no significant improvement in FTOE in the group transfused due to clinician concern, whereas there was in all other subgroups.[231] Insofar as the transfusion decision still included haemoglobin there is a high risk of bias towards the standard treatment approach in this study.

Trauma

There were seven studies evaluating NIRS and transfusion in the trauma setting.[174,175,189,190,203,208,216] All were observational. Two of these evaluated NIRS as a predictor of massive transfusion (defined variously as more than 10 or 3 units) rather than the need for any transfusion.[189,203] While this may be a valuable indicator, it is may be measuring more than the primary issue of tissue hypoxia due to anaemia (such as vasoconstriction). Excluding these two studies, all studies used a single device measuring muscle oxygen saturation (table 3.3). One studied compared fresh and older stored blood on tissue oxygenation in the trauma setting, and showed no change with fresh red cells, but a decrease with older red cells.[190] All other studies examined the relationship between StO_2 and the need for transfusion and despite using the same instrument the three studies that examined a cut-point StO_2 reading all used differing thresholds, from 65% to 75%. Different cut-points may partly explain the variability in sensitivity and specificity shown in figure 3.4 (A).

Table 3.3: Included trauma studies

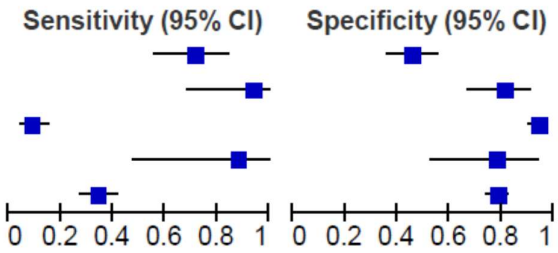
Study	N	Transfusion (n)	Study type	Intervention	Device	Site	Measurement	Comments
Beekley 2010[174]	147	42	Observational	Trauma	InSpectra	Muscle - thenar	StO_2	Comparison of minimum StO_2 in transfused or untransfused
Carlile 2015[175]	62	15	Observational	Trauma	InSpectra	Muscle - thenar	StO_2	Comparison of StO_2<75%
Khasawneh 2014[189]	325	113	Observational	Trauma	InSpectra	Muscle - thenar	StO_2	Comparison of STOs < 65%, transfusion and massive transfusion
Kiraly 2009[190]	32	46	Observational	Transfusion	InSpectra	Muscle - thenar	StO_2 AUC	Comparison of StO_2 drop with age of blood r=0.5 (p<0.05) - older blood led to a fall in oxygenation, fresher blood did not
Moore 2008[203]	383	NA	Observational	Trauma	InSpectra	Muscle - thenar	StO_2	Comparison of massive transfusion v not massively transfused
Reisner 2016[208]	487	NA	Observational	Trauma	Careguide 1100	Muscle - deltoid	StO_2	Comparison of transfused > or <3 units
Smith 2008[216]	26	8	Observational	Trauma	InSpectra	Muscle - thenar	StO_2	Comparison of < or > 70%

Figure 3.4: Sensitivity and specificity for StO_2 for transfusion (A) or massive transfusion (B) in trauma.

A

Predicting blood transfusion in trauma

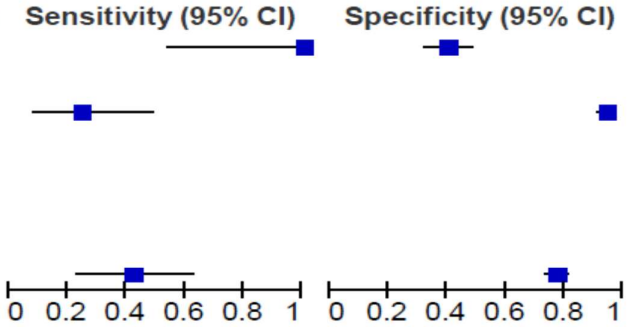
Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Beekley 2010	30	57	12	48	0.71 [0.55, 0.84]	0.46 [0.36, 0.56]
Carlile 2015	14	9	1	38	0.93 [0.68, 1.00]	0.81 [0.67, 0.91]
Khasawneh 2014	10	13	103	199	0.09 [0.04, 0.16]	0.94 [0.90, 0.97]
Smith 2008	7	4	1	14	0.88 [0.47, 1.00]	0.78 [0.52, 0.94]
Total, Trauma any transfusion	61	83	117	299	0.34 [0.27, 0.42]	0.78 [0.74, 0.82]



B

Predicting MT

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Beekley 2010	6	84	0	57	1.00 [0.54, 1.00]	0.40 [0.32, 0.49]
Carlile 2015	0	0	0	0	Not estimable	Not estimable
Khasawneh 2014	5	18	15	287	0.25 [0.09, 0.49]	0.94 [0.91, 0.96]
Moore 2008	0	0	0	0	Not estimable	Not estimable
Reisner 2016	0	0	0	0	Not estimable	Not estimable
Smith 2008	0	0	0	0	Not estimable	Not estimable
Stowell 2017	0	0	0	0	Not estimable	Not estimable
Total MT	11	102	15	344	0.42 [0.23, 0.63]	0.77 [0.73, 0.81]



While three studies showed a sensitivity above 70%, the weighted average is considerably lower due to the larger number of patients in the Khasawneh et al retrospective study.[174,175,189,216] Whereas the other retrospective studies chose a cut-point for StO_2 based on the need for transfusion, this study selected a value (65%) based on the need for massive transfusion. It was therefore better at predicting high volume blood users (massive transfusion) than transfusion *per se*, and significantly lower than the values derived predicting any transfusion. Only the Carlile study set the StO_2 cut-point (75%) prospectively, with the others deriving cut-points (70-78%) from their data retrospectively.[175] Notably, it was only in the trauma setting that studies reported on the sensitivity and specificity of StO_2 for predicting the need for transfusion.

Results for massive transfusion have also been reported by two of these studies, using the same criteria, and again show differences likely due to the different cut-points due to the ways they have selected them (figure 3.4 (B)).[174,189]

Paediatrics

There were six studies that used NIRS in the assessment of childhood anaemia or transfusion, beyond the neonatal setting.[181,184,186,195,214,220] All except one used cerebral tissue oxygenation, with two using muscle oxygenation either alone[214] or in addition to cerebral readings.[186] One study examined the change in tissue oxygenation before and after acute normovolaemic haemodilution immediately prior to surgery[220] and the others all examined the change with transfusion. One study was a pre and post intervention cohort study comparing transfusion rates before and after implementation of a protocol that included transfusion in response to low StO_2 levels in cardiac surgery.[195] There were at least three different devices used amongst the studies, with the device not being specified in one study. No studies determined a StO_2 threshold at which transfusion was indicated prospectively or determined an optimal value, such as from a receiver-operator characteristics curve. The studies are summarised in table 3.4.

The introduction of NIRS into paediatric cardiac surgery protocols reported by Lejus et al made no difference to the number of children transfused, but the haemoglobin concentration at which transfusion was initiated was higher with NIRS, suggesting it may have been useful for earlier recognition of tissue oxygen impairment.[195] The overall volume of red cells transfused was lower though, suggesting that continuous monitoring StO_2 may provide anaesthetists reassurance not to transfuse higher volumes of red cells. It is unclear whether the introduction of tissue oxygen monitoring was the cause of these changes, and whether transfusions after implementation were more or less appropriate than prior to implementation. With the same caveats, it is also interesting to note that the introduction of NIRS reduced intensive care length of stay.

There were four studies reporting on change in StO_2 with transfusion, with three showing a benefit.[181,186,214] The study that did not show an improvement in StO_2 with transfusion examined children on extracorporeal membrane oxygenation without a red cell transfusion protocol.[184] The authors noted that most of the children were not anaemic at the time of transfusion and there was no evidence of impaired oxygen delivery, perhaps explaining why transfusion also did not seem to improve StO_2. However, no change in StO_2 was seen also in the study that reported on changes before and after acute normovolaemic haemodilution.[220] Significant changes in StO_2 were reported by the other studies, including severe anaemia,[181] perioperative[186] and oncology outpatient settings.[214] It is interesting that the study that showed the largest improvement in StO_2 was in the oncology outpatient setting.[214] This may be due to the lack of confounding factors contributing to oxygen delivery in this group, such as sickling and malaria[181] or changes in blood pressure. Higher red cell

Table 3.4: Paediatric studies reporting StO_2 changes with changes in red cell masses

Study	N	Number Transfused	Study type	Setting	Intervention	Device	Site	Measurement	Change	r	p	Comments
Dhabangi 2016[181]	120	120	Observational	Severe anaemia	Transfusion	Equanox 7600	Cerebral	StO_2	6%		<0.001	Sickle cell disease and malaria accounted for most with StO_2 <65%
										0.2828		r2 = 0.08 for Hb and StO_2 correlation
Fiser 2014[184]	45	45	Observational	ECMO	Transfusion	Not stated	Cerebral	StO_2	0.56%		<0.05	ECMO. Most had normal Hb without evidence that O2 delivery was impaired. 95% CI 0.21-0.91
Hakim 2016[186]	50	33	Observational	Spinal Surgery	Transfusion	Invos	Muscle - deltoid	StO_2	3%		0.002	
							Cerebral	StO_2	8%		0.001	
Lejus 2015[195]	91	88	Case control	Cardiac surgery	Cardiac surgery	Invos	Cerebral	StO_2				NIRS (56) v no NIRS (35). Higher Hb at first transfusion with NIRS
Schenkman 2017[214]	16	5	Observational	Oncology outpatients	Transfusion	In house	Muscle - hand	StO_2	22%		<0.05	

Tobias 2011[220]	12		Observational	Peri-operative	Normovolaemic haemodilution	Invos 3100	Cerebral	StO_2	-4%		NS	

volumes transfused to minimise the need for repeated transfusion-related attendances may also be a factor, however post-transfusion haematocrits were not especially high (26.8-31.8%). It should also be noted that the study enrolled small numbers of transfusion episodes, so it possible that the results may represent outlying values.

Trials with NIRS guided transfusion

There were four randomised studies that used NIRS parameters prospectively in transfusion algorithms in cardiac surgery,[227] neurosurgical intensive care[194] and in neonatal intensive care.[231] Together they randomised 540 patients to NIRS-based or standard of care protocols. None showed a significant difference in clinical outcomes, including neurocognitive outcomes measured in one study.[210] The two cardiac surgical studies each used the same NIRS device, but had different NIRS-based transfusion criteria. Only one study, which had a more liberal NIRS-based transfusion strategy and a more restrictive haematocrit-based control arm, showed a significant difference in the number of people transfused, but not in the number of red cells transfused.[227] These studies are summarised in table 3.5 and figure 3.5 (A), showing a non-significant reduction in the number of people transfused under NIRS-based protocols (OR: 0.71 (0.46-1.10)).

The number of red cells transfused per patient was reported in three studies and showed a significant overall reduction of 0.44 (0.09-0.79) units per patient (figure 3.5(B)).

Methodological quality was assessed and judged to be low risk of bias in two studies,[210,227] but two had uncertain to high risk of bias due to unblinded clinicians, resulting in transfusion of patients in NIRS based protocols when they did not meet criteria or through possible bias in patient selection.[194,230] When only studies with a low risk of bias are included the odds ratio for transfusion was 0.62 (0.30-1.28).

Correlation with StO_2 and haemoglobin

There were seven studies that determined at least one correlation with haemoglobin concentration and StO_2. These were in the settings of major surgery (abdominal,[185] cardiac,[191] aortic,[222] spinal[222,224]

and hip replacements[233]), cardiac arrest[169] and subarachnoid haemorrhage.[205] All used the same device for measuring StO_2. Most studies found a positive correlation with cerebral StO_2. One study also measured StO_2 in the gastrocnemius and did not show a correlation with haemoglobin with gastrocnemius or cerebral StO_2, although there was a trend to a correlation with cerebral oxygenation.[224] The number of patients in this study was small (n=10). A preceding study had shown a correlation with haemoglobin and gastrocnemius muscle StO_2.[222] The correlations with haemoglobin, while present, were generally not strong, and are listed in table 3.6.

There were three studies reporting a correlation with haemoglobin changes and changes in StO_2, which are shown in table 3.7. Although all studies used the same measurement device, only two small studies, by the same group, demonstrated a positive correlation in the setting of major surgery.[223,224] This correlation was weak and only present in cerebral, not gastrocnemius muscle. The larger study, in the setting of cardiac surgery, showed no change with haemodilutional anaemia.[219] In this setting, maintaining the blood volume may help to sustain intravascular volume, and other factors, such as the use of inotropes and anaesthetic agents may influence local blood flow.

Additional studies

There were 23 additional studies in adults (table 3.8) that reported on differences between anaemic and non-anaemic groups, or changes in individuals before or after blood transfusion or blood loss due to surgery or venesection. While most studies occurred in acute settings, transfusions in haematology outpatients[158,234] and haemodialysis[187] involved chronic anaemia. In seven comparisons with StO_2 downslope at the onset of vascular occlusion, two reported significant changes in the rate of StO_2 fall, but in contradictory directions.[157,158,178,182,200] This compares with two of six where the upslope was measured, which were concordant with StO_2 changes.[157,158,178,182,211] There are no data to support the use of vascular occlusion testing over baseline StO_2 and there is a high degree of uncertainty as to whether dynamic testing may contribute to anaemia assessment.

With regards to the site of measurement, cerebral and muscle were the predominant tissues examined. Six studies examined both, with four[186,221-223] finding concordant results and two detecting cerebral changes with altered haemoglobin levels that were not significantly different when measured in muscle.[209,218] These two studies each examined small subgroups and used different devices to measure StO_2 at the different sites. Five neonatal studies examined simultaneous readings in cerebral and renal or splanchnic sites, and found them to be concordant.[168,180,202,213,215,217]

There were fewer studies using FTOE in adults than there were in the paediatric and neonatal setting. The FTOE appeared to have similar efficacy at separating haemoglobin concentration differences to StO_2. The former may be preferable when changes in arterial saturation may be expected, as in lung disease, and when changes in inspired oxygen concentration are anticipated.

The overall results suggest that NIRS changes were more reliable for measuring haemoglobin changes in neonates than in adults. In neonatal studies the haemoglobin increments are generally greater following transfusion than in adults, which may lead to a more substantial increase in tissue oxygenation. Foetal haemoglobin (HbF) is supplemented by adult (HbA) when transfusing neonates, with the former having higher oxygen affinity. Although these changes should be considered whenever evaluating technologies for transfusion triggers in neonates, the transition is unlikely to have an impact in this case. When haemoglobin is fully saturated in the lungs, oxygen delivery will be the same for HbA and HbF, with HbA more readily transferring oxygen to tissue cells.

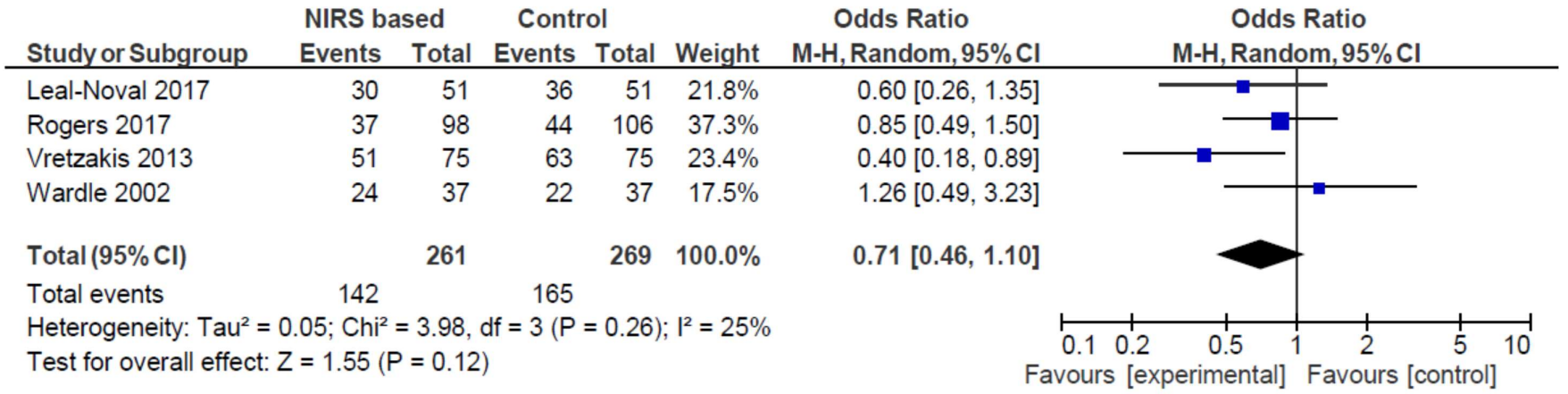
Table 3.5 Randomised trials comparing NIRS-based transfusion algorithms with haemoglobin or haematocrit-based triggers.

Study	N	Setting	Device	Site	Comparison	Result
Leal-Noval 2017[194]	102	Neurological ICU	Invos	Cerebral	Transfusion with Hb<85g/L v StO_2<60%	30 (59%) transfused in NIRS v 36 (71%) in Hb based protocol. P=0.15. 1.0 v 1.5 red cells transfused per patient P=0.04
Rogers 2017[210]	204	Cardiac surgery	Invos	Cerebral	Transfusion with Hct<23% vs Cerebral StO_2<50%, <70% of baseline levels or Hct<18%.	No difference in rates of transfusion (38% NIRS v 41% conventional), neurocognitive outcomes, LOS or ICU LOS.
Vretzakis 2013[227]	150	Cardiac surgery	Invos	Cerebral	StO_2<60% or >20% fall from baseline and low Hct vs low Hct only (<21%, but variable target during bypass)	46 (61%) transfused in NIRS v 55 (73%) in Hct based protocol. P=0.029
Wardle 2002[231]	74	Neonatal ICU	Not stated	Muscle - forearm	Transfusion based on standard Hb and symptom protocol or FTOE >47% during partial venous occlusion study	Transfused in NIRS = 24 (64%) v 22 (59%). 1.5 v 2.3 red cells transfused per patient p=0.64

Abbreviations: FTOE- fractional tissue oxygen extraction, Hb- haemoglobin, Hct – haematocrit, ICU – intensive care, LOS – Length of stay, NIRS – near infrared spectroscopy

Figure 3.5 Randomised controls trials of NIRs compared with standard of care, showing number of patients transfused (A) and number of transfusions per patient (B).

A



B

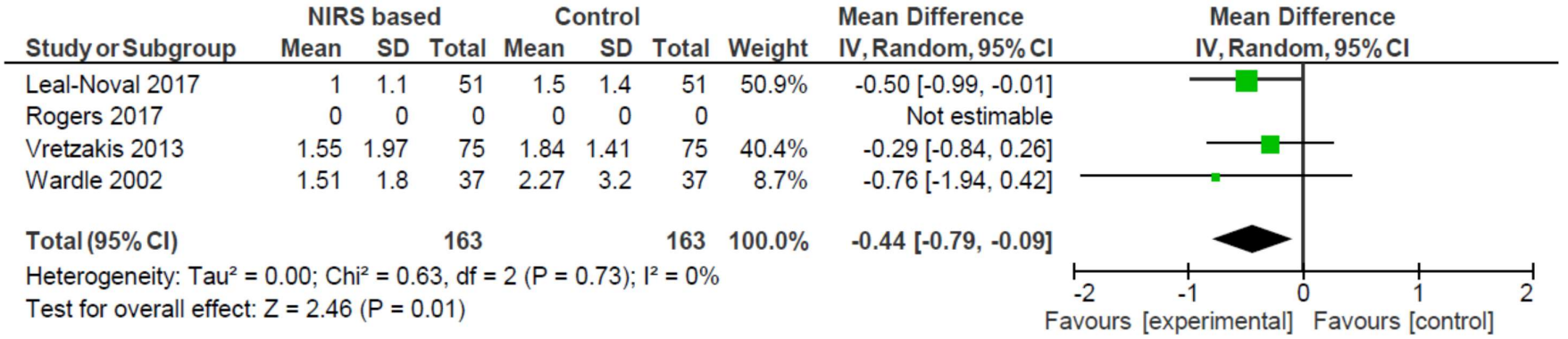


Table 3.6 Correlations with haemoglobin and StO_2 in adults

Study	N	Study type	Setting	Intervention	Device	Site	r	p
Ameloot 2015[169]	82	Observational	Cardiac arrest		Fore-sight	Cerebral	0.91	10^-6
Green 2007[185]	46	Observational	Major abdominal Surgery	Surgical blood loss	Invos	Cerebral	0.37	0.011
Kobayashi 2017[191]	223	Observational	Cardiac surgery		Invos	Cerebral	0.38	<0.001
Naidech 2008[205]	6	Observational	Subarachnoid haemorrhage		Invos	Cerebral	Not stated	<0.001
Torella 2002b[222]	30	Observational	Aortic surgery	Acute normovolaemic haemodilution	Invos	Muscle - calf	0.63	<0.001
						Cerebral	0.76	<0.001
Torella 2004[224]	10	Observational	Aortic and spinal surgery	Surgical blood loss	Invos	Muscle - calf	0.06	0.69
						Cerebral	0.29	0.09
Yoshitani 2005[233] - Propofol	18	Randomised Controlled trial	Total hip replacement	Surgical blood loss	Invos	Cerebral	0.37	<0.001
Yoshitani 2005[233] - Sevoflurane	19						0.46	<0.001

Table 3.7: Correlations with change in haemoglobin (including transfusion or blood loss) and StO_2 in adults

Study	N	Setting	Intervention	Device	Site	Comments	r	p
Sung 2011[219]	151	Cardiac surgery	Haemodilution	Invos	Cerebral	Correlation for change in Hb with StO_2	0.015	0.26
Torella 2003[223]	29	Aortic and spinal surgery	Transfusion	Invos	Muscle - calf	Correlation for change in Hb with StO_2	0.08	0.47
					Cerebral	Correlation for change in Hb with StO_2	0.59	<0.001
Torella 2004[224]	10	Spinal surgery	Surgical blood loss	Invos	Muscle - calf	Correlation of StO_2 with blood loss	0.13	0.42
					Cerebral	Correlation of StO_2 with blood loss	0.44	0.004

Table 3.8: Comparisons of NIRS parameters before and after a change in haemoglobin, or in anaemic and non-anaemic adult populations

Study	N	Number Transfused	Study type	Setting	Intervention	Device	Site	Measurement	Reported change	P
Cem 2014[176]	30		Case-control	Cardiac surgery		Invos	Cerebral	StO_2	13	<0.001
Creteur 2009[157]	44	44	Observational	Intensive care	Transfusion	InSpectra	Thenar	StO_2 (%)	0	NS
								StO_2 downslope (%/min)	1	NS
								StO_2 upslope (%/min)	-0.3	NS
Damiani 2015 fresh blood[178]	20	10	Case-control	Sepsis	Transfusion	InSpectra	Thenar	StO_2 (%)	2	0.03
								StO_2 downslope (%/min)	0.5	0.03
								StO_2 upslope (%/min)	17	<0.01
Damiani 2015 old blood[178]	20	10						StO_2 (%)	2	0.4
								StO_2 downslope (%/min)	8	0.16
								StO_2 upslope (%/min)	23	0.32
Donati 2014[182] non-leukodepleted (same population as "fresh blood"[178])	20	10	Case-control	Sepsis	Transfusion	InSpectra	Thenar	StO_2 (%)	3	0.59

Donati 2014[182] leukodepleted								StO_2 downslope (%/min)	0.9	0.56
								StO_2 upslope (%/min)	8.3	0.03
Ito 2017[187]	41	16	Case-control	ESRF on haemodialysis	Transfusion	Invos	Cerebral	StO_2 (%)	3.9	<0.001
								FTOE	-0.04	0.002
Kowalsky 2011 – muscle exercise[192]	34		Randomised Controlled trial	Blood donation	Blood donation	Invos	Cerebral	StO_2 (%)	-3.87	NS
Kowalsky 2011 – control[192]	38							StO_2 (%)	-4.27	NS
McCredie 2017[197]	19	19	Observational	Traumatic brain injury	Transfusion	Fore-sight	Cerebral	StO_2 (%)	2	0.11
								FTOE	-0.02	0.29
Memtsoudis 2015[198]	28	28	Observational	Total knee replacement	Transfusion	CareGuide	Muscle-deltoid	StO_2 (%)	3.4	0.007
Menke 2004[199]	50		Observational	Blood donation	Venesection	Criticon	Cerebral	StO_2 (%)	-0.44	<0.01
Meznar 2009[200] – haemodynamically stable group	281		Case-control	Emergency department		Invos	Muscle	StO_2 (%)	4.5	0.003
								StO_2 downslope (%/min)	-3.1	0.017
Meznar 2009[200] - haemodynamically unstable group	58							StO_2 (%)	8.3	0.04
								StO_2 downslope (%/min)	-1.9	NS
Muthuchellappan 2018[204]	13	13	Observational	Brain injury	Transfusion	Equanox	Cerebral	StO_2 (%)	10.5	<0.05
Podbregar 2015[158]	24	24	Observational	Haematology outpatients	Transfusion	Equanox	Thenar	StO_2 (%)	2	0.1
								StO_2 downslope (%/min)	-0.9	0.29

								StO_2 upslope (%/min)	4	0.74
Roberson 2012 – 7 day old blood[209]	8	8	Observational	Normal volunteers	Transfusion	Fore-sight	Cerebral	StO_2 (%)	1.4	0.03
						InSpectra	Thenar	StO_2 (%)	-1.8	0.14
Roberson 2012 – 42 day old blood[209]	8	8				Fore-sight	Cerebral	StO_2 (%)	0.4	0.23
						InSpectra	Thenar	StO_2 (%)	-0.4	0.41
Sadaka 2011[211]	21	21	Observational	Sepsis	Transfusion	InSpectra	Thenar	StO_2 (%)	-0.4	0.69
								StO_2 upslope (%/min)	0.1	0.78
Stowell 2017 10 day old blood [218]	50	22	Randomised Controlled trial	Cardiac surgery		InSpectra	Thenar	StO_2 (%)	1.7	NS
		19				Fore-sight	Cerebral	StO_2 (%)	2.6	<0.05
Stowell 2017 21 day old blood[218]		26				InSpectra	Thenar	StO_2 (%)	0.8	NS
		28				Fore-sight	Cerebral	StO_2 (%)	1.6	<0.05
Sung 2011[219]	151	151	Observational	Cardiac surgery	Haemodilution	Invos	Cerebral	StO_2 (%)	-13	<0.001
Torella 2002[235]	40		Observational	Blood donation	Venesection	Invos	Cerebral	StO_2 (%)	-3 (95%CI -2 to -3.9)	<0.001
							Muscle - calf	StO_2 (%)	-2.7 (95% CI: -1.7 to -3.8)	<0.001
Torella 2002b[222]	30		Observational	Aortic surgery	Acute normovolaemic haemodilution	Invos	Cerebral	StO_2 (%)	8	<0.001
							Muscle - calf	StO_2 (%)	5.5	<0.001
Torella 2003[223]	29	29	Observational	Aortic and spinal surgery	Transfusion	Invos	Cerebral	StO_2 (%)	4.2	<0.001
							Muscle - calf	StO_2 (%)	1.6	0.016
Torella 2004[223]	10	8	Observational	Major surgery	Surgical blood loss	Invos	Cerebral	StO_2 (%)	-8.4	0.004
Walz 2017[228]	20	20	Observational	Spinal Surgery	Transfusion	CareGuide	Muscle - deltoid	StO_2 (%)	1.29	0.0002

Yoshitani 2005 - Sevoflurane[233]	19		Randomised Controlled trial	Total hip replacement	Surgical blood loss	Invos	Cerebral	StO_2 (%)	-7	<0.05
Yoshitani 2005 - Propofol[233]	18								-6	<0.05
Yuruk 2012[234]	20	20	Observational	Haematology outpatients	Transfusion	InSpectra	Thenar	StO_2 (%)	5	0.002
							Sublingual	StO_2 (%)	5	<0.0001

Abbreviations: FTOE – fractional tissue oxygen extraction, StO_2 – Tissue oxygen saturation

Discussion

Near infrared spectroscopy detection of tissue oxygenation has the potential to measure oxygen delivery and ultimately assist in appropriate transfusion decision making. Oxygen delivery is dependent on respiratory, cardiac and vascular factors in addition to haemoglobin. As a transfusion trigger, compared to current, largely haemoglobin concentration based transfusion strategies, tissue oxygenation has the potential to determine which anaemic patients are compromised by anaemia and therefore may benefit from transfusion, taking into account the other factors that influence oxygen delivery. However, for the same reasons it lacks specificity for anaemia. This review brings together studies evaluating the effect of anaemia and transfusion on tissue oxygenation to determine its potential as a transfusion trigger.

There have been previous systematic reviews with NIRS. Serraino et al reviewed NIRS use in cardiac surgery.[236] They identified 10 randomised trials and concluded that there was neither harm nor benefit in NIRS-based algorithms, including no overall benefit in red cell transfusions. There was however a reduction in red cell transfusions when NIRS was used in two studies specifically to implement more restrictive transfusion practices, the objective of the current review. [210,227] When combined with randomised studies in neurosurgical intensive care[194] and neonatal intensive care,[231] NIRS-based transfusion strategies do not show a statistically significant reduction in the number of people transfused.

Despite this, the randomised trials have shown the potential for NIRS to assist with transfusion making decisions. As haemoglobin is not the only factor impacting on NIRS measurements, transfusion should not be the only response. However, if there is no reduction in tissue oxygenation then transfusion may not be required. These data suggest that this approach has the potential to reduce red cell requirements. Furthermore, this may be done without a risk of adverse events. In particular in the cardiac setting, Rogers undertook neurocognitive outcome assessments and found that the NIRS-

based protocols were safe.[210] The randomised studies however, lacked consistency in terms of the algorithms, including measurement cut-points.

Other systematic reviews with NIRS include its role in cerebral perioperative monitoring,[237] intensive care,[238] neonatal intensive care,[239] bone circulation,[240] vascular integrity of surgical reconstruction[241] and during normal exercise.[242] The heterogeneity of uses for tissue oxygenation measurement reflects the different physiological processes that contribute to tissue oxygenation and the variety of interventions that may be required to improve low saturation levels. Transfusion may not be the right approach to correct poor tissue oxygenation.

Measuring with NIRS requires clinicians to reconsider their traditional approaches to transfusion. As reflected by the high rate of protocol violations in the pilot randomised study conducted by Wardle and colleagues,[231] clinicians may be reluctant to tolerate untreated anaemia despite the real-time evidence of adequate tissue oxygenation. A belief in the current paradigm, as noted in the introductory chapter to this thesis, is an issue in developing new approaches and conducting the necessary clinical trials. However, the concerns regarding NIRS may not be completely unfounded.

There is no standard test measured by NIRS for making transfusion decisions. There are different measurements that can be made with NIRS, including StO_2, FTOE and changes during functional manoeuvres, particularly vascular occlusion testing. The latter may provide further information on vascular perfusion and adaptation, but requires additional time and testing by complete vascular occlusion. They are only applicable to measurements in muscle. As yet there are insufficient data to support one measure over another, and in particular an advantage of dynamic testing, such as with the vascular occlusion test, has not been established. StO_2 remains the most commonly used measure and appears suitable for most circumstances. FTOE appears to produce similar results and may be preferable where there is poor arterial oxygen saturation, such as in lung disease, and in particular when this is expected to change during observation.

There is also no standard site to measure NIRS for transfusion decision-making. While cerebral sites have predominated, muscle has also been widely used, with renal and splanchnic sites also used in infants. While all sites were comparable in neonates, cerebral data predominated. There was limited discordance (in two studies[209,218]) between muscle and cerebral sites, with the cerebral sites picking up changes where expected that were not detected by muscle, but both of these studies also used different instruments for the different sites. Although the cerebral site picked up more changes than muscle, these results overall present low-quality evidence not strongly supporting one site over another. A particular anatomical site cannot be recommended for measurements, but investigators should consider the treatment aims and sites at risk when selecting tissue sites for evaluation.

The lack of defined normal ranges and action triggers, and the variation in measurement wavelengths, inter-optode distance (and hence measurement depth) remain impediments to widespread implementation of NIRS for transfusion, although they are in common use in cardiac surgery despite the still limited evidence there.[243] Where available, direct tissue methods are preferred over NIRS in neurosurgical intensive care units.[244] The absolute difference between devices in most of the cited comparison studies is small compared with the inter and intraindividual variation. Further work should consider the role of functional changes in NIRS parameters that may differentiate better those with critical hypoxia.

All these studies examined patients in hospital or at rest. While transfusions in hospitalised patients are usually made about patients in bed, outpatients require enough haemoglobin to cope with the additional oxygen delivery demands with activity. In clinical settings, patients are often able to cope at rest, but complain of fatigue or dyspnoea limiting their performance capacity or endurance, even with activities of daily living. The lack of data on changes with StO_2 with exercise is addressed in the next study in this chapter.

Conclusions

There is currently insufficient data to recommend clinical use of NIRS to guide red cell transfusion. There is limited evidence that NIRS based transfusion protocols may be useful in restricting transfusion to those that have demonstrably poor tissue oxygenation that has not been corrected by adequate cardiorespiratory support. Better standardisation of sites, measurement standards and identification of ranges may assist in further research to define the role of NIRS in transfusion protocols.

Changes in Muscle Oxygen Saturation during exercise with anaemia

Aims:

The overall aim of this study is to explore whether dynamic testing of muscle oxygen saturation has the potential to correctly identify impairment due to anaemia. Specifically, it aims:

- To determine the normal pattern and values of gastrocnemius muscle oxygen saturation during 6MWT;
- To determine the effect of anaemia on gastrocnemius muscle oxygen saturation at rest and during 6MWT;
- To determine the effect of anaemia on forearm flexor muscle oxygen saturation at rest and during 20 second forced finger flexion;
- To determine whether correction of anaemia changes gastrocnemius muscle oxygen saturation during 6MWT within individuals.

Hypotheses:

- Anaemic patients will have greater impairment to muscle oxygen saturation (SMO2) as measured by lower nadirs of THb during 6MWT and isometric contraction.
- Anaemic patients will have slower recovery of SMO2 from nadir following isometric contraction and during 6MWT.
- Blood flow, as measured by THb will improve to a lesser extent with anaemia than controls during 6MWT.
- 6MWT distance, corrected for age and biometrics, will correlate with anaemia.
- That impairment in SMO2 during 6MWT and after isometric contraction will correlate with corrected performance in 6MWT.

Methods

This was an observational study, where participants undertook a 6 minute walk test and a brief isometric hand grip exercise while muscle oxygen saturation was measured by a non-invasive near infrared saturation monitor. Where participants had a significant change in haemoglobin concentration the tests could be repeated and compared.

Population

- The study enrolled two groups:
    - Normal controls (n=30): These included either:
        - a) Patients attending clinic without anaemia or;
        - b) other people (such as patient relatives, staff) without known anaemia or symptoms of anaemia (even if their Hb is unknown).
    - Anaemic participants: These were further divided into two groups, to ensure adequate numbers of participants with varying Hb levels. A minimum of 30 tests performed with Hb<80g/L and 30 with Hb 80-120g/L were planned.

Participants were recruited from hospital inpatient wards and outpatient clinics and selected based on Hb levels. Relatives were also approached. Staff were not approached in order to avoid the perception of coercion, however staff working in the areas seeing the research did express an interest and were given information on the study, some of whom offered to participate.

In order to be eligible participants had to meet the following inclusion and exclusion criteria:

- Inclusion Criteria:
    - 18 years of age or older
    - Able to understand the study and give fully informed consent in English or with a recognized heath care interpreter
    - Agree to participate and have signed the participant consent form
    - Meet one of the Hb recruitment bands:

- Hb<80g/L
- Hb >80g/L and <120g/L or
- Hb >120g/L or Hb unknown (presumed normal) in normal volunteers

- Exclusion Criteria:
    - Unable or unwilling to give informed consent
    - Recent deterioration in health due to a condition other than anaemia or bone marrow failure which may impact on exercise performance, including but not limited, to acute cardiac failure, pneumonia, exacerbation of chronic airways disease or major surgery. Participants with chronic cardiorespiratory conditions were not excluded
    - Recent (within 21 days) cardiac arrhythmia (other than chronic or recurrent atrial tachycardias), myocardial infarction, unstable angina or stroke
    - Controls with symptoms possibly attributable to anaemia, unless a recent haemoglobin has demonstrated a normal Hb and an alternative cause for symptoms is identified
    - In the opinion of the investigator, are unlikely to be able to walk independently for 6 minutes (but may participate in isometric test alone)
    - Conditions which may interfere with oximetry measurements (eg. methaemoglobinaemia)

All study procedures were conducted by the principal investigator, after obtaining written informed consent, in accordance with the study protocol approved by the ACT Human Research Ethics Committee and in compliance with the Declaration of Helsinki.

Study Procedures

A brief clinical history was obtained, either from the participant or their medical record. Measurement of baseline observations (pulse rate, arterial oxygen saturation and blood pressure) was conducted along with baseline symptoms of fatigue and dyspnoea measured by the modified Borg scale.[245] Skin fold thickness was measured using a calliper at NIRS measurement sites.

To measure StO_2, a portable wireless NIRS monitor (Moxy, Fortiori Design, Minnesota USA) was used. This device is designed to measure muscle oxygen saturation during exercise. It measures NIR light at four wavelengths between 630nm and 850nm wavelengths.[246] Sampling was at 2Hz, however for analysis all data (except the starting level) were 3s rolling means. For the 6MWT, the NIRS monitor was attached to the posterior calf over the gastrocnemius muscle and fixed into position with tubular bandages while the participant was at rest. For infection control purposes, the device was enclosed in a small clear plastic bag. Data from the portable monitor was transmitted directly to a portable laptop computer running exercise data collection and analysis software (Peripedal, Indiana USA) displaying real-time StO_2 and total haemoglobin (THb) readings. The 6MWT was commenced only when the resting StO_2 reached a steady state.

The 6MWT was conducted following recognised guidelines.[245] Participants walked back and forth along a measured 30m indoor corridor for a total of six minutes accompanied by the investigator. Instructions were given prior to starting and periodically during the test to ensure that symptoms of cardiorespiratory compromise were reported as well as update and encourage participants. They were instructed to walk as far as they could in six minutes, which may not necessarily mean beginning at the fastest possible pace. The walk could be stopped or paused at any time by the participant. During the test the investigator tracked the progress of the patient. Data were collected on the laptop, however the participants were kept blinded to the results during all tests to avoid biofeedback. The total distance travelled is measured by the investigator after six minutes and observations repeated,

including the modified Borg fatigue and dyspnoea scales. All procedures were carried out indoors in a clinical environment with emergency facilities available.

The 20s isometric handgrip involved the participant having the NIRS device held against the forearm flexors and recording through the laptop computer, as in the 6MWT. Either the 6MWT or the isometric exercise could be performed first, provided the participant was able to recover completely between tests. With the NIRS monitor in place and the arm rests on a pillow or arm rest, participants compressed a foam rubber ball ("stress ball") held within the palm of the hand for 20s, with the investigator offering encouragement throughout. They were asked to cease squeezing the ball and to leave the hand and forearm at rest immediately afterwards, with continuous reading thereafter until the StO_2 readings appeared to have reached a stable level.

Where a participant had a transfusion, or there was otherwise a change in haemoglobin concentration of more than 2g/L, repeat testing could be performed. Testing could be repeated with haemoglobin changes or pre and post transfusion for up to 4 tests.

Statistical analysis

Without prior studies, there were no data upon which to base power calculations. As an exploratory study, 30 participants were expected to undertake testing with normal haemoglobins, 30 with significant anaemia (Hb<80g/L) and 30 with Hb 80-120g/L. Recruitment for this initial study stopped once 30 participants undertook testing with presumed (controls) or known normal (>120g/L) haemoglobin concentrations. Descriptive statistics were applied to controls and anaemic groups. Data from the NIRS monitor was extracted to Excel and mean StO_2 and THb curves created for combined controls. The mean control curves for both the 6MWT test and isometric forearm were examined to determine significant features of the curves for quantification. These values were then determined for all tests for all participants.

Paired saturation samples from patients with changes in haemoglobin levels were assessed with paired t-tests. Where more than two tests were performed by one patient, they were paired

chronologically (ie: tests 1 & 2 and tests 3 & 4). Between group comparisons of continuous variables were assessed by unpaired t tests. For descriptive purposes, a clinically meaningful change in 6MWT distance was defined as an increase of 14m or more.[160] The effect of anaemia on the 6MWT distance and NIRS parameters were explored by Pearson correlation and significant parameters entered into stepwise multiple linear regression against the end points of 6MWT distance and haemoglobin concentration. In order to correct for participant biometrics, a relative 6MWT distance was calculated using the formulae proposed by Enright.[162] These equations were calculated by regression from healthy subjects aged 40 to 80 years. For men this was Distance (in m) = 7.57 x Height (in cm) – 5.02 x age (in years) – 1.76 x weight (in kg) – 3.09. For women it was Distance (in m) = 2.11 x height (in cm) – 2.29 x weight (in kg) – 5.78 x age (in years) + 667. The relative walk distance was the ratio of the actual distance walked to the expected distance. Pearson correlations between the 6MWT distance both actual and corrected, and with haemoglobin, were determined for the parameters identified on the StO_2 curves.

The process was repeated for the THb. The Moxy produces a THb, summing the oxygenated and deoxygenated readings and giving a result in g/L. However as noted above, these units are arbitrary, and it is likely that THb represents a significant component of myoglobin. During the study, large changes in THb were occasionally seen, usually as fractions of previous values, and were typically associated with sudden changes also in StO_2. As the THb was otherwise relatively constant these fluctuations were interpreted as read failures and *all data* from these timepoints were removed.

Data were plotted to determine whether some parameters may prove beneficial in further studies. Unless otherwise indicated, analyses were conducted in SPSS V24.0 (IBM). Findings were considered significant when $p<0.05$, however as this study is exploratory it is acknowledged that some findings may be considered hypothesis generating rather than conclusive and no correction for multiple analyses applied.

Results

There were 74 participants enrolled. The results of one (control) participant were excluded due to technical failure and another was recruited only for the isometric exercise test. There were 18 participants performing more than one set of exercise tests, with a total of 94 isometric exercise tests and 93 6MWT included. Where a patient had more than one set of assessments performed with haemoglobin levels more than and less than 120g/L they were included in the anaemic patient group for analysis (n=2).

All eligible participants completed the 6MWT. One sat to rest during the 6MWT (on two separate occasions). Walking aids were allowed and three participants walked with the aid of a stick, while one used crutches for assistance. Isometric test were performed on the dominant arm in 80 (85%) of cases. The most common reason for the non-dominant arm use was intravenous access in the dominant side. There was no significant difference in any of the measured parameters when the dominant and non-dominant performed tests were compared by independent t-tests. The majority of participants were Caucasian with two of Asian, one African and one Indigenous Australian. Characteristics of patients, controls and patients with normal haemoglobins are shown in table 3.9.

Table 3.9: Participant characteristics

	Controls (n=15)	**Non-anaemic patients (n=12)**	**Patients with anaemia (n=45)**
Median age in years (range)	64 (23-88)	62 (28-91)	64 (27-91)
Gender female (%)	10 (67%)	3 (25%)	21 (47%)
Median haemoglobin (g/L, range)	(135-150)*	141 (124-172)	82 (48-150)^
Median 6MWT distance in metres (range)	509 (359-727)	484 (300-669)	360 (147-698)
Diagnoses, number (%)	NA		
Non Hodgkin Lymphoma		3	14
Acute leukaemia		1	9
Chronic leukaemia		2	6
Myeloma		1	7
Myelodysplastic syndrome		5	
Myeloproliferative neoplasm		1	
Iron deficiency		1	5
Haemochromatosis		2	
Anaemia of chronic disease			1

*Only 2 controls had recent haemoglobin levels available. ^ Patients were counted as anaemic for the study if any test they performed was with haemoglobin <120g/L. From Crispin 2020,[247] used under licence.

The 6MWT relative walk distances were plotted for each group and compared (figure 3.6). There were no significant differences between the patients with normal haemoglobins and the normal controls (table 3.10), suggesting that haemoglobin was impacting on the relative walk distances.

Figure 3.6: Distance walked in 6MWT relative to expected distance based on Enright et al (relative walk distance), by participant grouping.

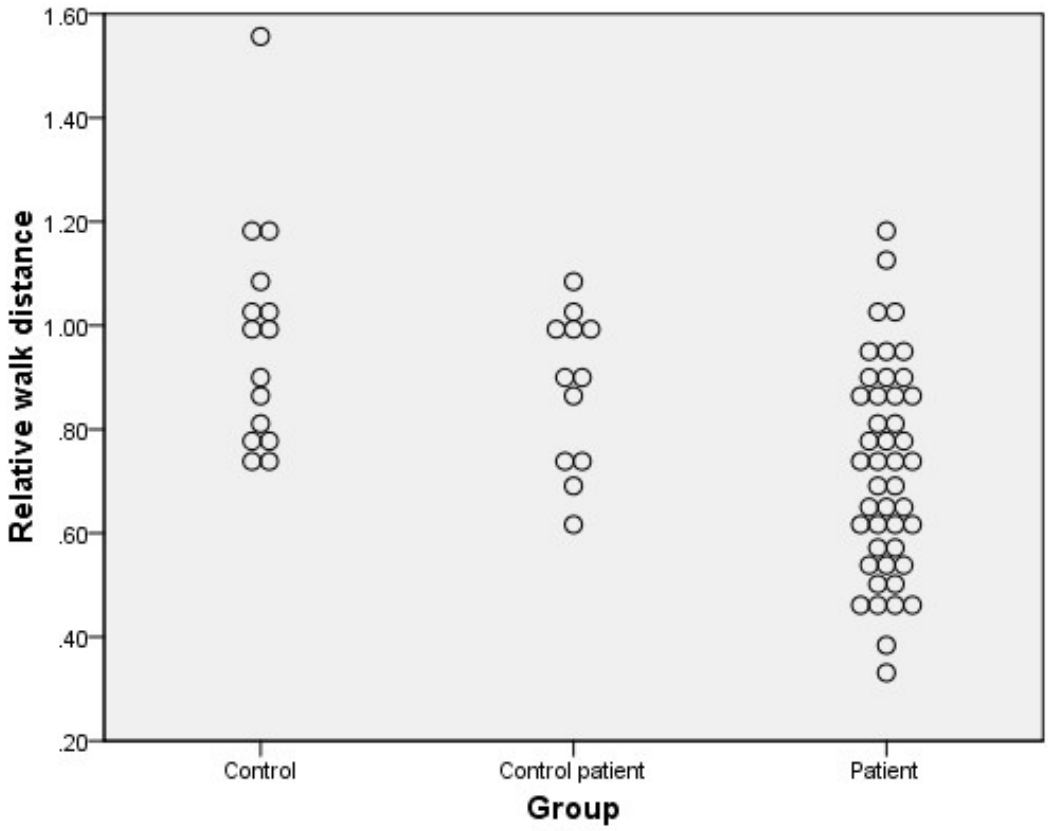


Table 3.10: Comparison of relative walk distances by participant group

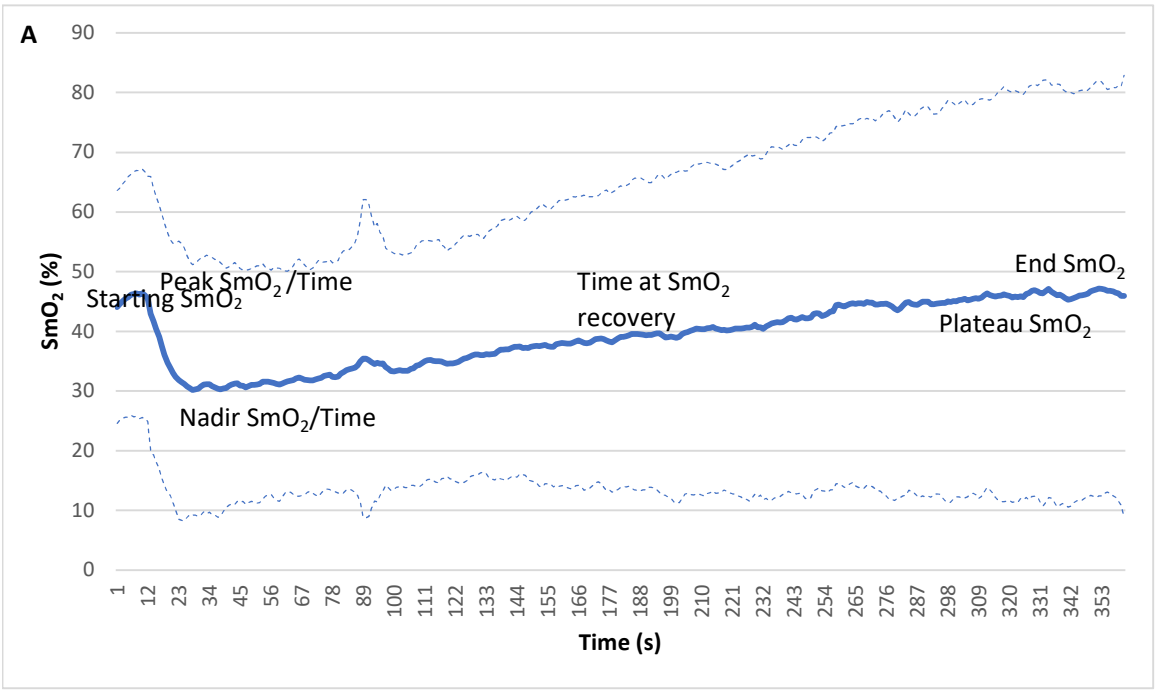
						Comparison (p)	
	N	**Mean**	**SEM**	**95% CI lower**	**95% CI upper**	**Control patients**	**Patients**
Control	15	0.9728	0.05617	0.862707	1.082893	0.218	<0.001
Control patients	12	0.8795	0.0431	0.795024	0.963976		0.013
Patients	45	0.7185	0.3009	0.128736	1.308264		
All controls	27	0.9131	0.03709	0.840404	0.985796		<0.001

The means of the absolute values at each time point in the 6MWT for controls were plotted (figure 3.7 (A)). In order to look at changes during the test rather than variation in baseline readings, the same data were graphed as mean changes from baseline (figure 3.7(B)). These were examined to determine significant patterns and points to be quantified for each participant. This was repeated for THb values. The typical pattern in the combined control curves demonstrated an initial brief peak suggesting increased blood flow as exercise begins, followed by a decline as oxygen utilisation exceeds supply. As walking continued there was a gradual improvement in tissue oxygen, presumably related to increased blood flow in response to exercise, with some participants reaching a plateau later in the test. The THb curve had a rapid fall, then a slight rise to a plateau before another more gradual fall. It is interesting that at the onset of exercise there was a rapid increase in SmO_2 with a simultaneous fall in THb, the latter suggesting a reduced amount of haem protein within the measurement field. Presumably this is due to vasoconstriction, with increased flow accounting for the increased local oxygen levels despite an immediate peak in oxygen usage. The variables are shown in figures 3.7 and 3.8 for SmO_2 and THb, respectively.

The identified parameters for analysis were defined as:

- Starting (or baseline): The absolute mean of SmO2 or THb. This is measured over 1s, whereas all other values are 3s rolling means
- Nadir: The lowest SmO_2 or THb during the exercise test
- Nadir time: The time (in s) at which the nadir occurs
- Time at first recovery: The time at which the SmO_2 or THb returns to baseline after nadir
- Plateau level: The first SmO_2 level where the curve flattens, reflecting a steady state during exercise
- End SmO_2: The SmO_2 at 360s of 6MWT

Figure 3.7: Mean (+/- 95%) SmO_2 values in controls during 6MWT expressed as absolute (a) values or change from baselines (b) confidence interval. Characteristics of the curves for which data were extracted are shown. Definitions are in the text.



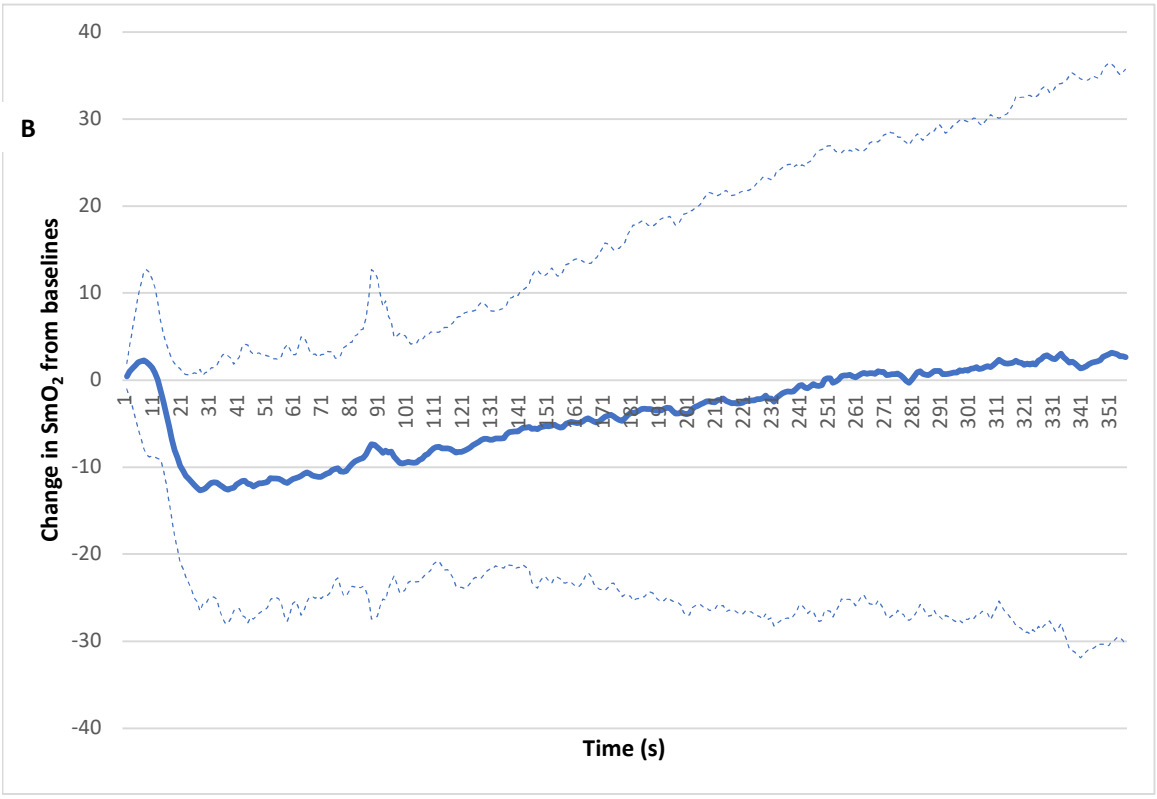


Figure 3.8: Mean (+/- 95% confidence interval) THb values in controls during 6MWT expressed as absolute (A) values or change from baselines (B). Characteristics of the curves for which data were extracted are shown. Definitions are in the text.

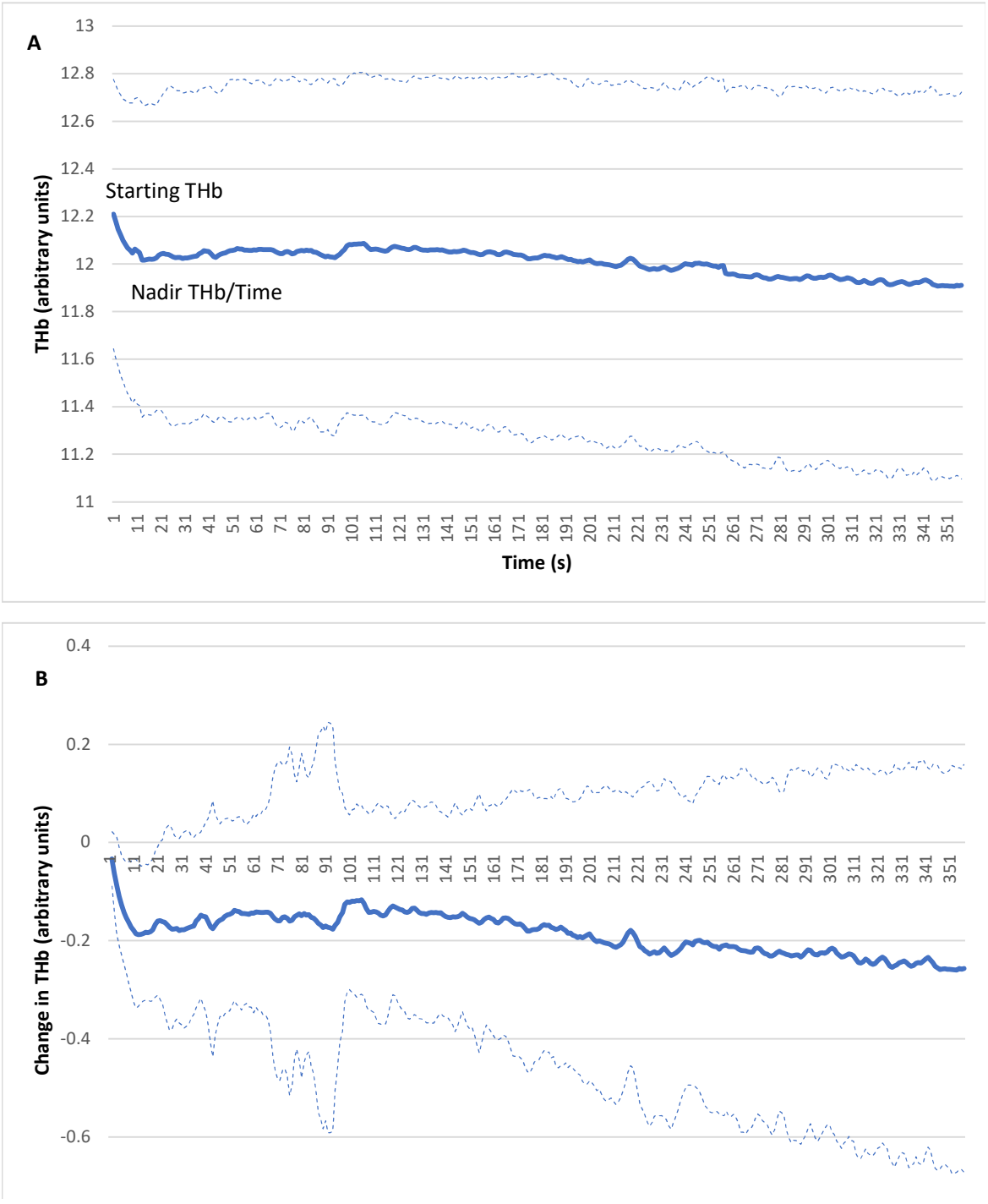
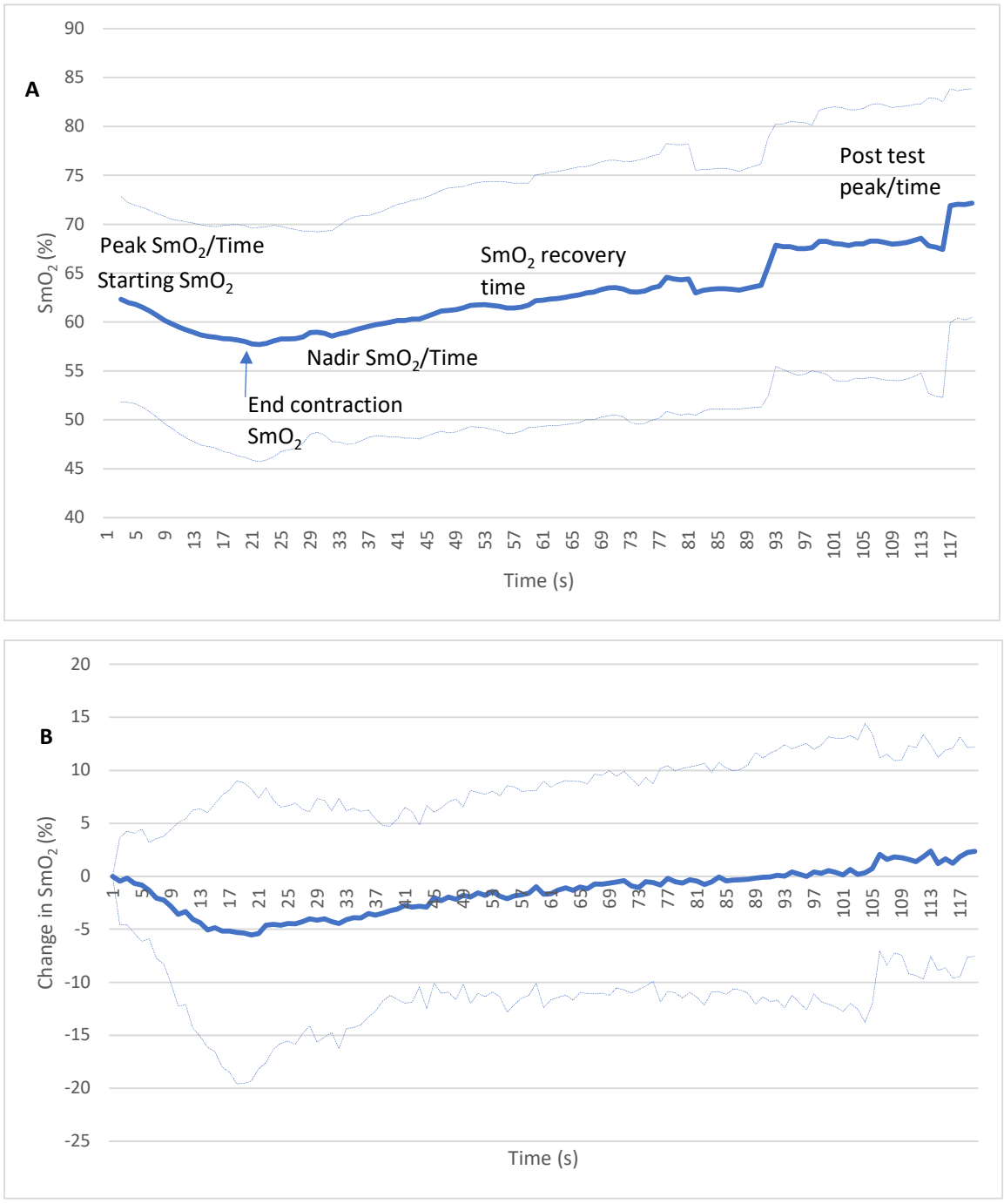


Figure 3.9: Mean (+/- 1SD) SmO_2 values in controls during isometric contraction, expressed as absolute (a) values or change from baselines (b). Characteristics of the curves for which data were extracted are shown. Definitions are in the text.



Isometric tests from control patients were analysed in the same way. While the test was to have contraction continue for 20s, 8 participants maintained contraction for 21-23s. The mean at each time

point was plotted, and the mean of the change from baseline (figure 3.9). This showed a linear fall in SmO_2 during the isometric contraction phase before a gradual recovery.

Parameters identified from these control curves were:

- Starting (or baseline): The SmO2 or THb immediately prior to contraction
- Nadir: The lowest SmO2 or THb value during isometric contraction
- Nadir time: The time (in s) of nadir value
- Peak SmO_2: during contraction: The maximum SmO_2 value during contraction
- Time of peak SmO_2: The time at which the maximum SmO2 occurred
- End: The value of SmO_2 at the end of contraction
- Recovery time: The time (in s) after nadir at which the SmO2 returns to the starting value
- Post test peak: The maximum value after completion of the isometric contraction
- Post test peak time: The time (in s from the start) of the post test peak

Using the changes during exercise, values for each exercise were obtained and univariate correlations performed. These are included in table 3.11. Linear regression against haemoglobin concentration using all walk tests with known haemoglobin concentrations showed a stronger association with actual walk distance than corrected distance, so actual walk distance was favoured over corrected distance in further analyses.

Higher dyspnoea and fatigue scores correlated with greater symptoms of fatigue and dyspnoea prior to undertaking the 6MWT. Fatigue after the exercise also correlated with haemoglobin, but the changes in fatigue and dyspnoea were not associated with the haemoglobin concentration. There was also a correlation with pulse prior to exercise and haemoglobin concentration. These changes affirm the physiological impact of haemoglobin in this cohort.

Table 3.11: Univariate correlations in 6MWT data

Variable 1	Variable 2	All tests		Including only first test (n=72)	
		Pearson correlation	**P**	**Pearson correlation**	**p**
Corrected walk distance	Starting SMO2	**0.251**	**0.015**		
	Nadir SMO2	0.127	0.224		
	Nadir SMO2 time	-0.143	0.172		
	Recovery time	0.129	0.496		
	Plateau	0.235	0.058		
	Plateau time	0.167	0.169		
	Starting Hb	-0.062	0.554		
	Absolute starting Hb	-0.053	0.616		
	Nadir Hb	-0.157	0.133		
	Nadir Hb Time	0.097	0.359		
	Recovery time Hb	-0.125	0.379		
	Pre-pulse	**-0.359**	**<0.001**		
	Pre-saturations	-0.186	0.074		
	Pre-fatigue	**-0.294**	**0.004**		
	Pre-dyspnoea	-0.155	0.138		
	Post-pulse	-0.075	0.477		
	Post-saturations	-0.094	0.368		
	Post-fatigue	**-0.453**	**<0.001**		
	Post-dyspnoea	-0.263	0.011		
	SMO2 fall	**0.204**	**0.05**		
	THb fall	0.189	0.07		
	Change in fatigue	**-0.277**	**0.007**		
	Change in dyspnoea	-0.17	0.104		
	Haemoglobin	**0.472**	**<0.001**		
Walk distance	Starting SMO2	**0.34**	**0.001**	**0.57**	**<0.001**
	Nadir SMO2	**0.258**	**0.013**	**0.26**	**0.026**
	Nadir SMO2 time	-0.169	0.105	-0.22	0.062
	Recovery time	-0.043	0.822	0.03	0.90
	Plateau	**0.400**	**0.001**	**0.41**	**0.003**
	Plateau time	0.118	0.335	0.16	0.25
	Starting Hb (3s)	-0.139	0.183	-0.018	0.89
	Nadir Hb	-0.2	0.055	-0.22	0.06
	Nadir Hb Time	0.149	0.157	0.137	0.26
	Recovery time Hb	-0.169	0.232	-0.139	0.41
	Pre-pulse	**-0.306**	**0.003**	**-0.33**	**0.004**
	Pre-fatigue	**-0.209**	**0.044**	**-0.30**	**0.011**
	Pre-dyspnoea	-0.109	0.292	-0.187	0.11
	Post-pulse	0.009	0.935	-0.27	0.82
	Post-saturations	-0.040	0.701		
	Post-fatigue	**-0.42**	**<0.001**	**-0.548**	**<0.001**
	Post-dyspnoea	**-0.291**	**0.005**	**-0.324**	**0.005**
	SMO2 fall	0.155	0.137	0.118	0.32

	THb fall	0.175	0.094		
	Change in fatigue	**-0.323**	**0.002**	**-0.361**	**0.002**
	Change in dyspnoea	**-0.252**	**0.015**	-0.219	0.06
	Haemoglobin			**0.567**	**<0.001**
Haemoglobin	Starting SMO2	**0.577**	**<0.001**		
	Nadir SMO2	**0.425**	**<0.001**		
	Nadir SMO2 time	-0.11	0.36		
	Recovery time	-0.306	0.25		
	Plateau	**0.483**	**<0.001**		
	Plateau time	-0.61	0.665		
	Starting Hb (3s)	0.016	0.896		
	Absolute starting Hb	0.022	0.853		
	Nadir Hb	-0.041	0.735		
	Nadir Hb Time	0.005	0.97		
	Recovery time Hb	-0.095	0.534		
	Pre-pulse	**-0.458**	**<0.001**		
	Pre-saturations	**-0.237**	**0.047**		
	Pre-fatigue	**-0.343**	**0.003**		
	Pre-dyspnoea	**-0.414**	**<0.001**		
	Post-pulse	-0.214	0.73		
	Post-saturations	-0.043	0.721		
	Post-fatigue	**-0.400**	**0.001**		
	Post-dyspnoea	**-0.335**	**0.004**		
	SMO2 fall	**0.28**	**0.018**		
	THb fall	0.081	0.500		
	Change in fatigue	0.025	-0.138		
	Change in dyspnoea	0.835	0.253		

The distance walked in the 6MWT was poorly correlated with baseline levels of fatigue (r=-0.21, p=0.04) and not with dyspnoea, but there was a smaller increase in fatigue and dyspnoea scores with longer distances walked.

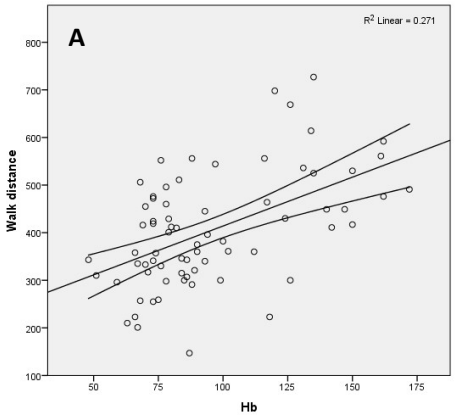
SmO_2 levels in gastrocnemius muscles at baseline correlated with the haemoglobin concentration and the distance walked over 6 minutes (figure 3.11 (B & C)), as did the nadir and plateau SmO_2 levels (figure 3.11(D & E)). There was a weak correlation with the absolute fall in SmO2 and haemoglobin (r=0.28, p=0.018), but not with the walk distance (r=0.155, p=0.14).

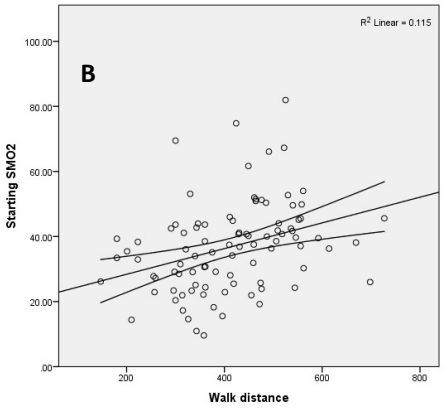
In order to evaluate the potential effect of multiple tests in the same patient, correlations were repeat using only the first test performed by each participant. In most cases (Table 3.11) this showed similar correlations, although the change in dyspnoea before and after exercise lost significance when only the first tests were considered.

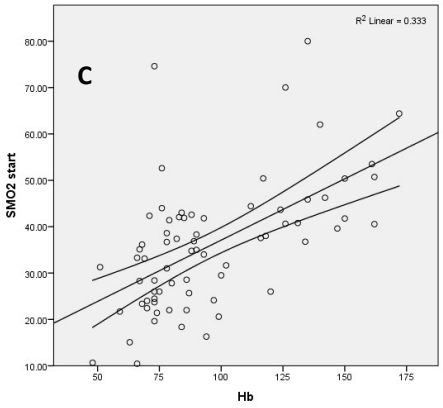
Including all the factors with a univariate correlation to walk distance into a multivariate stepwise linear regression model identified only the haemoglobin concentration as an independent predictor of the distance walked. Although walk distance, as the functional measure, was the primary dependent variable, multivariate linear regression was also performed with haemoglobin as the dependent variable. This showed the starting SmO2, pulse prior to exercise and walk distance were independently associated.

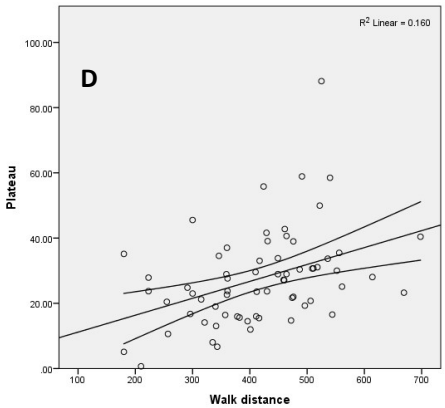
There were no correlations between NIRS estimates of total haemoglobin in the tissues and either the venous haemoglobin concentration or exercise capacity.

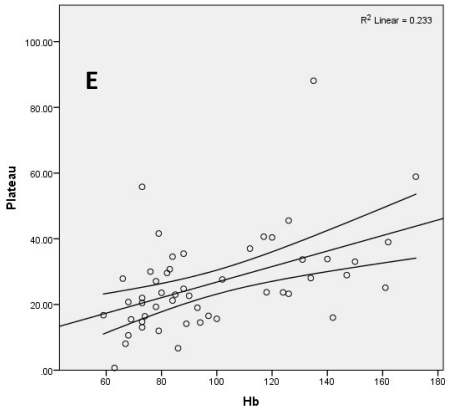
Figure 3.11: Correlations between haemoglobin (g/L), walk distance (m), pulse (beats/s) and the SmO_2(%) during the 6MWT. Abbreviations: Hb – Haemoglobin; SmO2 – Muscle oxygen saturation; 6MWT – Six minute walk test)

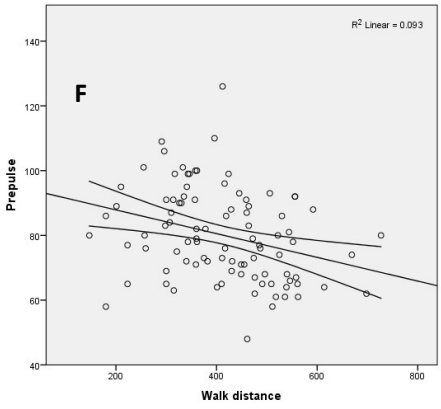


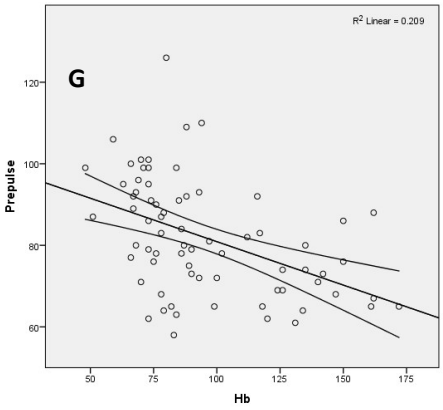












Change in SmO2 with individual changes in haemoglobin

There were 15 participants who had repeated exercise measurement following a change in haemoglobin, a total of 17 paired tests (ie: two participants completed four tests). Haemoglobin changes were either measured and quantified, or a haemoglobin increment was assumed if repeated testing was conducted immediately after transfusion. The haemoglobin for both sets of exercises was available in nine cases. There were 13 paired test sets pre and post transfusion, one where there was recovery of haemoglobin after an intervening five month period and three where the haemoglobin fell between the first and second tests. All analyses compared the higher with the lower haemoglobin irrespective of the chronological order of testing. Of the 17 paired tests, only 9 were accompanied by an increase in 6MWT distance of 14m or more.

There were significant improvements in physical signs and symptoms within individuals when they had a higher compared with a lower haemoglobin. The pulse rate was lower both before (mean of -9.4s, $p<0.001$) and after (-7.7s, $p=0.017$), despite walking a mean of 48.4m further with higher haemoglobins. Fatigue was reduced before (-0.76, $p=0.009$) and after (-1.18, $p=0.23$) exercise, but dyspnoea (-0.38, $p=0.27$, before) was not. Although there was a correlation across the population between baseline SmO2 and haemoglobin, there was no significant improvement in these paired samples between readings in the gastrocnemius with higher and lower haemoglobins (mean difference 5.3, $p=0.28$). Other SmO2 parameters were also not significantly different with higher and lower haemoglobin readings.

Patients who did not improve with higher haemoglobins

While the majority of participants improved their walk distance, examining those that did not is informative to determine potential limitations of this approach. Five patients did not show walk distance improvement of more than 14m with higher haemoglobins. One of these had the first test prior to treatment, with a haemoglobin of 112g/L, and the subsequent test performed 19 days later with a haemoglobin of 74g/L following chemotherapy for lymphoma, and had concurrent knee joint

osteoarthritis. The initial walk test of 360m did not significantly change despite intervening chemotherapy and fall in haemoglobin. It is possible that the concurrent arthritis impeded walk distance, so there was no significant further reduction despite intervening therapy and lower haemoglobin as systemic factors may not have been rate-limiting.

In the transfused group, one had an increase of 13m with a haemoglobin increment of 5g/L post transfusion. One had a decreased walk distance of 21m following a 2 unit transfusion (post transfusion haemoglobin not performed). She had a shuffling gait and early dementia as confounding factors. She reported more fatigue post transfusion (0.5 v 0) and there was no difference in pulse or dyspnoea prior to exercise. Post exercise, the pulse rate was lower following transfusion (104bpm pre v 96bpm post) and fatigue was reported as 1 prior to transfusion and 0 post transfusion. Another started with a haemoglobin of 67g/L and post transfusion (no haemoglobin obtained) had a reduced walk distance from 201 to 180m. There was no difference between the pulse (89/min v 86/min) or fatigue (0) prior to exercise, although dyspnoea was reported (1 v 0) pre but not post transfusion. Following exercise, fatigue was reported as 3 prior to transfusion and 2 post transfusion. The patient reported concurrent hip pain and had to rest during both exercises. He recommenced walking in the pre-transfusion walk, but not the second, as he stopped later (possibly indicating improved stamina). Calculating the actual time walking (4:43s v 4:17s) gives an almost identical pace of 42.6m/min pre-transfusion v 42.0m/s post transfusion. The final patient who did not improve had a haemoglobin of 76g/L followed by a 2 unit transfusion (no haemoglobin performed) and walk distance deteriorated from 552 to 518m (predicted 484m). Pre-exercise fatigue score was higher prior to than post transfusion (3 v 1), as was dyspnoea (4 v 1). Pulse was higher before transfusion, both before (78/min v 61/min) and after (106/min v 81/min) exercise, fatigue improved (5 v 2) and dyspnoea improved (5 v 2), but it is unclear whether these results reflect reduced capacity or reduced effort intensity.

Table 3.12: Comparison of 6MWT results in patients who had testing performed at different haemoglobin concentrations (change = test with higher - test with lower haemoglobin)

	All pairs (walk with higher Hb-lower Hb)			**Transfused pairs with change in walk distance >14m**		
Parameter	Mean change	N	P	Mean change	N	P
Haemoglobin (g/L)	20	9	0.003	12.5	4	0.046
Pre-pulse (/s)	-9.4	17	<0.001	-8.1	9	0.16
Pre-fatigue	-0.76	17	0.009	-1.11	9	0.012
Pre-dyspnoea	-0.38	17	0.272	-0.278	9	0.48
Walk distance (m)	48.41	17	0.009	65.78	9	0.004
Post-pulse (/s)	-7.65	17	0.017	-3.9	9	0.44
Post-fatigue	-1.18	17	0.007	-1.89	9	<0.001
Post-dyspnoea	-0.53	17	0.23	-0.56	9	0.47
Starting SMO_2 (%)	5.29	17	0.28	6.85	9	0.44
SMO_2 Nadir (%)	8.53	17	0.055	6.19	9	0.35
SMO_2 Nadir time (s)	-13.82	17	0.71	-28	9	0.62
SMO_2 recovery time (s)	-70.5	2	0.622			
SMO_2 plateau level (%)	5.22	6	0.407	2.7	3	0.66
SMO_2 plateau time (s)	-24.1	6	0.25	-2.67	3	0.94
THb start (g/dL)	-0.07	17	0.53	-0.24	9	0.128
THb Nadir (g/dL)	-0.28	17	0.28	-0.64	9	0.18
THb Nadir time	32.8	16	0.353	25.78	9	0.25
THb recovery time (s)	-5.44	8	0.891	16.4	5	0.544
Change in SMO_2 (%) (Initial-Nadir)	-3.24	17	0.29	0.66	9	0.88
SMO2 Nadir to end	-0.31	16	0.86	ND	ND	
Change in THb (g/dL)	0.22	17	0.335	0.40	9	0.352

The transfused participants had before and after testing chronologically close in most cases. This cohort would therefore be expected to have fewer changes in their underlying condition than where there has was a longer time between testing.

In order to determine whether baseline factors were predictive of improvement in walk distance, baseline values from the lower haemoglobin test of each pair were compared between groups that did or did not improve, defined as a >14m increase in walk distance (table 3.12). Baseline SmO2 was

the only factor predictive of an improvement in walk distance with Pearson correlation (r=-0.52, p=0.03, figure 3.12).

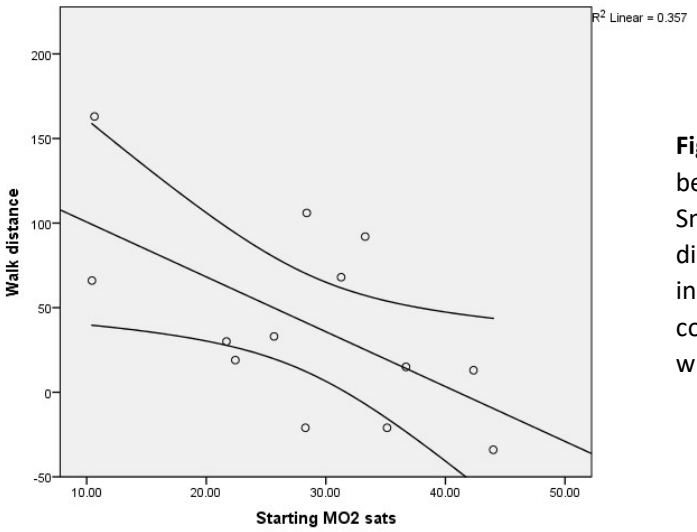


Figure 3.12: Correlation between starting gastrocnemius SmO2 and the improvement in distance walked following an increase in haemoglobin concentration in participants who has repeated 6MWT.

Isometric contraction

Correlations (Pearson) were performed to determine associations with haemoglobin concentration (when known) initially and walk distance (table 3.13).

As in the gastrocnemius muscle, there was a positive correlation between the SmO2 at rest in the forearm and the distance walked in the 6MWT (figure 4.14 (A)), as well as the haemoglobin concentration. There was a correlation between the SmO2 at rest measured in the gastrocnemius and forearm, however, there was a considerable degree of variability, highlighting a difficulty with SmO2 measurements showing significant regional variation (figure 3.13).

Table 3.13: Correlations between SmO_2 variables and haemoglobin concentration and the distance walked in 6MWT

Variable 1	Variable 2	Pearson correlation	P value
Haemoglobin	**PreSMO2**	**0.30**	**0.01**
	Nadir 3s	**0.30**	**0.011**
	Nadir 3s Time	0.196	0.1
	SMO2 (3s) peak during contraction	**0.32**	**0.007**
	Time @SMO2 peak during contraction	0.058	0.63
	SMO2 (3s)at the end of contraction	**0.235**	**0.047**
	SMO2 (3s) recovery time	-0.12	0.365
	Post peak (3s) SMO2	0.196	0.101
	Post peak time	**-0.270**	**0.023**
	SMO2 (3s) fall from start	-0.20	0.865
	SmO2 nadir to post exercise peak (3s)	0.025	0.832
	THb start	0.22	0.068
	THb Nadir (3s)	0.21	0.077
	THb nadir (3s) time	0.036	0.76
	THb recovery (3s)	0.229	0.13
6MWT distance	**PreSMO2**	**0.242**	**0.02**
	Nadir 3s	**0.295**	**0.004**
	Nadir 3s Time	0.056	0.594
	SMO2 (3s) peak during contraction	**0.267**	**0.01**
	Time @SMO2 peak during contraction	-0.001	0.99
	SMO2 (3s)at the end of contraction	**0.27**	**0.01**
	SMO2 (3s) recovery time	**-0.31**	**0.007**
	Post peak (3s) SMO2	0.12	0.26

	Post peak time	**-0.26**	**0.013**
	SMO2 (3s) fall from start	-0.11	0.28
	SmO2 nadir to post exercise peak (3s)	-0.06	0.569
	THb start	0.16	0.12
	THb Nadir (3s)	0.18	0.07
	THb nadir (3s) time	-0.013	0.9
	THb recovery (3s)	-0.70	0.59
6MWT pre SMO2	Isometric pre SMO2	0.483	<0.001
Skin fold thickness	**PreSMO2**	**0.22**	**0.034**
	THb start	**-0.59**	**<0.001**
	THb nadir 3s	**-0.58**	**<0.001**
	THb nadir 3s time	-0.09	0.38

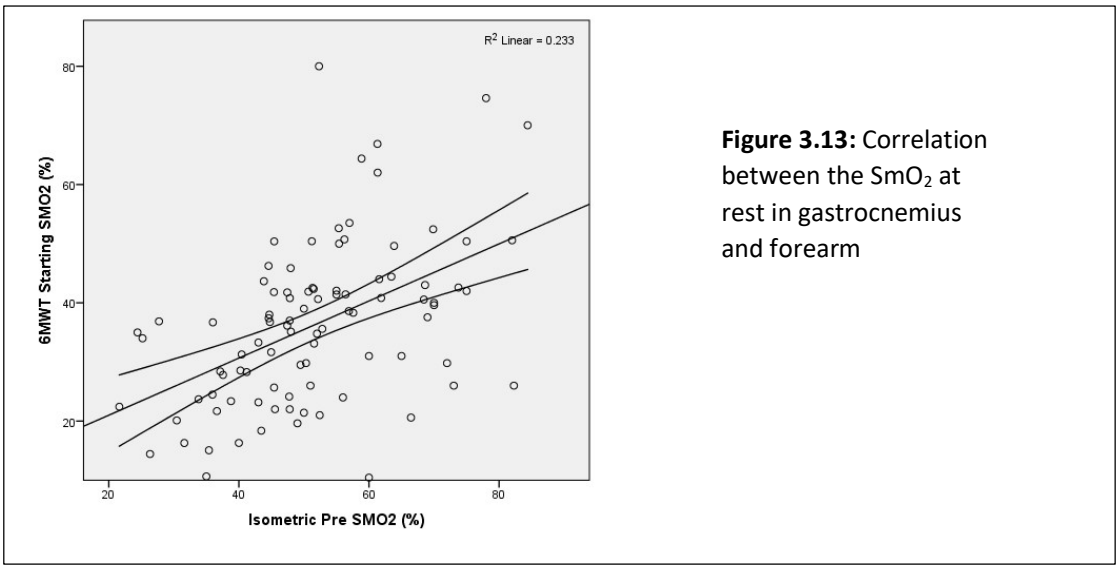


Figure 3.13: Correlation between the SmO_2 at rest in gastrocnemius and forearm

There were correlations with walk distance and the peak SmO2 during isometric contraction, which represents a very brief increase as the muscle contracts, nadir SmO2 and the SmO2 at the end of isometric contraction (3.14(B)). These are not independent and likely represent, as with the 6MWT,

changes during contraction, which are not markedly different with varying haemoglobin levels superimposed upon a change in baseline SmO2, which does vary with haemoglobin.

The time at which SmO2 recovered was shorter in participants who were able to walk farther in the 6MWT, as was the time at which the peak SmO2 occurred after the contraction (Figure 3.14 (C)). The post contraction peak time was negatively correlated with haemoglobin, but the recovery time was not. These results suggest that anaemia impairs local tissue oxygen recover after brief isometric exercise.

When significant factors were placed into a stepwise linear regression model the time of SmO2 recovery and the early SmO2 peak level during contraction remained independently predictive, with an adjusted R^2 of 0.166.

Table 3.14: Comparison of isometric results in patients who had testing performed at different haemoglobins (change = test with higher - test with lower haemoglobin)

	All pairs (walk with higher Hb-lower Hb)			Transfused pairs with change in walk distance >14m		
Parameter	Mean change	N	P	Mean change	N	P
Haemoglobin (g/L)	20	9	0.003	22	6	0.01
Walk distance (m)	48.41	17	0.009	78.9	11	0.002
Starting SMO_2 (%)	4.66	16	0.12	3.27	11	0.41
SmO_2 peak during contraction (%)	4.33	16	0.111	3.82	11	0.31
SmO_2 Nadir (%)	6.9	16	0.13	8.21	11	0.19
SmO_2 sats at end contraction (%)	10.19	16	0.03	8.87	11	0.18
SmO_2 post test peak (%)	1.13	16	0.78	-2.59	11	0.64
SmO_2 post test peak time (s)	-4.75	16	0.56	-2	11	0.85
SmO_2 fall (%)	-2.2	16	0.25	-2.67	3	0.94

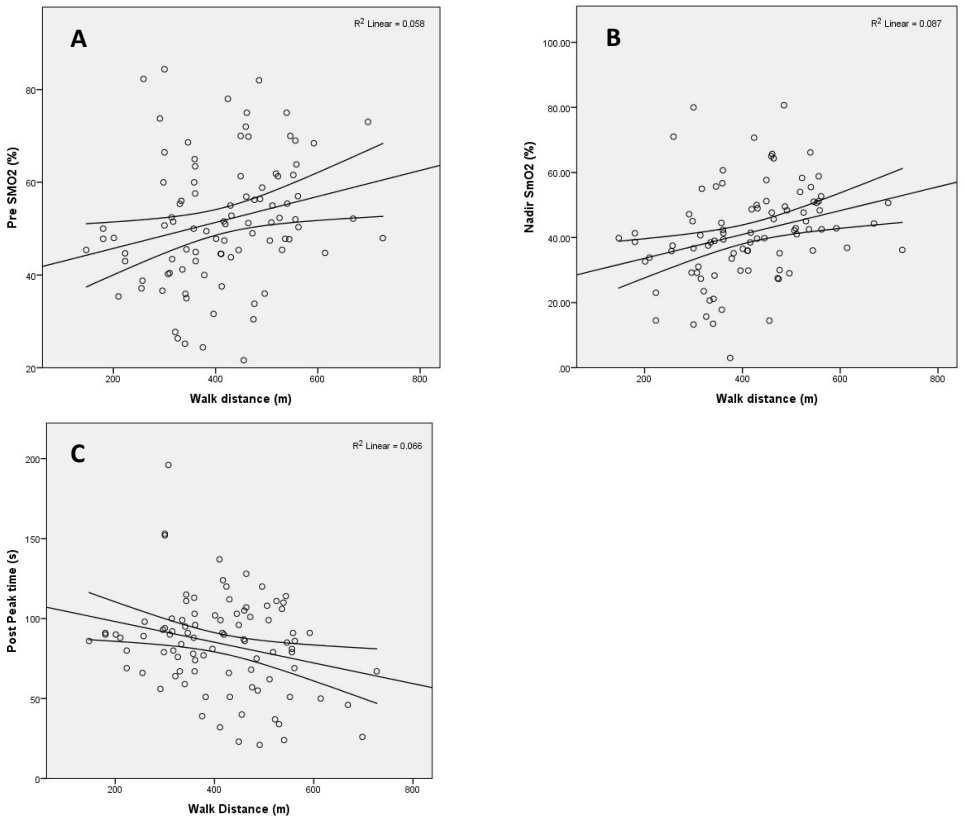
Participants who had a change in the haemoglobin and repeat testing then had the tests compared (higher – lower haemoglobin tests) with paired T tests. The only significant difference with improved

haemoglobin was with a higher SmO_2 at the end of contraction. There were no significant changes when the analysis was restricted to the population that had an improved walking distance of more than 14m. These results are shown in table 3.14.

Skin fold thickness

As the depth of overlying subcutaneous tissue influences the amount of haemoglobin and myoglobin in the NIR light path, the results were examined to determine whether skin fold thickness impacted on NIRS measurements. Skin fold thickness over the gastrocnemius showed correlation with the baseline THb (r=0.54, p<0.001) but not SMO2 (r=0.074, p=0.48). Tests in which read errors occurred

Figure 3.14: Correlations between haemoglobin, SmO2 during isometric contraction and distance walked in the 6MWT.



(where there were large shifts in THb, usually as a fraction of the previous THb reading) were also more common with increasing skin fold thickness (p=0.007, Kruskal-Wallis test). Skin fold thickness in

the forearm showed a similar finding, correlating with THb ($r=0.59$, $p<0.001$), but not SmO2. These results suggest that SmO2 remains a valid reading with higher skin fold depth, although the amount of haemoglobin (or myoglobin) within the light path is reduced with increased subcutaneous tissue.

Discussion

Anaemia tolerance is variable and there are limited data on how this may be objectively assessed. This study further explored tissue oxygen saturation as a measure of anaemia tolerance. There have been many studies correlating tissue oxygen saturation with haemoglobin concentration, as outlined in the systematic review in the first part of this chapter. While the weight of evidence points to an increasing level of tissue oxygen saturation with increasing haemoglobin, the test has struggled to obtain a place in routine clinical practice. Levels, whichever site is used, are usually done at rest. Some groups have examined the changes in StO_2 with venous occlusion tests,[157,158,178,182] attempting to measure dynamic changes in flow that may be different with varying levels of haemoglobin. This study is the first to examine the dynamic effect of exercise on SmO_2 with differing haemoglobin concentrations. Simultaneously, it evaluated the impact of SmO_2 on 6MWT distance, a submaximal exercise measure that correlates with functional capacity. While there were clear correlations with SmO_2 and haemoglobin at rest, SmO_2 changes during the 6MWT correlated with the amount of work done (as measured by walk distance). Baseline SmO_2 levels correlated with the 6MWT distance, but provided no advantage over conventional haemoglobin concentration readings for the ability to predict functional capacity.

It was hypothesised that with lower oxygen a delivery capacity in anaemia, the fall in oxygen delivery during exercise would be greater in anaemia than with a normal haemoglobin and that this would correlate with a shorter distance walked. This could then be used as an objective measure of the effect of anaemia within tissues. There was no association between the fall in SmO_2 and distance walked and paradoxically there was a positive correlation between the fall in SmO_2 during the 6MWT and haemoglobin concentration. This unexpected finding was likely due to greater work being undertaken in those with higher haemoglobin concentrations. The determining factor of tissue oxygenation during submaximal exercise then was not oxygen delivery due to changes in haemoglobin concentration, but the rate of work done during exercise.

The study also examined changes in tissue oxygenation during isometric exercise. This was expected to be different. A contracting muscle belly has increased oxygen utilisation and also impaired oxygen delivery. Small vessels with thin or non-elastic walls within contracting muscle will be compressed. This was clearly demonstrated in the forearm SmO_2 curves with a steady decrease in SmO_2 during the short sustained isometric contraction (after a very brief peak immediately upon contraction). Oxygen delivery was expected to be impaired due to poor flow of blood rather than the haemoglobin concentration, and this was the case, with no correlation between the fall in SmO_2 and haemoglobin concentration.

This test may be considered similar to a vascular occlusion test, but rather than impairing arterial flow to reduce tissue oxygen concentration, it uses both impaired microvascular flow and increased oxygen consumption to reduce tissue oxygenation. By contrast, some studies have shown a greater rate of fall in the SmO_2 during the vascular occlusion test.[157,178,182] If occlusion is complete, the rate of fall should be equal to the rate of consumption and therefore be independent of haemoglobin, except for a small allowance for the oxygen already on haemoglobin within the tissue (box 1) . It is possible that the higher ratio of myoglobin (oxygen using tissue) to haemoglobin (oxygen carrying tissue) may lead to a greater rate of fall following occlusion, but not with isometric contraction when haemoglobin is forced out of muscle.

The rate of recovery is more consistent than the rate of fall following vascular occlusion.[182] In both the vascular occlusion test and the current isometric concentration study there is a degree of rebound hyperaemia, with an increase in SmO_2 above the baseline readings. The time to reach the peak SmO_2 after completion of contraction was shorter with higher haemoglobin concentrations in the present study, which aligns with higher recovery rates following vascular occlusion tests.

Box 1. Is there a difference between the effect of anaemia on StO2 downslope in isometric contraction and vascular occlusion?

Theoretically with vascular occlusion blood remains trapped within the tissue vascular bed, whereas in isometric contraction blood is forced out from small vessels.

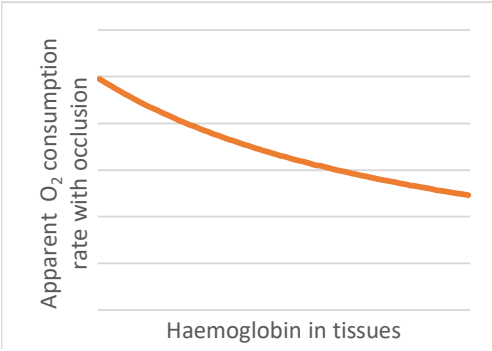
Haemoglobin bound oxygen is therefore available for muscles during vascular occlusion but not isometric contraction.

The combined rate of O_2 consumption (C_C) within the light path is approximated by:

$$Cc = \frac{CmxM + ChxH + CzxZ}{M + H + Z}$$

where M, H and Z are the quantities of myoglobin, haemoglobin and non-globin tissues, respectively and Cm, Ch and Cz represent their O_2 consumption rates.

Assuming, M and Z are constant, as are their O2 consumptions, there is a non-linear inverse correlation with the amount of haemoglobin in tissues, so apparent oxygen consumption rate increases with anaemia when blood remains in the tissues, with the effect reduced when haemoglobin is removed, as in isometric contraction.



The isometric contraction used in this study could be the basis for an alternative to the vascular occlusion test. Isometric concentration has the ability to be shorter, due to the combined effect of impaired microvascular flow and increased oxygen demand. While comfort has not been measured for either test, the isometric contraction was generally well tolerated and within the capabilities of even frail patients without any distress. Having a tight tourniquet, above systolic blood pressure for three to six minutes is likely to be more distressing. However, the isometric concentration is not suitable where the patient is unable to cooperate, as often seen in the critical care setting. It is also not standardised, as the amount of work may vary with each contraction. Further improvements to the test could be made by standardising the force of contraction using a manometer or using a firmer object which is compressed to a certain depth by a known force.

There are potential advantages to measuring muscle oxygenation dynamically during an exercise or occlusive challenge. The technique can measure the effect of arterial or venous insufficiency[248-251] and has been used in chronic airways disease and heart failure.[252] Further optimisation may therefore be of value, but the optimal approach is not yet known.

This study confirmed the association between SmO_2 at rest and haemoglobin concentration noted in the preceding systematic review. It also confirmed the recently reported association between 6MWT distance and haemoglobin concentration.[164] However, neither of these appears adequate to assess the need for transfusion due to the relatively large variation in values between individuals compared with the changes seen due to haemoglobin. Other factors play a role in determining the level of both. While there were also correlations between SmO_2 and haemoglobin, or 6MWT distance at other points during both the isometric and submaximal exercises, in multivariate analysis these were not significant and it appears that they may be considered as the sum of the baseline SmO_2 and the changes during exercise, which are dependent on effort rather than haemoglobin.

Patient reported symptoms

Fatigue and dyspnoea are subjective symptoms measured in this study using a modified Borg scale. Across the population, both symptoms increased in anaemia and were reduced following transfusion. Walk distance was negatively associated with the change in fatigue and dyspnoea scores, indicating that people who experienced a greater increase in these symptoms walked shorter distances. As with SmO_2, the degree of variability, particularly in a population with multiple concurrent causes for these non-specific symptoms, means that these symptoms alone are unsuitable for use solely as transfusion triggers. Nevertheless, the study confirms the validity of simple scales in the assessment of anaemia.

Can multiple variables combine to make a transfusion trigger?

Given several factors with correlations to haemoglobin and exercise distance it would be useful to be able to add these factors together to determine a score that could be used to predict benefit from transfusion. In addition to the factors mentioned above, the pulse rate also correlated negatively with haemoglobin, and transfusion reduced it. Adding pulse along with the other significant factors into multivariate analysis showed a high degree of covariance with haemoglobin being the only significant independent determinant of walk distance. Adding multiple factors will therefore not improve the predictive value.

Limitations

The 6MWT used as the functional endpoint in this study has multiple factors that can influence the outcome, including the degree of effort from participants. While there are correlations with haemoglobin and symptoms that confirm its validity as a functional endpoint in this study, the measured factors account for a relatively small proportion of the variability of 6MWT distance. Even when inter-individual factors are reduced by comparing patients with themselves at different haemoglobin levels, there is a high degree of variability. Despite a known increase in total red cell volume, some patients did not increase their walk distance. Comorbidities, such as arthritis may reduce the specificity of the 6MWT to the effects of anaemia. Some participants had quite high 6MWT values, perhaps related to fitness training or personality traits, and this reduces the sensitivity of the 6MWT for anaemia. This latter group however may have little functional impairment and therefore the test could arguably be more appropriate as test for the functional effects of anaemia than the haemoglobin.

Skinfold thickness is a known potential confounder for NIRS measurements.[253] In this study the skinfold thickness, reflecting the adipose tissue layer, was measured and correlated negatively with the THb. The THb was not associated with haemoglobin concentration or functional outcomes, and likely represents the volume of haemoglobin and myoglobin within the light path. As no impact of skinfold thickness on SmO2 was found, the impact of this is thought to be negligible, but cannot be completely excluded.

Physiological implications

The results suggest that there is no floor to muscle oxygenation during the 6MWT limiting exercise. It is likely that exercise capacity in anaemia is limited by factors other than muscle oxygenation. Based on these results, tissue oxygenation does not appear to be the limiting factor in the exercising muscles. The 6MWT is submaximal, so participants are not being pushed to the point of peak exertion. The amount of work done is dependent on their *perception* of effort and this study suggests that this is

not based on hypoxia in the exercising muscles. There was a correlation though with haemoglobin, and so there is clearly an effect. Submaximal, not just maximal exercise capacity is limited by anaemia.

Alternative explanations for this could be changes in cerebral oxygenation during exercise. This has been associated with altered perception of effort.[254,255] This may vary between standard exercise tests to exhaustion and self-paced exercise, as there is evidence that cerebral oxygenation may not be limiting in the self-paced setting[256] or be the cause for reduced tolerance of exercise in chronic airways disease.[257] A similar possibility arises from reduced supply to non-primary target muscles,[258] such as respiratory muscles, which may have impaired flow[259] and be associated with exercise tolerance via sympathetic feedback.[260] If tissue hypoxia is not identified in the exercising muscles then metabolism would be expected to remain aerobic. Without a transition to anaerobic metabolism, the change in carbon dioxide production would be proportional to the amount of work being performed, so changes in carbon dioxide, or acidaemia are unlikely to account for the decrease in submaximal exercise. Participants with anaemia had higher pulse rates at rest and a higher pulse rate at rest was associated with a shorter walk distance. By contrast, the pulse rate at the end of the 6MWT was not significantly correlated with haemoglobin concentrations or the distance walked. It is known that cardiac output increases with anaemia. This suggests that increased cardiovascular effort may be the factor that determines the perception of submaximal exercise effort, and with anaemia already requiring a degree of cardiovascular adaptation, the amount of exercise required to increase the perception of exercise effort is reduced.

Conclusions

These exploratory studies demonstrated correlations with clinically relevant outcomes and SmO_2. However, on the basis of the data presented, SmO_2 is unable to predict transfusion needs. Improvement in its predictive value could be possible with more standardised approaches. In the 6MWT, the SmO2 fall depends on the effort. While the intention was to use the 6MWT as a functional endpoint, a more standardised effort, such as a fixed walking pace could, or fixed muscle contraction

pressure in the isometric exercise, could reduce variability. It is my view that this is unlikely to reduce confounders to the point where 6MWT becomes a useful tool for predicting transfusion, although it remains a useful endpoint in clinical studies. While the same may apply to NIRS based measurements, there remains scope to explore optimal tissue and site and significant further work to determine whether more standardised dynamic testing could increase the utility of tissue oxygen measurement as a tool for assessing transfusion requirements.

Chapter 4. Managing thrombocytopenia: exploring new approaches to measurement and treatment

Introduction

Patient blood management has largely focused on mechanisms to improve red cell numbers and prevent unnecessary transfusions. One of the key principles of PBM is to limit the amount of blood lost. Often referred to as the second pillar of PBM, maintaining haemostasis can be summarised by the three features outlined by Virchow: blood flow, blood vessel integrity and blood constituents. While these are generally cited in the context of pathological thrombosis, they are relevant in preventing excessive blood loss.

Blood flow

Blood flow in PBM terms requires efforts to reduce the haemodynamic factors that enhance blood loss. Earlier trends to give large volumes of crystalloid as early aggressive resuscitation have given way to permissive hypotension.[261] Trauma specialists have been encouraged to reduce initial resuscitation fluids with the aim of reducing blood flow to reduce bleeding without compromising normal tissue.[49,262] Randomised trials have been performed and although logistical factors may have confounded the results, the evidence weighs towards less aggressive fluid resuscitation.[263,264]

The same principle may contribute to the risks associated with higher red cell transfusion targets in bleeding patients. In the setting of upper gastrointestinal haemorrhage, a lower haemoglobin target to initiate transfusion led to improved outcomes with reduced bleeding, fewer cases needing to return to the endoscopy suite and improved survival.[265] This survival advantage has been questioned and may relate to differing prevalence of portal hypertension between studies. It remains clear that there is no advantage from a liberal strategy and harm is probable when liberal transfusion increases or maintains portal hypertension in the context of liver disease and gastrointestinal haemorrhage.[266]

The concept of blood flow is also relevant to our understanding of primary haemostasis. While blood is always flowing in vivo, most of our tests of haemostasis are performed in static plasma, or

occasionally whole blood. Yet primary haemostasis is critically dependent on blood rheology, which is well established for the effect of platelets and von Willebrand factor.[267]

Vessel wall and integrity

Loss of blood vessel integrity requires early surgical attention to control bleeding. This is widely accepted in the trauma setting.[49] Ultimately, intervention to localise and control bleeding is in most circumstances the best way to prevent excessive blood loss, rather than delaying to fix coagulation disorders. But the blood vessel wall integrity also plays a role in haemostasis by integrating the cellular and enzymatic components of the blood. The endothelium normally inhibits coagulation while exposure of subendothelial collagen and tissue factor promotes thrombus formation. As with blood flow, these factors are not measured by traditional coagulation assays.

Blood constituents

The blood constituents are the third essential consideration in control and prevention of bleeding required in a PBM approach. Factors affecting the ability of blood to clot include cellular factors, especially platelets, and molecular factors within the plasma. The interaction between the two and the physical factors that affect that, particularly temperature in addition to fluid shear, are essential to the maintenance of haemostasis.

Cellular factors in haemostasis traditionally refers primarily to platelets. Rheologically, red cells may also have a role to play as they contribute to whole blood viscosity, opening small vessels and potentially also the margination of the platelets.[268,269] Platelets circulate mostly inactive, and at sites of exposed subendothelium roll and bind to exposed tissues via von Willebrand factor and collagen, predominantly through GPIb-IX-V and GPVI, respectively. Ligand engagement of these receptors triggers outside in signalling, platelet activation and a change in the GPIIb-IIIa complex to an active conformation to bind fibrinogen, leading to platelet aggregation. Platelet activation simultaneously results in changes to the platelet membrane structure, externalising the inner phosphatidylserine membrane and expanding the membrane through recruitment of the canalicular network. The result is an aggregate of platelets, linked together by fibrinogen to bridge injured vessels while the negatively

charged activated platelet surface serves as a cofactor for the enzymatic proteins of the coagulation system.

The coagulation system contains the major molecular factors leading to clot formation. Coagulation inhibitors and fibrinolytic and antifibrinolytic proteins are also relevant to maintaining haemostasis, with the overall haemostatic effect dependent on the balance between these processes. The coagulation system itself is a series of zymogens. Activated primarily by tissue factor, thrombin is produced by the sequential activation of zymogens in conjunction with calcium and phospholipid on the activated platelet surface. These reactions are also sensitive to temperature and pH, with the impact of acidosis and hypothermia standing alongside coagulopathy in the "lethal triad" that pre-empts death in critical bleeding scenarios.[270] Thrombin serves a key role, cleaving fibrinogen to insoluble fibrin, to make formed clot. Thrombin also activates the intrinsic pathway of coagulation – a positive feedback loop enhancing the coagulation cascade – as well as platelets, promoting degranulation, release of soluble agonists and further platelet recruitment. In addition, thrombin also activates coagulation inhibitors that regulate these positive feedback loops.

Transfusion for haemostasis

Platelets are transfused to patients who are bleeding with thrombocytopenia or prophylactically in the setting of severe thrombocytopenia during chemotherapy (usually platelets $<10x10^9/L$).[271,272] Platelet transfusions are not always given prophylactically for severe thrombocytopenia. For example, in immune mediated thrombocytopenia transfused platelets are rapidly removed by the immune system, eliminating any potential value from prophylactic transfusion. Many clinicians also choose not to transfuse to long term thrombocytopenic patients, as a benefit from prophylactic transfusion has not been demonstrated outside the setting of recent chemotherapy or stem cell transplant. The reduction in bleeding risk is small even when one is demonstrated[272,273] and are therefore likely to be surpassed by the difficulties of attending for frequent platelet transfusions and risk of developing immune responses to platelets, which could lead to refractoriness to future platelet transfusions.

Even in the setting of prophylaxis in patients undergoing chemotherapy, there remains some controversy. A randomized clinical trial comparing platelet transfusion prophylactically at a platelet count of less than $10x10^9$/L failed to meet its pre-specified primary endpoint of non-inferiority, but the majority of bleeds were minor (grade 2) and the overall rate of serious bleeding was low.[271] A prior randomized study of prophylactic versus therapeutic platelet transfusion also showed a low rate of serious bleeding, with none seen in autologous transplantation.[272] The benefit of prophylaxis is small and may be confined to subgroups, such as acute leukaemia and allogeneic transplantation, but may be of no, or limited, benefit in autologous stem cell transplants.[271,272]

Alternatives to platelet transfusion have been suggested in thrombocytopenia. Thrombopoietin and analogues have been used in leukaemia and bone marrow transplant related thrombocytopenia, however did not significantly reduce the time to platelet count recovery.[274] These agents limited efficacy in reducing platelet transfusions. Tranexamic acid has been used in thrombocytopenia due to haematological malignancies and myelodysplastic syndrome,[275] but there is unclear evidence of efficacy with further studies underway.[276,277] The potential for fibrinogen infusion to reduce bleeding in thrombocytopenia is an intriguing option that deserves further evaluation.

There are two prior in vitro studies demonstrating that fibrinogen concentrate may improve haemostasis in whole blood derived from thrombocytopenic patients, with the effect measured by thromboelastography being similar to the effect of platelet transfusion.[278,279] These findings were supported by a correlation between fibrinogen levels and thromboelastography amplitudes even in thrombocytopenic patients across a population.[280] A porcine study in vivo has shown similar results, suggesting fibrinogen may be able to compensate for thrombocytopenia.[281] Another study has found fibrinogen improves clotting ex-vivo in blood from clopidogrel treated patients.[282] Human studies have not been undertaken in vivo in the setting of thrombocytopenia, but these in vitro and animal studies

suggest that additional fibrinogen supplementation may be effective at improving haemostasis in thrombocytopenia patients.

Cryoprecipitate is widely considered equivalent to fibrinogen concentrate as a source of fibrinogen, although the need for cryoprecipitate has been questioned with the availability of factor concentrates.[283,284] Although often considered therapeutically equivalent, cryoprecipitate has additional coagulation factors, including factor VIII (FVIII), von Willebrand factor (VWF), factor XIII (FXIII) and fibronectin. There may be differences in fibrinogen recovery after transfusion, depending on the source, leading to theoretical superiority of concentrate as a fibrinogen source.[285] This was found in the FIESTY study[286] (presented at Blood, 2018), where *a priori* dose equivalence of fibrinogen concentrate was calculated for a pilot randomised study of fibrinogen or cryoprecipitate in trauma. Despite expected equivalent doses of fibrinogen, the fibrinogen increment was higher in the arm given concentrate.

A systematic review of the comparative efficacy of fibrinogen concentrate and cryoprecipitate concluded that there was insufficient evidence to favour one over the other.[287] This included only one randomised controlled trial, a small study in paediatric cardiac surgery, which showed similar rates of blood loss with the two products.[288] Since then, a further large multi-centre randomised controlled trial has indicated non-inferiority of fibrinogen concentrate in the cardiac surgical setting.[289] The full results of the FIESTY study are also awaited, although as a pilot feasibility study this was not powered to show equivalence.[286]

The evidence for the utility of cryopreserved platelets is also limited and these could be another product to improve haemostasis, with their use currently restricted to deployed military settings.[290] Cryopreserved platelet are manufactured by adding dimethyl sulfoxide (DMSO) to prepared donor platelet products. Platelet bags are then centrifuged and the supernatant discarded to leave a small platelet rich product of approximately 30mL. These are then frozen at -80^{o}C and stored. When required frozen platelets are thawed, resuspended in thawed plasma or saline and transfused.[291,292] This product is not identical to platelets stored at room temperature.

Freezing and thawing leads to fracturing of the platelets into smaller microparticles and the exposure of negatively charged phospholipids,[292,293] with a proportion being activated in this process[294]. This manifests as platelets with a higher proportion of platelets expressing activated surface proteins, shortened clotting times in viscoelastic tests[295] and increased fibrin deposition in shear dependent flow channel experiments.[291] Cryopreserved platelets show a reduction in the platelet increment in vivo,[296] platelet aggregation in response to agonists[297] and reduced platelet aggregation in flow channels (indicating a reduction in collagen mediated adhesion, activation and aggregation).[293,297,298] Microparticles are also increased and activated.[299] While the recovery of platelets at 24 hours is lower with cryopreserved platelets than room temperature stored platelets, their subsequent survival is adequate.[300,301] While this appears to indicate a prothrombotic tendency following cryopreserved platelet transfusion, there may be rapid clearance of many of the prothrombotic platelets and the surviving platelets may be less responsive to agonists. It is unclear how the overall effect should be measured and for how long the haemostatic effect persists following transfusion.

There are limited clinical data to show a benefit from cryopreserved platelets. Bleeding cessation was achieved in 58% of bleeding thrombocytopenic patients in a dose escalation study.[296] A pilot study in cardiac surgery has shown feasibility. While similar outcomes for blood loss were seen between patients given room temperature or cryopreserved platelets, the study was not powered for equivalence.[302] While thrombosis remains a concern with the use of cryopreserved platelets, no thrombosis has been reported with the use of cryopreserved platelets despite a review of over 3000 transfused units[301] or in the subsequent studies cited above.

Measurement

The lack of an assay that serves as a tool to detect an increased risk of bleeding to help target transfusion for haemostasis is a broad issue. In the two randomised studies comparing prophylactic platelet transfusion with therapy only platelet transfusion the criteria used to initiate prophylactic transfusion was a platelet count of $<10x10^9$/L as measured by impedance blood counters.[271,272]

Although this is standard practice, and the currently best available test to determine bleeding risk, it has marked limitations. Uhl and colleagues found that there was no difference in the bleeding risk associated with platelet counts between the range of 6 to 80 x 10^9/L in analysis of their study on the effect of platelet transfusion dose, and lower platelet counts were not predictive of bleeding in autologous stem cell transplants.[79] Despite the two prophylactic versus transfusion only studies showing a decrease in bleeding overall, serious bleeds were less common in the prophylactic group, but in the majority of severe bleeds occurred despite the platelet counts being above 10x10^9/L (and thus may not have been prevented by a prophylactic transfusion strategy)[271,272]. This may indicate that other factors are more significant determinants of bleeding risk, or that the method used to determine the risk group by conventional platelet counters are inadequate.

Impedance counters

Conventionally, the number of platelets is measured by changes in electrical impedance as blood cells are passed through a laminar flow chamber in blood counters.[303] Particles of a certain size (<20fL) are counted as platelets. There are known limitations to this method, with larger platelets not counted and smaller platelets, or platelet fragments, also not counted. The latter may be particularly relevant as they are formed in a wide variety of conditions associated with platelet activation and are in many cases already activated. They may therefore more actively promote clot formation than platelets measured by conventional cell counters. The variation in measured platelet counts at the threshold of transfusion is also another limitation to the value of platelet count estimation by impedance counters as a transfusion trigger.[304]

Typically, the success of platelet transfusion has been measured by increments in platelet counts, corrected for estimated blood volume, as the corrected count increment (CCI).[305] The CCI is however a surrogate marker, as the intention of the transfusion is to reduce bleeding, either currently or into the future. Undertaking studies to show a statistically robust beneficial effect on bleeding from platelet transfusion involves a large number of patients, and is difficult in unstable target populations, such as trauma.

Although the platelet count is most frequently used as a surrogate outcome, the studies cited above indicate that a low platelet count correlates poorly with bleeding risk. Other surrogate markers have been proposed, and may be more predictive, for example whole blood viscoelastometry,[306] the haematocrit (proportion of the blood that is red cells, by volume), prothrombin time (PT) or activated partial thromboplastin time (APTT).[79] Viscoelastometry measures the ability of whole blood to clot in the presence of tissue factor or other trigger. It may represent a more holistic approach than tests that identify deficiencies in only one aspect of coagulation, such as the platelet count, fibrinogen, PT or APTT (the latter two of which were developed for the purpose of anticoagulant monitoring, but have been adopted, perhaps inappropriately, as measures of the adequacy or otherwise of the coagulation system). Every test has limitations and strengths. Thromboelastometry is sensitive to coagulation factor deficiency, particularly fibrinogen (by far the most plentiful clotting factor in the blood), platelets and some anticoagulants, and is being increasingly used in transfusion protocols in trauma,[307] cardiac surgery[308] and liver disease[309], with success.[310] These protocols typically lead to a greater emphasis on fibrinogen replacement. In severe thrombocytopenia it has been shown to correlate better with bleeding scores than the platelet count.[311-313] There is therefore a sound basis for using thromboelastometry to assess bleeding risk, although not all studies have confirmed this association.[314]

Flow Cytometry

Flow cytometry is a commonly used clinical test looking at the expression of cell surface and some intracellular proteins and may be used to quantify (if used with calibrated counting beads) and evaluate platelet surface markers. It can reliably quantify platelet activation by measuring degranulation and changes in levels of active GPIIbIIIa, most notably in samples from people with extremely low ($<5x10^9$/L) platelet levels. By prior exposure to platelet agonists flow cytometry can also determine the ability of platelets to respond to triggers in their environment and in doing so identify intrinsic platelet functional pathway defects.

Clot waveform analysis

The standard anticoagulation tests PT and APTT have been in clinical use for many years. The clotting times produced are measured by some modern analysers through changes in light transmission as the thrombus forms in in plasma. While typically the result is reported as a single clotting time, with the end point on analysers being the time of maximal acceleration of the change in light transmission, there is information to be gained from evaluation of clot formation kinetics. This has been termed clot waveform analysis.

Clot waveform analysis has been used to detect changes in coagulation beyond the information provided in the single PT or APTT value. Changes are detected in diffuse intravascular coagulation earlier than in other coagulation parameters, with some changes due to the effect of C reactive protein precipitating with lipoproteins in the presence of calcium.[315,316] APTT clot waveforms are different in lupus anticoagulant and coagulation deficiencies, and between patients with coagulation deficiencies.[317-320] Light transmission waveforms are readily available on a number of commercial analysers with optically measured endpoints, but are not usually part of diagnostic algorithms. The exception is the use of PT derived fibrinogen, based on calibrated change in the maximum amplitude of change in light transmission. This has a good correlation with fibrinogen concentrations, although occasional errors are noted, so that it is not recommended as routine method for determining fibrinogen in clinical laboratories.[321]

The effect of microparticles on clot waveforms has not been studied. During plasma preparation, centrifugation aims to remove cells, making the PT and APTT independent of cellular components. However, microparticles are more difficult to remove and require ultracentrifugation (>100000g). Cryopreserved platelets have higher concentrations of microparticles than fresh stored platelets. Partly for this reason, the platelet increments are poor following transfusion, but if the whole blood propensity to clot is being evaluated, clot waveform analysis has potential to measure this effect with standard plasma separation procedures and this has not yet been experimentally addressed. The

impact of additional coagulation factors in this more wholistic assay following transfusion is also unknown.

Platelets under shear

It would be highly advantageous to correlate bleeding risk with coagulation assays, however, although often clinically assumed, this has been difficult to achieve. Each of the assays in clinical use measures usually only one aspect in the process of coagulation. While rotational thromboelastometry has been used to evaluate whole blood coagulation in the setting of trauma and surgery and has been used to predict bleeding in a number of other conditions, including thrombocytopenia, it also has well-known limitations. One factor not measured in these assays is the response to fluid shear stress. While rotational thromboelastometry does introduce motion not present in coagulation assays that detect the endpoint through changes in light transmission, it does not subject them to significant shear.

Shear is important in platelet activation and adhesion. Changes in the structure of GPIb with shear expose the VWF binding site to increase VWF binding.[322] Similarly, VWF undergoes structural changes as it stretches from a globular to an extended form under shear to expose the GPIb binding site within the VWF A1 domain.[323] Yet few assays in clinical use are performed under shear to measure the physiological impact of these changes. The deposition of platelets and clot under high shear stress has been assessed with a cone and plate analyser (Impact-R®) and shown predictive value for bleeding in thrombocytopenia.[324,325] While shear and agonist induced channel occlusion (eg. Platelet function analyser, PFA-100®) is in use, it is sensitive to thrombocytopenia and anaemia and so unsuitable for examination of haemostasis in pancytopenic patients.[326]

Shear induced platelet aggregation is performed in microfluidic channels, however it remains a research tool primarily to investigate the function of platelets.[327] Microfluidic channels have whole blood or platelet rich plasma pass through at known shear stress and measure platelet activation and adhesion to the channel surface, pre-coated usually with collagen.[328] In most cases, coagulation is

inhibited so that the measured effect is primarily platelet function.[329] An assay using higher shear rates that incorporates coagulation could have the capacity to separate patients at risk of bleeding in severe thrombocytopenia. It may also have the capacity to measure the total effect of cryopreserved platelets where platelet derived microparticle numbers are high and are known to adhere to the collagen surface.[330] Microfluidic channels have been shown to detect changes in von Willebrand disorder and haemophilia, however few studies are performed in severe thrombocytopenia.[327,331] With improvements in microfluidics, such tests could be readily extended for use beyond the research arena into the clinical laboratory.[331]

Platelets transfusion in thrombocytopenia

Platelets prepared for transfusion are stored at room temperature. Refrigerated platelets are permanently impaired from activation. In order to prevent bacterial contamination of platelet products at room temperature storage they are therefore only stored for five to seven days, unlike refrigerated red cells (42 days) or frozen plasma products (12 months). This very limited shelf life means that platelets are only available for transfusion for very limited time-frames. Transfusion laboratories need to keep adequate stocks to meet demands, especially when needing to supply blood to haematological malignancy, major trauma and cardiac surgical patients. The need for adequate stocks to meet immediate demands with a short shelf-life product leads to high wastage rates with associated high costs, despite careful utilisation policies. Strategies that decrease the bleeding risk (as opposed to simply increasing the measured platelet numbers) that don't involve room temperature stored products would enhance availability of the products and reduce wastage of blood collected from volunteer donors.

Cryoprecipitate, fibrinogen and cryopreserved platelets have the potential to improve haemostasis and can all be stored for long periods. Cryoprecipitate is used primarily as a source of fibrinogen in Australia. Cryoprecipitate contains, in addition to fibrinogen, VWF, factor VIII, factor XIII and fibronectin and could have advantages over fibrinogen concentrate alone. If efficacy of frozen products for thrombocytopenic patients is demonstrated this would significantly increase the

availability of haemostatic therapies in times of need. Frozen products are stored in regional hospitals where platelets are not routinely kept due to their shorter lifespan. They could also act as a buffer in larger hospitals, and thereby reduce the need for stock holdings of the labile room temperature stored platelet, which may reduce wastage.

In order to establish the effectiveness of cryopreserved platelets or cryoprecipitate in thrombocytopenic patients a clinical trial with bleeding as the endpoint is required. This would require a large number of patients. Although the in vitro studies suggest that this may be warranted, the effect on measures of haemostasis following transfusion should first be established before proceeding to such a trial, and any patient characteristics that may predict efficacy of the different products should be explored.

In this chapter, I will compare the effect of cryoprecipitate, fibrinogen and cryopreserved platelets in vitro on haemostatic assays, using the thromboelastogram as a surrogate for bleeding risk. Based on these results, a clinical trial of the effect of cryoprecipitate in vivo will be presented. Although the study will use the well-established thromboelastogram as the primary endpoint, novel approaches to measure the haemostatic effect of transfusion in thrombocytopenic patients will be explored. These will include the effects on platelet and clot formation in a flow channel under shear, APTT and PT clot waveform analysis, cell surface antigen expression to determine activation and responses to agonists by flow cytometry.

Effects of coagulation factors in vitro on tissue factor pathway activated thromboelastometry

Objectives

- This study will determine the effect of addition of fibrinogen concentrate and cryoprecipitate to thrombocytopenic whole blood on the thromboelastogram.
- The secondary objectives are:
    - To confirm the comparable effects of platelets and fibrinogen replacement on thromboelastogram results as shown in previous studies;[278,279]
    - To examine the ability of additional FVIII/VWF and FXIII concentrates to correct any thromboelastometry differences found between cryoprecipitate and fibrinogen replacement;
    - To determine the impact of cryopreserved platelets on Extem parameters and compare these with the effect of room temperature stored platelets and cryoprecipitate.

Hypotheses

- That cryoprecipitate will improve haemostasis as measured by tissue factor activated (Extem) clotting time (CT) and/or maximum clot firmness (MCF) in blood from patients with thrombocytopenia.
- That cryoprecipitate will reduce CT when compared with platelets or fibrinogen due to the effect of VWF/FVIII.
- Therefore, that addition of VWF/ FVIII concentrate to fibrinogen will improve Extem CT and MCF to be equivalent to cryoprecipitate.
- That FXIII supplementation will not improve coagulation as measured by Extem.

Methods:

The study was approved by the ACT Health (ETHLR18.106) and ANU (2018/484) Human Research Ethics Committees. Patients were recruited from Canberra Hospital Haematology Department. After

informed consent was obtained, 5.4mL blood was drawn from 8 thrombocytopenic patients (platelets < $20x10^9$/L) into 2 citrate tubes (2.7mL Vacutainer, Becton Dickinson Franklin Lakes NJ USA).

Platelets for transfusion prepared by the Australian Red Cross Blood Service (now Lifeblood) were collected from ACT Pathology and Capital Pathology in Canberra at day 6-7 of storage. These conditions were chosen so as not impact on clinical blood supplies as platelet bags are discarded after day 5 within Australia however platelets up to 7 days old are licensed in other jurisdictions. (At the time of writing, day 7 platelet bag expiry has been given regulatory approval in Australia, but is not yet implemented.) The discarding of bags at day 5 rather than day 7 has been based on microbiological rather than functional considerations, so these platelets were considered representative of the functional capacity of transfused platelets. All platelet units were inspected for spoilage prior to use. All platelets had blood cultures taken within the Blood Service prior to release according to standard operating procedures and none were flagged positive. Apheresis or pooled platelets were available, with the volume of platelets adjusted for this in vitro study to model the transfusion or a single unit as required.

Each sample had Extem (with coagulation activated by tissue factor to assess the extrinsic pathway) performed on the ROTEM Delta (Haemoview Diagnostics, Brisbane, Australia) with dilutions based on the transfusion of 100mg/kg fibrinogen (or equivalent fibrinogen dose from cryoprecipitate) to a 70kg person with approximate 5000mL blood volume. The samples were prepared in the following dilutions:

1. Whole Blood Neat 330µL
2. Whole Blood 308µL + Platelets 22µL (equivalent to 1 transfused unit; 218/12ul for apheresis platelets)
3. Whole Blood 308µL + Cryopreserved platelets 22µL (equivalent to 1 transfused unit; 218/12ul for apheresis platelets)
4. Whole blood 284 µL +Cryoprecipitate 46 µL (equivalent to 20u (700mL) transfusion

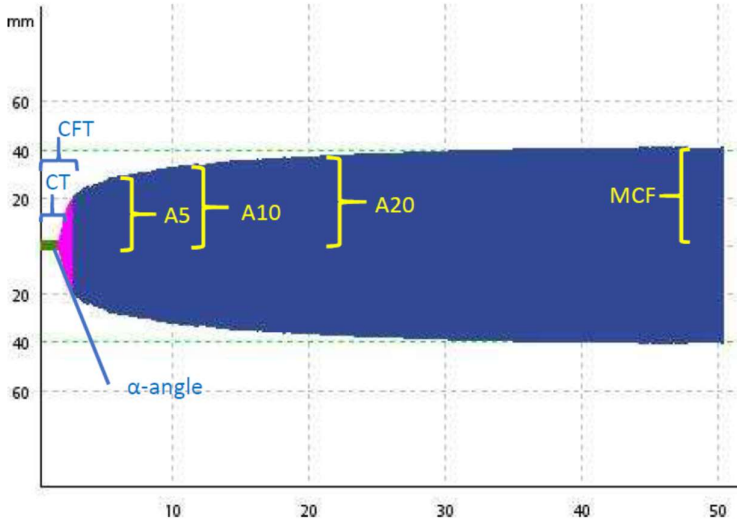
5. Whole blood 307µL + 23µL (0.42mg) fibrinogen (equivalent to 100mg/kg)
6. Whole blood 328 µL + 2.1µL FXIII (equivalent to 20 bags cryoprecipitate giving, and increase of 0.41u/mL(WB) FXIII)
7. Whole blood 326 µL + 3.9µL FVIII concentrate (equivalent to 20U cryoprecipitate, giving an increase of 0.59u/mL(WB))

Each test was run for at least 30 minutes. Additional concentrations were used as available during later studies to explore dosage effects. Mixing was performed in the test cuvette as each test was performed, with the last step in each process being a mixing step after the addition of starting reagent (including $CaCl_2$). The thromboelastometry parameters are shown in figure 4.1.

Human derived fibrinogen 20mg/mL concentrate (RiaSTAP, CSL Behring), FVIII (50iu/mL) / VWF (130iu/mL) concentrate (Product code 330W1985, CSL Behring), FXIII 62.5IU/mL concentrate (Fibrogammin, CSL Behring) were reconstituted according to the manufacturer's directions, aliquoted and stored at -20^{o}C until required. These products were all supplied by CSL-Behring, Melbourne Australia.

All patients had blood counts performed using usual laboratory procedures within ACT Pathology (Canberra) on DXH800 analysers. Coagulation studies were not performed as part of the protocol, but data on coagulation studies, if performed, were from ACT Pathology performed on ACL TOP 700 CTS1 (Werfen, Barcelona) with Recombiplastin (PT), APTT-SS (APTT) and QFA Thrombin (fibrinogen) in accordance with usual laboratory procedures.

Figure 4.1: Rotem (Extem curve demonstrating measured parameter) and their interpretation.



CT – initiation until 2mm amplitude (initiation of fibrin formation (Extem 38-79s)
Alpha angle– angle of curve at 2mm amplitude (Extem 63-83)
CFT (clot formation time) – time between 2 and 20mm amplitude (Extem 34-159s)
A5 / A10 / A20 – amplitudes at respective time (minutes) post CT (A20 Extem 50-71s)
MCF – Maximum amplitude (Extem 50-72s)
CLI 30 Reduction in amplitude from MCF to 30 min post CT. (Extem 94-100%)
ML is maximum reduction at end of test.

Cryopreserved platelets

Cryopreserved platelets were prepared using a method modified from Johnson et al.[332] Group O RhD negative pooled platelets on day 6 of storage were selected. DMSO 25% solution 40mL was prepared in a 50mL syringe by dilution with 0.9%NaCl. Excess volume was removed from the platelet bag to ensure a volume between 167-200mL, then the DMSO cryoprotectant solution was added during platelet agitation slowly over 5 minutes. Using a gentle, slow withdrawal through a Luer lock connector, platelet / DMSO solution was withdrawn from the spiked platelet bag and 1.8mL dripped into cryopreservation vials. Cryovials were centrifuged at 4200rpm (2485g, EBA 20, Hettich Tuttlingen Germany with E1624 rack) for 10 minutes. Excess supernatant was removed and discarded, then platelets gently resuspended in the remaining plasma DMSO solution. Platelets were then placed in a -80^{o}C freezer.

To thaw, cryopreserved platelets were placed in a 37^{o}C water bath. They were then reconstituted in by the addition of thawed plasma to make a total volume of 1.8mL.

Thawed plasma

Plasma was prepared by the Australian Red Cross Blood Service from voluntary blood donors into citrate, phosphate dextrose as per their standard manufacturing practice.[333] AB plasma was thawed

for clinical use, but discarded after 5 days in accordance with national guidelines for Extended Life Plasma.[334] Samples were aliquoted a re-frozen for later use in these experiments for the reconstitution of frozen platelets.

Cryoprecipitate`

This was also salvaged from clinically prepared thawed cryoprecipitate collected by the Blood Service, but not used. It was aliquoted and re-frozen. Samples were thawed immediately prior to use.

Study population

Participants all met the following prospective *inclusion criteria*:

1. Over 18 years of age
2. Able to give informed consent
3. Have a platelet count of $<20x10^9/L$

The following *exclusion criteria* were also applied:

1. Any cognitive or language barrier to consent
2. Unwilling to provide consent
3. Known concurrent coagulopathy in addition to thrombocytopenia
4. Anticoagulant therapy
5. Specific antiplatelet therapy

Statistical considerations

The primary endpoint was the change in MCF from baseline following the addition of cryoprecipitate and platelets. With a mean pre-transfusion MCF of 37mm, a standard deviation of 10mm (for the population) and an expected post treatment MCF of 48mm, it was calculated that 8 patients were needed, based on a 2 sided paired t test, to demonstrate with 80% power a difference at $p<0.05$ with transfusion in vivo as was found previously in vitro.[279] Power calculations were performed using GPower 3.1 statistical software.[335] Paired t tests were used with and alpha of 5% to compare results from individuals. Unpaired t tests were used to compare results between groups. Secondary endpoints included changes in CT, alpha angle, earlier amplitudes at 5, 10 and 20 minutes (A5, A10

and A10, respectively) and clot lysis parameters (LI30 and ML). Analyses were performed using Excel spreadsheet (Microsoft) and Prism statistical and graphing software (Graphpad, San Diego, CA USA).

Results

The eight patients had a median age of 63.5 years (range 36 to 68). Four were female. They had a mean platelet count of 13 (range 10-16). Of the six patients who had a fibrinogen level performed, four were elevated and two were normal (range 2 to 7.8g/L). These same six patients also had results for PT, which was normal to mildly prolonged (range 12-18s) and APTT, which were all within established normal limits. All patients were anaemic (haemoglobin range 74 to 107g/L). Five patients had acute myeloid leukaemia, with the remainder having acute lymphoblastic leukaemia, multiple myeloma and diffuse large B cell lymphoma (one of each). All patients had received cytotoxic chemotherapy.

Initial values

Extem values were significantly impaired compared with expected normal ranges for people without thrombocytopenia or impaired coagulation. The mean CT was increased to 80.5 s (NR: 42-74) as was the CFT at 166s (NR: 46-148). The amplitudes were also reduced with the A20 and MCF having mean values of 36.5s (NR: 50-69) and 39.1s (NR: 49-71) respectively.

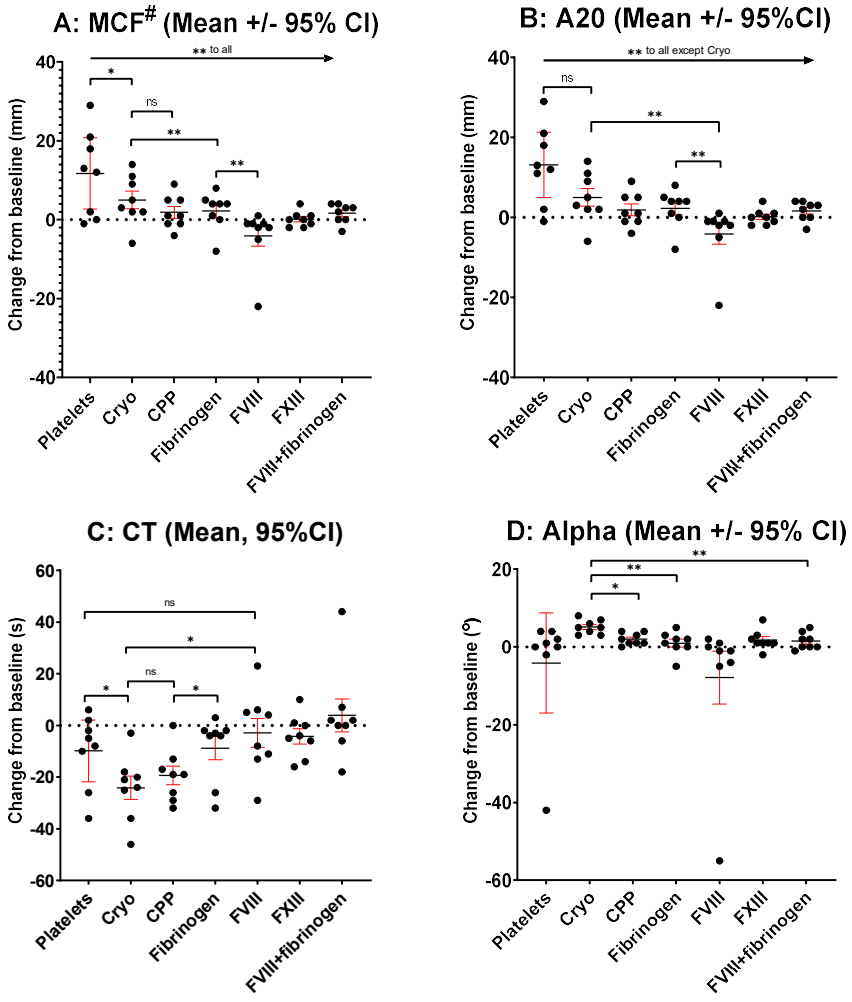


Figure 4.2: Change of thromboelastometry values from baseline with in vitro addition of plasma and coagulation products. Cryo - cryoprecipitate, CPP - Cryopreserved platelets, NS - not significant, * p<0.05, **p<0.01, ***p<0.001, # - MCF includes anticipated or measured MCF values

Platelet concentrates

Platelet concentrates significantly increased the Extem MCF by a mean of 14.75mm (p=0.001, figure 4.2A). However, MCF requires the clot to reach maximum amplitude and a majority of samples did not reach this point. The Rotem calculates an anticipated MCF, which has been used in this analysis when MCF was not available. The A20 is the amplitude at 20 minutes after clot starts to form. These are all actual values and therefore used as the primary endpoint, being the closest measured value to the pre-specified MCF. A20 also showed a significant improvement, of 13.1mm (p<0.01) following the addition of platelets (figure 4.2B). The platelets also increased the amplitude at 5 and 10 minutes,

although this was only statistically significant at 10 minutes. The amplitudes with the addition of platelets were just below the lower limit of normal (A20 49.6s with NR 50-69s). Platelets did not show a statistically significant change in CT (shortened by 9s, p=0.09, figure 4.2C), although the mean CT (70.s) did return to within the normal range, or alpha angle (figure 4.2D). There was also no statistically significant change in CFT (p=0.67), although the mean after transfusion (140.8s) was also within the normal range. Inspecting these CFT data suggested they were non-parametric. A Wilcoxin signed rank sum test for the difference before and after platelet transfusion was also not significant.

Cryoprecipitate

While there was an improvement in the mean A20 (and MCF) following cryoprecipitate transfusion of 5mm, this did not reach statistical significance (p=0.06, figure 4.2A and 4.2B). The A5 was significantly higher by 7.3mm (p=0.02) however, although there was improvement in the amplitudes at later times, these did not reach statistical significance, and did not correct to normal range values. Cryoprecipitate significantly reduced the CT by 24.1s (p=0.001, figure 4.2C) and increased the alpha angle by 5.1^{o} (p=0.001, figure 4.2D), which resulted in a mean level (81.8^{o}) just above the normal reference range (63-81^{o}). The CFT was shortened by 82.6s (p<0.05). When compared with platelet transfusions, cryoprecipitate had a significantly greater improvement in the CT by 14.3s (p<0.05, figure 4.2C). Extem amplitudes were similar at 5 minutes, with increasing difference over time so that the estimated MCF was statistically different by 10mm (p<0.05) with higher amplitudes when platelets were added (figures 4.2 A and 4.2B).

Cryopreserved platelets

Cryopreserved platelets had no impact on the Extem amplitudes (Figures 4.2A and 4.2B). They decreased the CT by 19.4s (p=0.001, figure 4.2C) and increased the alpha angle by 2^{o} (p<0.01, figure 4.2D). The CFT decreased by 37s (p=0.02). The mean values for CT alpha angle and CFT after transfusion were all within the reference ranges. The pattern of these results is similar to cryoprecipitate, rather than room temperature stored platelets, reducing the time of onset of clot formation, although cryopreserved platelets had no effect on Extem amplitudes.

Fibrinogen concentrate

Fibrinogen had an effect only on the A10, reducing it by 3.8mm ($p<0.05$), without a significant change from baseline for other amplitudes (figures 4.2A and 4.2B), although a similar trend was observed in the A5. Although there was a mild reduction in the CT of 8.8s, this did not reach statistical significance ($p<0.1$, figure 4.2C). The mean (71.8s) was however within normal reference values. Cryoprecipitate was significantly more effective at decreasing the CT (by 15.4s, $p=0.01$, figure 4.2C) and increasing the alpha angle (by 4.1^{o}, $p<0.01$, figure 4.2D) than fibrinogen. Although the fibrinogen had lower amplitudes than cryoprecipitate, these did not reach statistical significance.

FVIII and FXIII

Exogenous addition of either FVIII or FXIII had no impact on any Extem parameters (figure 4.2). The combination of FVIII and fibrinogen showed a significant change from baseline with the A5 and CFT, but the results were not significantly different from fibrinogen alone. The combination of fibrinogen and FVIII, designed to replicate the effect of cryoprecipitate, remained significantly inferior to cryoprecipitate for both the CT and alpha angle.

The difference between Extem values where fibrinogen or cryoprecipitate were added consistently significant for most parameters. While fibrinogen is a standardised product, cryoprecipitate is a biological product with the concentration dependent on the donor and manufacturing steps. Based on values from the Blood Service, fibrinogen concentration was expected to be 10.3g/L in cryoprecipitate, however this was measured in the available cryoprecipitate as 34.4g/L. Anticipated FVIII levels were 423iu/dL and measured at 274iu/dL.

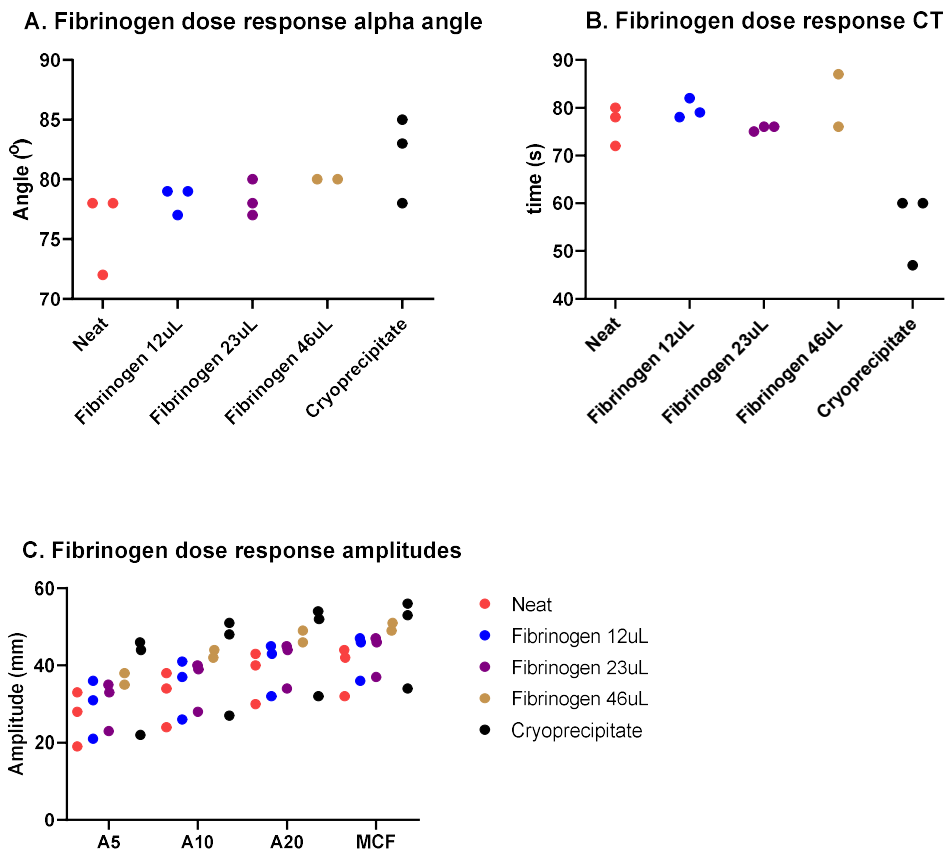
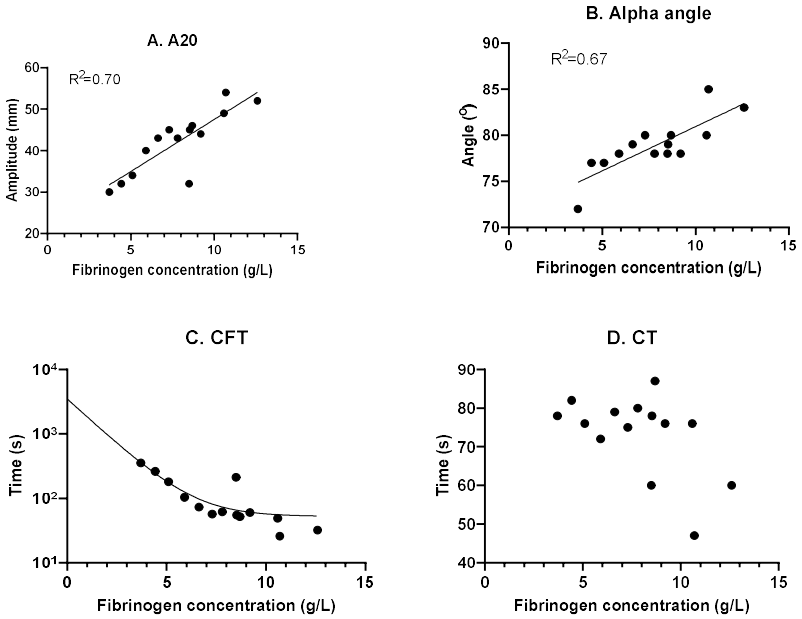


Figure 4.3. Fibrinogen has been added to samples increasing concentrations (patients 6-8) and shows a dose response curve for the alpha angle (A) and amplitudes (C), suggesting the benefit of cryoprecipitate over fibrinogen may be consistent with increased fibrinogen concentration alone. By contrast, there was no dose-response curve with CT (B), suggesting a unique effect of cryoprecipitate on CT was not simply due to increased fibrinogen concentration.

Additional fibrinogen dilutions (12uL and 46uL, equating to 50 and 200mg/kg, respectively) were performed in three samples. Although there are too few samples to be definitive, the results plotted in figure 4.3 demonstrate trends that are consistent with a dose response curve for the addition of fibrinogen for the amplitude and alpha angles. There was no dose response in the CT to fibrinogen, which showed a reduction with cryoprecipitate, but not with fibrinogen addition at any concentration.

Figure 4.4: Correlations between the final calculated fibrinogen concentrations within the samples and Extem parameters, for samples 6-8 where escalating fibrinogen dosing was performed. Correlations between A20 (A) and alpha angle (B) were linear. CFT showed a strong logarithmic correlation (C), but CT was not associated with the final fibrinogen concentration.



To confirm this, using the measured values of the patient samples, the final fibrinogen concentration was calculated after addition of the different fibrinogen doses. Including the fibrinogen and cryoprecipitate tested samples, the amount of fibrinogen was compared with absolute Extem values (Figure 4.4). Pearson correlation showed significant correlations between fibrinogen concentration and amplitudes at all time points ($p<0.001$ for all) with $R^2 = 0.70$ for A20 (figure 4.4A). The alpha angle was also significantly associated with fibrinogen concentration ($R^2=0.67$, $p<0.001$, figure 4.4B). The CT was not associated with fibrinogen concentration ($R^2=0.28$, $p=0.54$, figure 4.4D). The CFT correlated negatively with the fibrinogen concentration, however the association was non-linear, with a strong logarithmic correlation (R2= 0.80, figure 4.4C). These remained significant when only samples with fibrinogen concentrate, not cryoprecipitate, were included in the model.

Sensitivity analysis excluding patients 1 &2

The first two samples were tested on the same day and as the first run samples may have been prone to more process errors. Therefore, a repeat analysis was performed with the samples from this day

excluded. Predominantly, this strengthened the conclusions noted above. However, the addition of platelets was similar to the effect of cryoprecipitate in this sub-analysis, but remained superior to other products with respect to the effect on clot amplitudes. Cryoprecipitate and fibrinogen showed significant improvements in amplitudes at all timepoints (A5-A20) over neat samples, affirming the conclusions of the full analysis. Other conclusions remained unchanged.

Following these results, cryoprecipitate rather than fibrinogen concentrate was used in the subsequent in vivo study. Cryoprecipitate was chosen because it is the standard source of fibrinogen in Australia and in this in vitro study it showed non-inferiority to platelet transfusion on Extem amplitudes and had additional benefits on alpha angle and CT that may add to haemostasis.

Transfusion of cryoprecipitate to improve haemostasis in severe thrombocytopenia

Objectives

- This study determined the effect of cryoprecipitate transfusion and compare with subsequent platelet transfusion, on whole blood thromboelastometry and clot formation in flow chambers, platelet surface marker expression and platelet activation by flow cytometry in thrombocytopenic haematology patients;
- This study evaluated how baseline coagulation characteristics influenced the effect of cryoprecipitate concentrate on whole blood coagulation as measured by thromboelastometry.

Hypotheses

- That platelet transfusion and cryoprecipitate transfusion will both be effective at correcting the maximal clot firmness (MCF) of Extem thromboelastometry readings in haematological malignancy or marrow failure patients with severe thrombocytopenia;
- That the degree of MCF correction is dependent on baseline fibrinogen in patients with severe thrombocytopenia.

Methods

Patient recruitment

The study was approved by the ACTH Health Human Research Ethics Committee (2018.ETH.00188) and Australian National University Human Research Ethics Committee (2019/98) and conducted in accordance with the NHMRC Statement on the Conduct of Human Research and the Declaration of Helsinki. It was prospectively registered with the Australian and New Zealand Clinical Trials Register (ACTRN12619000322134, with the universal trial number U1111-1229-1474).

Patients under the care of the Haematology Department in Canberra Hospital who were due for prophylactic platelet transfusion were approached to consider participation. Potential participants were screened against the inclusion and exclusion criteria and eight eligible patients who agreed to participate provided written informed consent.

The inclusion criteria were that participants must:

- Have a diagnosis of a haematological neoplasm as defined in the WHO Classification of Haematological Neoplasms 2008 or aplastic anaemia;
- Be 18 years or older at the date of consent;
- Be able to provide informed consent, directly or by means of a facilitator or interpreter for those unable to read or understand English, respectively;
- Have a platelet count as measured by a clinical laboratory of $10x10^9$/L or less;
- Have no, or only minor (CTC grade 1 or 2) bleeding;
- Have no religious or other objection to blood product transfusion.

Participants were excluded if they:

- Were unable to provide fully informed consent by reason of intellectual, mental or physical disability, or poor understanding of English, except where it can be corrected, such as by the use of a health care interpreter;
- Had suspected immune thrombocytopenic purpura, thrombotic thrombocytopenic purpura or heparin induced thrombocytopenia;
- Had acute promyelocytic leukaemia;
- Had paroxysmal nocturnal haemoglobinuria;
- Had established diffuse intravascular coagulation;
- Had serious active bleeding, defined as grade 3 or above by CTC Criteria;
- Were planned for a clinical procedure within 72 hours of enrolment that may require prophylactic platelet transfusion;
- Had prophylactic transfusions prescribed at a platelet count higher than $10x10^9$/L;
- Had antiplatelet therapy within the previous 5 days;
- Had anti-thymocyte globulin therapy within the previous 7 days;
- Had immune mediated refractoriness to platelet transfusion (HLA or HPA mediated);

- Had prior venous or arterial thrombosis (proven or suspected transient ischaemic attack, stroke or acute coronary syndrome) within three months;
- Had a prior idiopathic venous thrombosis;
- Had a history of long-term anticoagulant therapy at the time of the study, an indication for long term anticoagulation (eg. atrial fibrillation, mechanical prosthetic valve), or where anticoagulation is usually prescribed, but has been ceased or withheld due to thrombocytopenia.
- Had clinical signs or symptoms suspicious for recent venous thromboembolism, unless investigations excluded this as a cause or an alternative cause has been established.

The exclusion criteria were devised to exclude patient groups where platelet, plasma and cryoprecipitate transfusion regimens differ from standard prophylaxis, who were at risk of thrombosis, were on medications that could interfere with outcome assessments or were likely to be refractory to platelet transfusion.

Interventions

Participants had baseline bloods taken prior to transfusion. They were transfused the equivalent of 20 single donor units of cryoprecipitate (manufactured as pools of 2 or equivalent apheresis product), equivalent to 2 standard adult doses. The dose of cryoprecipitate was based on approximately 100mg/kg fibrinogen[278,279] for a 70kg recipient and an expected fibrinogen dose of 357mg/unit[333] (Blood Component Information Circular of Information, Red Cross Blood Service, 2018). A bleeding assessment was done at the time of enrolment by the investigators and on each of two subsequent days. Bleeding was measured using a tool based on the WHO CTC and validated by Dyer et al[336] prior to the first transfusion and on each subsequent day of blood sampling.

Following cryoprecipitate transfusion, participants were transfused a unit of platelets in accordance with standard of care. Further blood tests were collected after each transfusion. At each time point, blood was collected into EDTA for full blood count (DxH800, Beckman Coulter, Brea CA, USA) and for

all other assays into citrate. The coagulation assays performed were PT (Recombiplastin , performed on ACL TOP 700 CTS1, Werfen Barcelona), APTT (APTT-SS performed on ACL TOP 700 CTS1, Werfen Barcelona), fibrinogen (QFA Thrombin, performed on ACL TOP 700 CTS1, Werfen Barcelona), thromboelastometry (ROTEM) with tissue factor activator (EXTEM), with cytochalasin C to inhibit platelet function as a measure of fibrinogen (FIBTEM) and without activator (NATEM). These assays were performed as per manufacturers' instructions through ACT Pathology, except for ROTEM, which was performed by the investigator. Additional samples for flow cytometry and thrombus formation on collagen under shear stress were taken.

Thromboelastometry

Samples were performed on a ROTEM Delta according to manufacturer's procedures. Natem, Extem and Fibtem were performed. All tests were commenced within four hours of sampling. Natem was performed to evaluate the CT, which is longer in Natem, in anticipation that changes may be more apparent with this assay, which involves only the addition of calcium to citrated whole blood without clot activator. Extem and Fibtem were performed in order to determine the differential effect of the platelet contribution to thrombus formation, which is calculated as: $MCF_{Extem} - MCF_{Fibtem}$. Alternative approaches have been to use $MCE_{Extem} - MCE_{Fibtem}$ or $A10_{Extem} - A10_{Fibtem}$ and these were also tested.[337,338]

Flow Cytometry

Citrated whole blood was diluted 4:1 with Tris-Saline-EDTA, except for tubes with CD62p, where Tris-Saline containing 2.5mM $CaCl_2$ was used. One tube was pre-activated with collagen 2µL of 1mg/mL for 5 minutes at 37^{o}C prior to labelling with antibody-fluorochrome conjugates as per table 4.1. Samples were incubated in the dark at room temperature for 30 minutes then diluted with further Tris saline.

Samples were labelled with the following antibody conjugates: PE-IgG1 isotype (Mouse IgG1 control PE IC002P R&D systems, R&D Systems, Minneapolis, MN, USA); FITC IgG1 isotype (Mouse IgG1 FITC isotype control (B11/6) ab91356 Abcam, Cambridge, UK); Alexa 488 IgG control (Goat anti-mouse IgG

Table 4.1. Flow cytometry antibodies and fluorochromes used.

Tube		TS-EDTA (µL)	Citrated blood (µL)	Collagen (µL)	Incubation 1 (mins)	Antibody (µL)	Incubation 2 (mins; RT, dark)	Dilution TS-EDTA (µL)
1 Unstained		80µL	20µL	-	-	-	-	150
2 PE IgG1 Iso		80µL	20µL	-	-	5 µL	30	150
3 FITC IgG1 Iso		80µL	20µL	-	-	4 µL	30	150
4 Alexa 488 IgG Iso		80µL	20µL	-	-	1 µL	30	150
5 AK2-FITC		80µL	20µL	-	-	4 µL	30	150
6 1G5-Alexa488		80µL	20µL	-	-	1 µL	30	150
7 CD41a-PE		80µL	20µL	-	-	10 µL	30	150
8 CD9-PE		80µL	20µL	-	-	5 µL	30	150
9 P-Selectin-PE / AK2-FITC		80µL (Ts-$CaCl_2$)	20µL			PE 1 µL AK2 4 µ	30	400 TS-$CaCl_2$
10 Activation P-Selectin / AK2-FITC		80µL (Ts-$CaCl_2$)	20µL	2 µL	5 at 37°C	PE 1 µL AK2 4 µL	30	400 TS $CACl_2$

AF488 ab150113 Abcam, Cambridge, UK); FITC-AK2 (CD42b (AK2) FITC mouse IgG1 Invitrogen MA1-82266, Invitrogen, Waltham, MA, USA); Alexa488-1G5 (Anti-GPVI Ms mAb IgG2a Goat anti-mouse IgG AF488 ab150113 Abcam, Cambridge, UK); PE-CD41a (α2b) (Ms mAb to CD41 IgG1 (MEM-06) PE ab134372 Abcam, Cambridge, UK); PE-anti-CD9 (Anti-h CD9 PE mouse IgG2b FAB1880P R&D systems, R&D Systems, Minneapolis, MN, USA); PE-P-selectin (ab119104 Abcam Cambridge ,UK).

Samples were analysed on the FACSCalibur flow cytometer (BD Biosciences San Jose CA, USA). Flow cytometry files were analysed in Flowing Software v. 2.5.1 (Terho, P, University of Turku, Finland, available at: http://flowingsoftware.btk.fi/). Platelets were gated on forward (FSC) and side (SSC) light scatter characteristics and these confirmed by back-gating from CD41. Proportions of gated events were determined and characterised with descriptive statistics. In order to test whether cryoprecipitate may be activating, or otherwise changing platelet surface antigen expression, paired t-tests were performed between the platelets before and after cryoprecipitate transfusion.

Clot waveform

Clot waveform curves for the APTT, PT and fibrinogen were collected for all samples. These were examined for qualitative differences and then described with a view to quantifying any significant changes before and after transfusion of cryoprecipitate or platelets. Quantitative changes were measured in accordance with recommended unified terminology[339] and compared prior to treatment and following transfusion of each product with paired t-tests.

Statistical analysis

The primary endpoint was the change in MCF from baseline following transfusion of each product (cryoprecipitate and platelets). With a mean pre-transfusion MCF of 37mm, a standard deviation of 10mm (for the population) and an expected post treatment MCF of 48mm, 8 patients were estimated to be needed, based on a 2 sided paired t test, to demonstrate a difference with 80% power at $p<0.05$ with transfusion in vivo as was found in vitro[279]. [Note that in order to compare post fibrinogen with post platelets with a difference of 5mm or less, 63 patients in each arm would be required.] Data were plotted for all pre-transfusion patients and an estimate of the distribution made. Paired t tests were

used with an alpha of 5% to compare sequential results within each group. Non-parametric statistics were used (Wilcoxin signed rank test) if the assumption of normality appears to be false on inspection of the plotted data.

Results

Population and baseline results

There were 18 patients screened and approached for participation. From these, three had no venous access device for blood collection, three had been transfused, one was unable to be enrolled for logistical reasons, two failed screening (current sepsis and prior venous thromboembolism) and one patient declined to participate. There were eight patients recruited, all undergoing chemotherapy, six for acute myeloid leukaemia and two for non-Hodgkin lymphoma. The median age was 65.5 years (range 42-71) and five were male. All patients were anaemic, with a median haemoglobin concentration of 67.5g/L (62-98), and leukopenic with a median white cell count of $0.55x10^9$/L (0.1-2.9). While serious bleeding requiring immediate platelet transfusion was an exclusion criteria, four patients had minor bleeding and four were asymptomatic from thrombocytopenia.

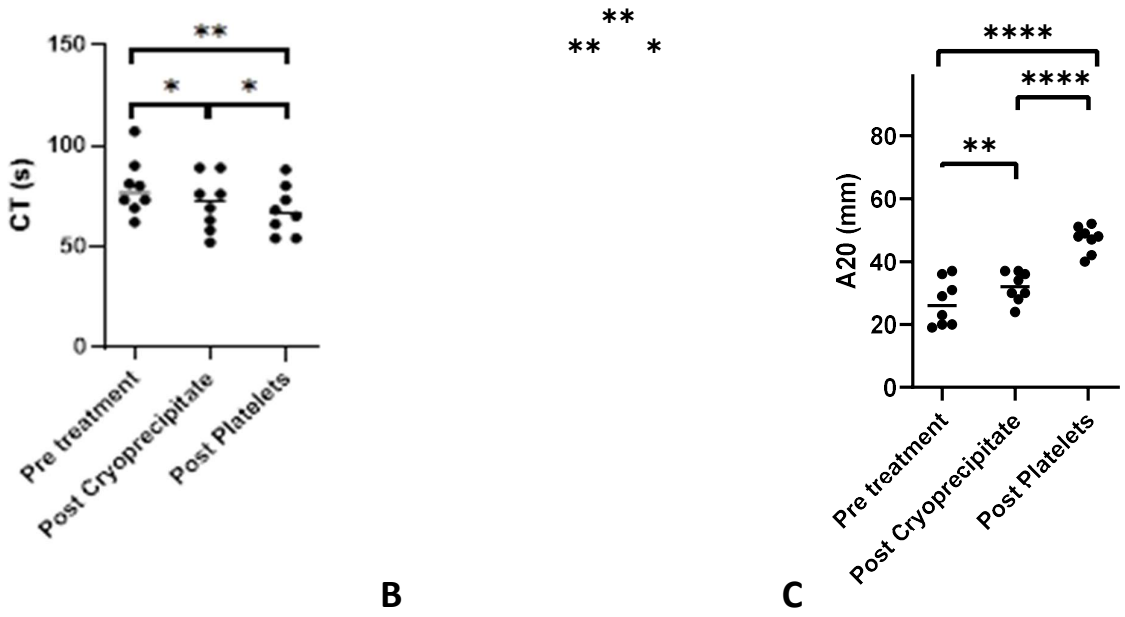
The median platelet count at the time of transfusion was $6.5x10^9$/L (3-9). The APTT was normal in all patients. The PT was prolonged in two patients with an overall median of 14s (12-22). Fibrinogen was reduced in one patient and elevated in four. The median fibrinogen was 4.4g/L (1.3-5.2). The Extem-CT had a median of 76.5s (62-107) and was prolonged in four patients, based on published ranges.[340] Extem alpha angles were predominantly normal with a median of 74.5^o (27-80) and only one patent having a low baseline value. The Extem A20 had a median of 26mm (19-37), with all baseline levels being reduced. Fibtem-CT levels had a median of 73.5s (54-97) and were prolonged in five patients. Consistent with higher fibrinogen levels, five patients had high Fibtem A20 levels, two were normal and the patient with low fibrinogen concentration had low Fibtem amplitudes. The median Fibtem A20 was 23mm (7-33). There was no significant fibrinolysis detected in any of the samples.

Thromboelastometry

Cryoprecipitate transfusion

Following cryoprecipitate transfusion there was a significant increase in fibrinogen (mean increase of 1.59g/L, $p<10^{-4}$) with a mean increase in Fibtem A20 of 7.5mm and all post-cryoprecipitate transfusion values being above the reference range. The Fibtem CT was also shortened by a mean of 8.38s (p=0.01) and the alpha angle increased by a mean of 4^{o} (p=0.003). There was a degree of haemodilution seen with cryoprecipitate transfusion with a mean fall in haemoglobin concentration of 6.4g/L (range 2-11). Consistent with this, the platelet count also fell in five patients and remained unchanged in two. One patient had an increase in platelet count (by $21x10^{9}$/L) in the post cryoprecipitate sample compared with the pre-transfusion sample, which was unexpected over such a short period. Their post platelet transfusion count was $56x10^{9}$/L suggesting that the post cryoprecipitate count was likely to be correct. The Fibtem maximum rate of clot formation increased from a mean of 16.5mm/min to 25.9mm/min (p<0.001).

Figure 4.5: Improvements in thromboelastometry (Extem) parameters following cryoprecipitate and subsequent platelet transfusion (CT: Clotting time, A20: amplitude at 20 minutes)



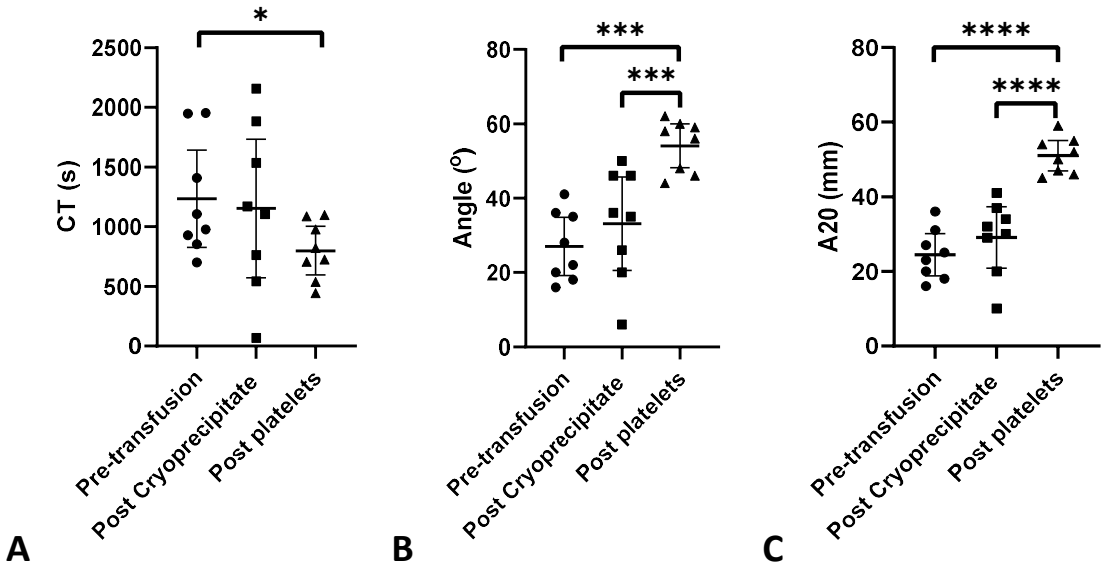
Extem showed a significant improvement in CT with a mean reduction of 7.9s (Figure 4.5A), similar to that seen in the Fibtem. The alpha angle was also increased (p=0.008, figure 4.5B), as were all the amplitudes (mean increase in A20 5.1mm, p<0.01, figure 4.5C). These improvements corrected the mean CT (71.5s) into the normal range, but other values did not completely correct. There was a significant increase in the maximum clot velocity from 16.5 to 25.9mm/min (p<0.001) and the AUC increased from 2825 to 3114 (p<0.001).

Platelet transfusion

Platelets were transfused after cryoprecipitate and as expected showed a significant increment in platelet count from a means of 6.5 pre-transfusion (p<0.001) and 7 post-cryoprecipitate (p<0.001) to $33.4x10^9$/L following platelet transfusion. There was further limited but measurable haemodilution with haemoglobin concentrations falling by a mean of 2.25g/L (p=0.005). While Natem values were unaffected by cryoprecipitate, there were significant improvements in amplitudes following platelet transfusion, with the A20 increasing by 26.5mm ($p<10^{-5}$) and 22.9s (p<0.001) when compared with

pre-treatment and post cryoprecipitate samples, respectively (figure 4.6C). There were also improvements in the alpha angle (mean increase of 21^{o} from post-cryoprecipitate sample, p<0.001, figure 4.6B), the maximum rate of clot formation (increasing by a mean of 4.5mm/min, p<10^{-4}) and NATEM AUC (p<0.01).

Figure 4.6: Improvements in thromboelastometry (Natem) parameters following cryoprecipitate and subsequent platelet transfusion (CT: Clotting time, A20: amplitude at 20 minutes)



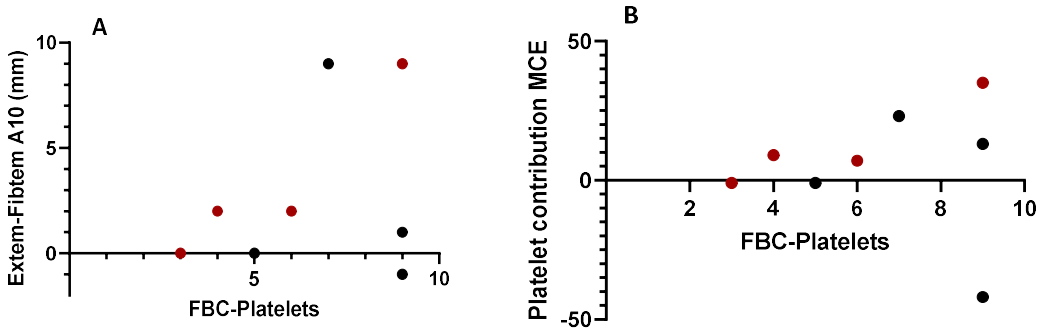
The Extem, when compared with post-cryoprecipitate samples, showed significant improvements in the CT (mean reduction of 3.6s, p<0.05), alpha angle (mean increase of 0.8^{o}, p=0.05, figure 4.5B) and all amplitudes (mean A20 increase of 15mm, p<10^{-4}, figure 4.5C). There was no change in the maximum velocity of clot formation, but there was a further increase in AUC of 1596 (p=1x10^{-5}). With the addition of platelets (immediately subsequent to the cryoprecipitate transfusion), A20 remained significantly below normal reference values (mean 32mm compared with reference range of 49-71mm).

The addition of platelets resulted in no significant changes to Fibtem parameters compared with the post cryoprecipitate samples.

Platelet component of thromboelastogram

The platelet component of the thromboelastogram was calculated by subtracting the Fibtem amplitude from Extem amplitude on tests conducted simultaneously on the same sample. These were compared with bleeding history, where any patient with minor bleeding or greater according to the scoring criteria was considered to be "bleeding." It has been previously shown in our group, including early participants in this study, that a low platelet count relative to the platelet component was associated with bleeding in thrombocytopenic patients, the majority of whom had immune thrombocytopenic purpura.[338] However this did not hold across this cohort, suggesting that the effect may be unique to ITP, possibly due to antibody interference in platelet function. Fibtem-Extem data are shown in figure 4.7, using values at A10, as this was the optimal timepoint previously reported (A) and at the same point using MCE as preferred by some authors as a better reflection of change in elasticity (B).

Figure 4.7: Rotem platelet component in patients with any bleeding (red) or not bleeding (black) plotted against platelet count. Platelet component did not differentiate the two whether measured by the Extem-Fibtem amplitude values directly (A) or by subtracting the MCE (B) of these curves.



Lysis

No samples had abnormal lysis results at 30 or 60 minutes, or an increased maximum lysis. Following platelet transfusion there was an increase in maximum lysis compared with pre-treatment and post cryoprecipitate samples (mean change of 3.1%, p=0.003). The maximum lysis values represent the maximum decrease in clot strength from its peak. These may represent fibrinolysis following platelet

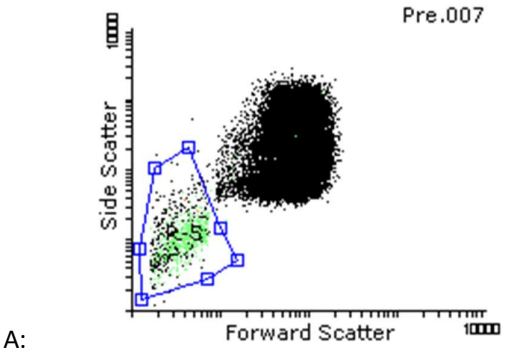
transfusion. However, post-platelet transfusion samples were performed last and had longer run times. As there was no significant change in lysis at earlier time points, the increased maximum could be due to a low level of fibrinolysis detectable over longer total run times.

Flow cytometry

Flow cytometry was not performed on participant 1 and P-selectin levels not assessed for participant 4. All other participants had samples tested before transfusion, after cryoprecipitate and after platelet transfusion.

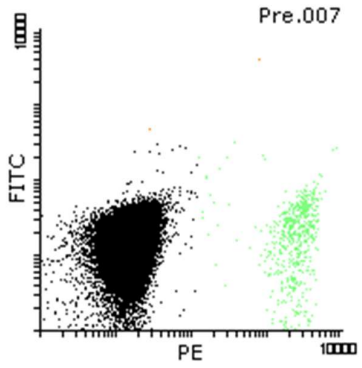
The gating strategy is shown in figure 4.8. At very low platelet counts, the low-forward scatter (FSC), low- side scatter (SSC) gate included a higher proportion of CD41a negative events, likely representing other cellular fragments, which were proportionately less at higher platelet counts (CD41a increased from a mean of 59.7% pre transfusion and 51.7% post-cryoprecipitate to 92.8% following platelet transfusion ($p<0.02$ for both comparisons). Values are expressed as a proportion of the total gate. However, to account for the change in proportion of platelets within this gate, comparisons were made after dividing the measured proportions by the proportion of CD41a positive events.

Figure 4.8: Whole blood flow Cytometry gating strategy, with representative plots (from participant number 2). Low FSC / SSC population gated in A. CD41a (PE) positive population identified in B (coloured green). Back-gating shows CD41a negative events (black), which are proportionately smaller following platelet transfusion (C).



A:

B: (CD41a-PE)



Following the transfusion of cryoprecipitate, the proportion of gated events expressing CD41a, CD42b (detected by AK-2 binding) and GPVI remained constant. There was no significant difference in the proportion of P-selectin expressing platelets unstimulated (25.1% pre-transfusion compared with 28.3% post cryoprecipitate, p=0.55), or following activation by collagen (from 54.2% to 28.9%, p=0.4).

Platelet transfusion resulted in an increase in the proportion of platelets within the gated region, consistent with an increase in platelet count compared with non-platelet fragments. P-selectin positive cells were significantly increased from 25.1% pre transfusion to 55.6% ($p<0.001$) and were also increased from post cryoprecipitate levels ($p<0.01$). There was also an increase in the mean GPVI expression following platelets transfusion (table 4.2).

Table 4.2: Changes in platelet antigens with transfusion

Antigen	Pre-transfusion		Post cryoprecipitate		Post platelet transfusion	
	Proportion positive (mean %)	Geometric mean	Proportion positive (%)	Geometric mean	Proportion positive (%)	Geometric mean
CD41	59.6	2068	51.7	2007	92.8*	2089
CD9	44.2	80.3	33.4	82.6	66.7†	43.5
GPVI	57.5	392	45.5	348	91.4*	429
CD42b	46.7	1233	45.1	933	92.6*	1018
P-selectin	16.4	127	15.0	93	51.0**	82

*Significantly different to both pre transfusion and post cryoprecipitate samples, but not significant when corrected for CD41 positive events. **Significantly different to both pre transfusion and post. † Significantly different from post-cryoprecipitate sample, but not significant when corrected for CD41 positive events.

Clot waveform analysis

Clot waveforms were available for seven of the eight transfused patients, with one sample having results only from after platelet transfusion (patient 3).

The APTT clot waveform was reviewed. Most waveforms had normal characteristics, and these were recorded as per recommended nomenclature (shown in figure 4.9). A number (participant numbers 1,3 (post platelet transfusion available only),4,5,8) showed a notch in the second derivative curve in the post clotting phase (figure 4.10). These did not resolve with cryoprecipitate or platelet transfusion. The cause of this notch is unknown. A previous report has associated irregular notches with other

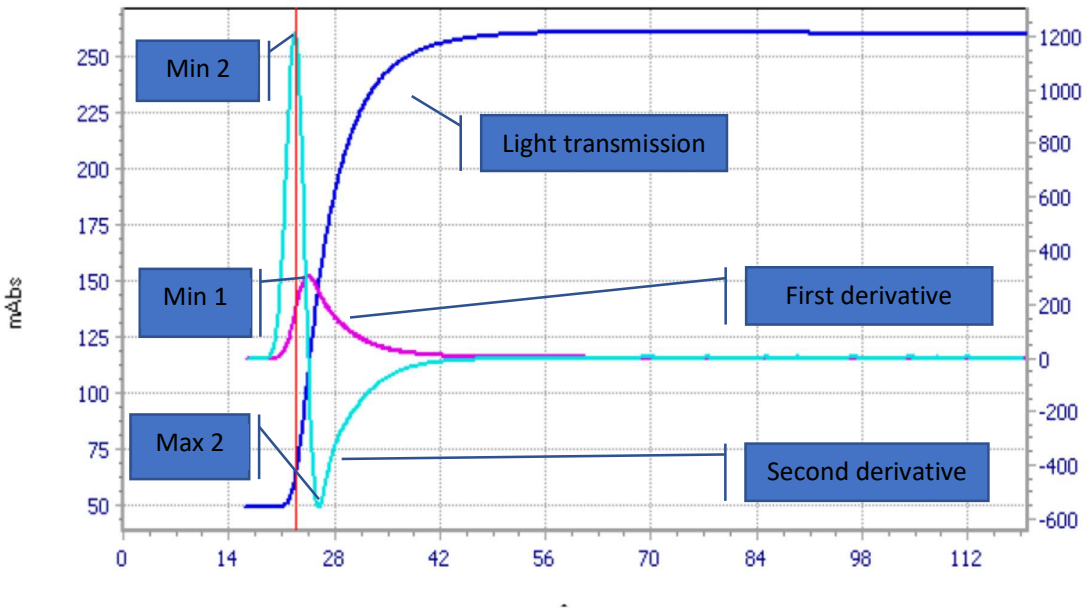


Figure 4.9: Representative typical clot waveform (Patient 7, pre-treatment) showing the measurements analysed. Red line is endpoint. Min 1 is the maximal velocity of rate the change in light transisission. Min 2 is the maximal acceleration in light transmission (the time of which is recorded as the APTT). Max 2 is the maximum rate of decceleration of light transmission. Nomencalture is based on Shima et al.

calcium dependent reactions, particularly C-reactive protein interactions with very low density lipoprotein.

There were significant changes in all the APTT clot waveform parameters following cryoprecipitate transfusion in all patients (Figure 4.11). These are apparent despite normal to high levels of fibrinogen prior to transfusion.

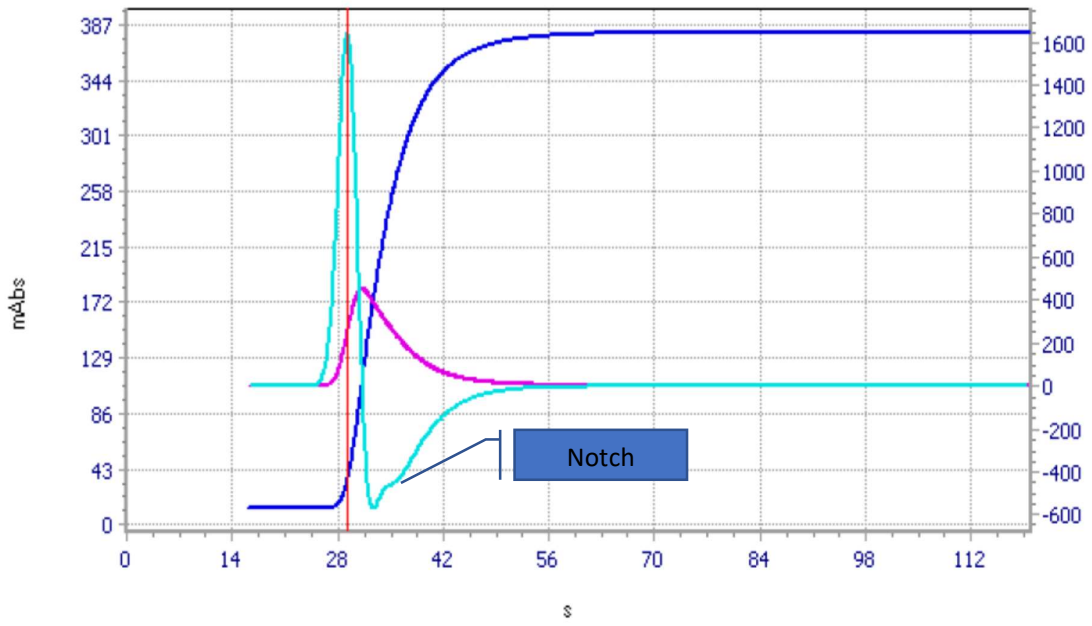


Figure 4.10: Representative clot waveform (Participant 1, pre-treatment) with a second derivative curve notch in the post clotting phase.

Following platelet transfusion there was a significant fall in the maximum clot velocity (p=0.002), without a significant change in other absorption parameters or fibrinogen concentration (0.01 g/L, p=0.88). This suggests that some of the changes may relate to short acting coagulation factors other than fibrinogen, possibly FVIII, which have decreased during the short time between cryoprecipitate and platelet transfusions.

The PT clot formation curves showed an increasing amplitude with increased fibrinogen, as expected, but there were no other significant changes in the PT or fibrinogen waveforms seen. Only the light transmission curve (no derivative curve) was available for fibrinogen, and only the transmission curve and first derivative curves was available for PT.

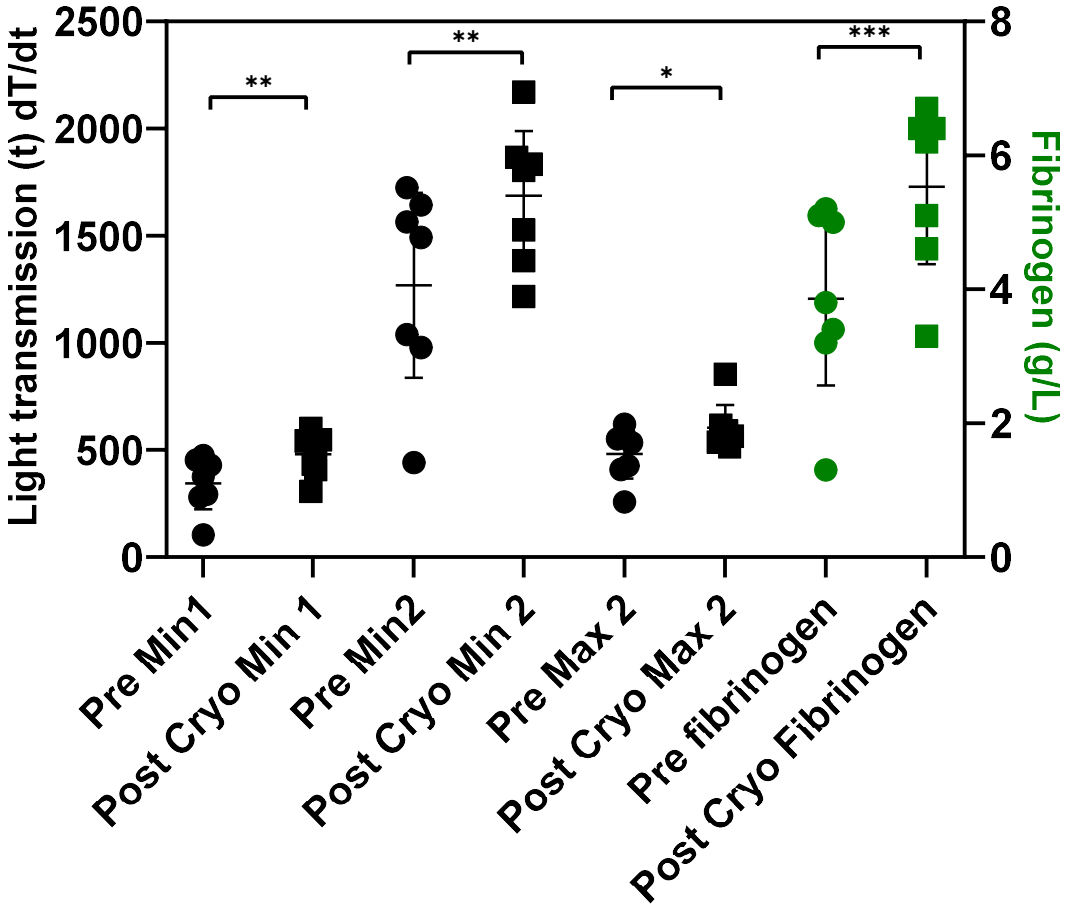


Figure 4.11: The effect of cryoprecipitate (cryo) transfusion on APTT clot curve parameters and fibrinogen concentration. Min1 is maximal rate of clot formation. Min 2 is maximal rate of acceleration of clot formation. Max 2 is maximal rate of clot deceleration. *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

In order to examine the potential for APTT waveforms to detect the effect of cryopreserved platelets, thawed ELP and donor plasma were obtained, and added to separate vials of thawed cryopreserved platelets. A control sample of ELP was also used.

Each sample had an APTT performed and clot waveform parameters are listed in Table 4.3. Samples were then centrifuged as per standard clinical practice to remove platelets. The results do not support an effect of CPP in increasing the rate of clot formation. The neat and centrifuged samples showed a higher rate of clot formation and higher rate of acceleration than samples with CPP. This is counter-intuitive, as CPPs are a rich source of phospholipid, an essential co-factor in the X-ase and

prothrombinase complexes of coagulation, and also increase thrombin generation.[341] Further exploration of a potential effect is necessary before excluding APTT waveforms as a measurement tool for CPP.

Table 4.3: Clot waveform analysis in donor blood (n=1) and extended life plasma (ELP) with cryopreserved platelets (CPP). Min1 is maximal rate of clot formation. Min 2 is maximal rate of acceleration of clot formation. Max 2 is maximal rate of clot deceleration.

Sample	APTT (s)	Min 1	Min 2	Max 2
Donor - CPP	33	223	855	-296
Donor CPP post centrifuge	35	232	904	-324
ELP – CPP	46	114	381	-185
ELP CPP post centrifuge	46	124	439	-220
ELP neat	37	149	543	-276

Adverse events

There were four patients with adverse events following transfusion, all of low imputability to the transfused products. A rash developed in one participant, however the time of onset 24 hours following cryoprecipitate and platelet transfusion suggests an alternative cause may have been implicated. No treatment was required. The three remaining adverse events were all infection-related with continuation of fevers in two and the development of cellulitis on the face in another. There were no deep vein or arterial thrombotic events within six weeks of study treatment, however one participant had a superficial vein thrombosis within a vein at the wrist, related to a recent canula, at day 47 following study transfusions. This was considered to be unrelated. No clinically significant bleeding was seen within 24 hours of transfusions.

Clot formation under shear

This study investigated thrombus formation in whole blood under fluid shear as a detection tool for haemostatic and platelet changes in severe thrombocytopenia.

Aims:

- To explore the ability of shear-based whole blood assays to detect increased bleeding risk.

Hypotheses:

- That whole blood coagulation within a high shear environment (as measured by the surface area of thrombosis on a collagen coated channel surface) will be sensitive to haemostatic changes at low platelet counts.

Methods

Blood was collected from haematology patients with severe thrombocytopenia participating in the two studies previously reported in this chapter, in accordance with the respective study protocols and ethics approvals. As noted above, participants in the first study had a single sample collected, with a platelet count of $<20x10^9$/L, and these were used when sample volume and availability of resources permitted. The second study was interventional. All participants had a baseline platelet count of $<10x10^9$/L and underwent transfusion of cryoprecipitate and platelets, with samples taken before and after transfusion. The intention was to optimise test parameters using the cohort from the first study and to compare samples before and after transfusion designed to enhance haemostasis. In order to test the assay's ability to detect changes, ex vivo addition of platelets and cryopreserved platelets to achieve ratios expected from transfusion were performed. In addition, some donor samples were used from normal volunteers to optimise the testing requirements.

Recalcified whole citrated (3.2%) blood was drawn through collagen-coated, glass-mounted polydimethylsiloxane (PDMS) channels under shear. PDMS channels were prepared in the laboratory of Prof Steve Lee (Engineering, ANU). Following blood collection, PDMS slides were coated by instilling collagen 100µl/mL (Horn; Takeda Austria) and incubating at room temperature for at least 30mins.

Fluidic channels were set up with a withdrawal pump drawing blood through the collagen coated channel. Prior to reaching the PDMS channel, calcium chloride ($CaCl_2$ 0.2M), was added through a sideline. The PDMS channel was mounted on a fluorescent microscope (Axio Observer, Zeiss Microscopy, Jena, Germany) focussing on the channel wall just after the inlet, aiming for minimal turbulence. All experiments were performed in an environmental chamber heated to 37^{o}C. Fluorescent and differential interference contrast (DIC) images were taken at 5s intervals during the recording phase. Images were captured with Zen photomicrographic software (Zeiss Microscopy, Jena, Germany).

Following priming and excluding air from lines, 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, ThermoFisher Scientific, Waltham, MA, USA) 1µL/mL was added to the citrated whole blood. Blood was aspirated at a rate of 58µL/min and $CaCl_2$ at 3.9 µL/min. The rates were determined to give an estimated wall shear of 1000^{-s} (equivalent to shear rates found in arterioles and capillaries) with a haematocrit of 21.7% and protein of 50g/L, and to reverse the effect of citrate with a 1:15 dilution.

Videofluoroscopy was started when blood reached the microscope field and continued for five minutes. When whole blood flow ceased it was replaced by phosphate buffered saline. Still images were taken along the length of the channel. Areas of thrombus formation were identified in photomicrographs and the thrombus surface area determined in the FIJI version of Image J software.[342] The change in thrombus volume was compared before and after transfusion by paired t tests in Prism (Graphpad, San Diego, CA, USA).

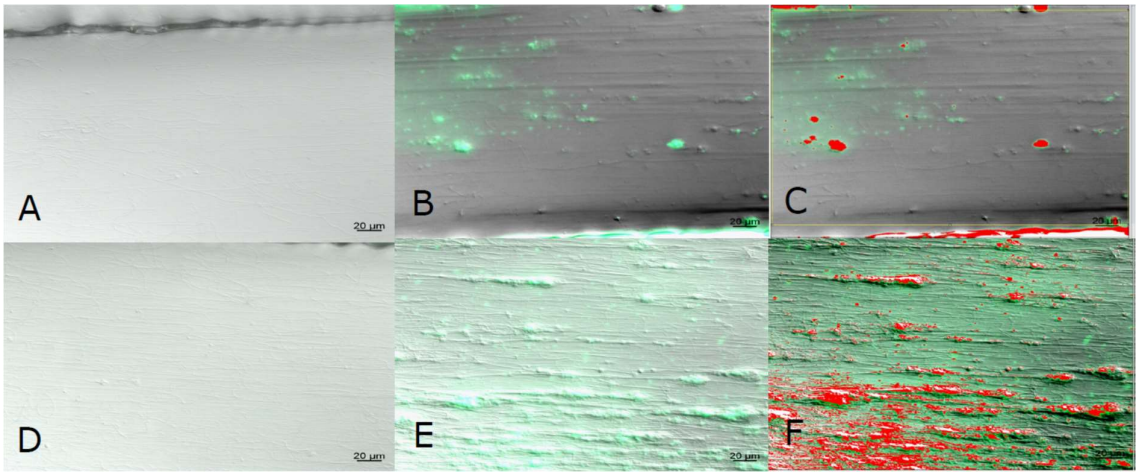
Results:

Studies were performed using blood from thrombocytopenic patients. Donor blood was used to create modified samples in a limited number of tests. Donor blood was centrifuged to remove platelets and reconstitute to approximate thrombocytopenic patients for assay development and thrombocytopenic patients were tested neat. These demonstrated the ability of the test conditions to detect thrombi as exemplified in Figure 4.12, which shows collagen coated channels (A and D) with

platelet rich thrombi at the end of the experiments (B and E). Ex-vivo addition of day 6 room temperature stored platelets showed a qualitative increase in thrombi (D to and F). The changes from baseline were measured in ImageJ, with representative capture technique illustrated in figure 4. C and F.

In another sample, donor blood was separated into platelet poor plasma and red cells, then reconstituted to a haematocrit of 0.21%, haemoglobin of 76g/L, leukocytes of $1.5x10^9$/L and platelet count of $27x10^9$/L. Under the testing conditions, there was a maximum of 0.72% platelet aggregation within any imaged field. Cryopreserved platelets were added (67µL to 1mL reconstituted blood). Under these same conditions there was increased platelet adhesion, with the maximum field having 1.38% surface area coverage. This suggests that cryopreserved platelets showed some adhesion to the collagen surface under shear, however additional studies are needed to investigate further. The change detected was small relative to the observed variability between samples, which may hinder the application to a clinical setting.

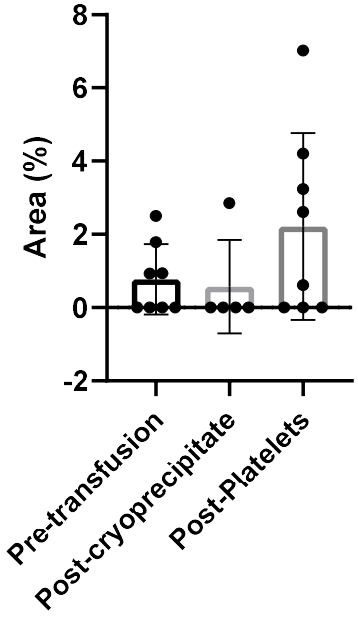
Figure 4.12 Platelet deposition on collagen coated channels under shear. Typical baseline slide areas are shown in panels A and D. Thrombocytopenic patient (acute lymphoblastic leukaemia with platelet count of $15x10^9$/L) had recalcified whole blood pumped to produce shear of $1000s^{-1}$ for 5 minutes through a collagen-coated channel. Adherent platelets are shown fluorescing (B) and the percentage of the fluorescing area calculated in ImageJ (C). The test was repeated following addition of 100µL of room temperatures stored group O, day 6 donor platelets to 1.5mL of whole blood (D-F).



These preliminary results provided the basis to proceed with patient samples pre and post transfusion. Variable light affected the thresholds in ImageJ, as seen in figure 4.12 Where abnormal fluorescence was noted (such as edges in figures 4.12 panels B and C), these were removed, and in analysis thresholds were applied to capture platelet thrombi uniformly.

There were eight patients who received transfusions for thrombocytopenia, with cryoprecipitate and platelet transfusions given sequentially as previously described. There were 37 investigations for platelet adhesion under shear. There were nine tests performed prior to transfusion (days 1 or 2 of the study protocol), when the platelet count was less than $10x10^9$/L. Fluorescence was minimal in 7 cases, but >1% in two cases. In one case there was significant fluorescence after transfusion of cryoprecipitate (2.85%), which had not been detected pre-transfusion, but in all other cases thrombi were not seen following cryoprecipitate transfusion. Of the 10 samples tested post platelet transfusion, 5 had significant thrombi seen, and one other had minimal thrombi present.

Figure 4.13: Platelet coated surface area on collagen under shear (1000^{-S}) before and after cryopreciptate and platelet transfusions



Although there was an apparent increase in platelet thrombi following the transfusion of platelets, this was not consistent, and when the eight samples that had data for both pre and post platelet transfusion samples were analysed, there was no significant difference following transfusion (p=0.3, Mann-Whitney test). Results are listed in table 4.4, with a summary of changes in surface area in figure 4.13.

Table 4.4: Platelet adhesion under shear (1000^{-s}) in patients transfused cryoprecipitate and platelets

Patient and timing (platelet count $x10^9$/L)	Pre	Post cryoprecipitate	Post platelets	Other conditions
Patient 1 day 1 (9)	No fluorescence Clotted during washout	No fluorescence. Bubbles and clot	Very few thrombi	
Patient 1 Day 2 (6)	No fluorescence		Measurable thrombi, maximum field 0.61%	
Patient 1 Day 3 (6)	No fluorescence			
Patient 2 Day 1 (5)	No fluorescence	No fluorescence	Measurable thrombi – maximum 2.61%	
Patient 2 day 2 (4)	No fluorescence	Not collected		With VWF 50% and collagen coating 0.80%
Patient 2 day 3 (28)	Some fluorescence, maximum 0.71%			With 50% VWF and collagen coating. Clotted prior to starting
Patient 2 day 4 (15)	Fluorescence maximum 1.43%			With VWF 100iu/mL after collagen – no fluorescence
Patient 3 day 1 (9)	No fluorescence	Several platelet aggregates, with a maximal area of 2.85%.	Moderate platelet thrombi, with maximum area 3.24%.	
Patient 3 day 2 (5)	Fluorescence maximum 2.5%		Numerous aggregates. Fluorescence maximum 4.2%	Added CPP. Maximum thrombi 1.82%
Patient 3 day 3 (12)	Fluorescence maximum 5.71%			
Patient 3 day 4 (7)	Minimal fluorescence			With VWF coating. Small aggregates, maximum area 0.58%
Patient 4 day 1 (4)	No fluorescence	Not available	Not available	
Patient 5 day 1 (6)	Minimal fluorescence. Maximum area 0.93%	No fluorescence	No fluorescence (sample clotted during test)	

Patient 6 day 1 (7)	Not available	Not available	Maximum fluorescence 2.45%	
Patient 7 day 1 (3)	Not available (channel failure)	Not available		
Patient 7 day 2 (4)	Minimal fluorescence.* Maximum area 0.94%		No flourescence.* Clotted near inlet.	*Sideline of $CaCl_2$ and thromboplastin in both tests
Patient 7 day 3	No fluorescence			CPP added. No fluorescence.
Patient 8 day 1 (9)	Thrombi seen. Maximum area 1.79%	Minimal fluorescence	Significant thrombi. 7.02%	

Discussion

These studies have examined the effect of coagulation products to improve haemostasis in thrombocytopenia. They suggest that transfusion of platelets remains the standard of care, but transfusion of cryoprecipitate may potentially facilitate haemostasis and could be used in a setting where fresh platelets are not available. They have also shown how different approaches to measuring the effect of thrombocytopenia and the impact of haemostatic correction may influence decisions about therapies. While these studies have explored the potential for shear-based assays to view haemostasis more holistically, current approaches are not ideal. At present the tools to test haemostasis are fragmented into components of the haemostatic system and none consider the contribution of the vasculature to haemostasis. Further studies examining bleeding as an endpoint are required.

Cryoprecipitate is the preferred option when room temperature platelets are not available

There is interest in cryopreserved platelets to stop bleeding in trauma and thrombocytopenia. Based on all the assays used in these studies, cryopreserved platelets have a potential role, appearing to enhance the time of onset of clot formation, but had little effect on clot strength. Cryoprecipitate, by contrast, improved both clot initiation and clot strength.

While the in vitro studies showed that platelet transfusion gave the maximal correction of clotting amplitudes, the results from cryoprecipitate were statistically no different when using measured values (excluding extrapolations to derive the MCF), but fibrinogen alone was inferior. This was in contrast to prior findings showing that fibrinogen replacement was equivalent or even superior to platelet transfusion.[278,279] These prior studies used fibrinogen concentrate added ex vivo in patients deemed to need platelet transfusion and the Extem parameters compared with a second blood draw after in vivo platelet transfusion. Loss of platelets post transfusion may account for some of the difference compared with the current study. In one of the prior studies the median platelet count was $86x10^9$/L and fibrinogen 1.94g/L, suggesting that the selected population was more likely to benefit from fibrinogen rather than platelet transfusion. The other study was conducted in a cohort with

severe thrombocytopenia (<20x10^9/L),[279] as in this study. Conducted by the same group, it used similar methodology and showed similar results, with higher ex vivo fibrinogen supplementation being superior to in vivo platelet transfusion. As with the patients in this study, fibrinogen levels were higher, with a median of 4.5g/L. The results of that study and this one, suggest a benefit from fibrinogen concentrate in thrombocytopenia that is dependent on fibrinogen dose, but improvement noted even with higher initial fibrinogen levels.

Cryoprecipitate shortened the CT when added to whole blood, whereas this was not seen with platelets in vitro. This is in contrast to the findings in a 40% blood dilution model where additional platelets reduced the CT.[343] In vivo, there was a small additional shortening of the CT, but no improvement in alpha angle with the addition of platelets.

With cryoprecipitate, clot formation was faster, as evidenced by the increased APTT clot waveform Min 1. The rate of acceleration of clot formation was also faster, as shown by the Min 2 and increased alpha angle in Extem with cryoprecipitate addition in vitro and in vivo. There was no additional improvement following the transfusion of platelets to patients in these parameters, which was predicted from the in vitro model showing no impact of platelet transfusion. Although platelets are involved in clot initiation through binding of von Willebrand factor and exposed collagen, in the Extem model, the measured effect of platelets appears to be primarily during the development of the clot, leading to a firmer clot in the presence of platelets than in their absence. It suggests that thromboelastometry is measuring the procoagulant effect of platelets.

The second study presented here sought to compare the effect of cryoprecipitate for thrombocytopenia in vivo. It confirmed a benefit of cryoprecipitate transfusion on Extem amplitudes. Platelet transfusion appeared to be superior in this severely thrombocytopenic cohort, although these were tested sequentially rather than compared in random order. While platelet transfusions appear more effective, the study design, prone to sequence bias, precludes certainty on this conclusion.

FVIII and FXIII are not effective in thrombocytopenia

FVIII and FXIII did not significantly impact Extem parameters in this model. The lack of FXIII effect is consistent with previous work in modelling trauma associated haemodilution.[344] As it is an acute phase reactant, FVIII levels may have been expected to be high in this cohort, although levels were not specifically measured, and a benefit in other settings is not excluded. However, the results do suggest that neither factor accounts for the observed differences between cryoprecipitate and fibrinogen.

Cryoprecipitate effective even with high fibrinogen levels

These studies suggest that increasing fibrinogen, even in patients with levels above the normal range can replace the platelet procoagulant effect to increase clot firmness. The high doses in the cryoprecipitate sample used in this in vitro study suggested that MCF levels could increase to near normal. These findings extend the findings of Ninnivagi and colleagues, who reported that the addition of platelets and fibrinogen were additive in a whole blood haemodilution model.[343]

The improvement in Rotem values went beyond changes to the amplitudes. Improvements in CT were not seen with fibrinogen concentrate in vitro. While an effect due to FVIII was postulated to account for this difference, addition of FVIII did not lead to improvement. FXIII was likewise not responsible. The possibility that changes to coagulation proteins during cryoprecipitate manufacture may activate platelets was explored, but no changes in platelet activation surface levels was seen. It remains possible that clot activation is promoted by other factors within cryoprecipitate. Galas and colleagues showed similar efficacy of fibrinogen and cryoprecipitate transfusion in paediatric cardiac surgery.[288] Surprisingly, both products led to improved FVII levels, but cryoprecipitate also improved prothrombin and FX levels. Although fibrinolysis parameters were not reported by Galas et al, cryoprecipitate can reduce hyperfibrinolysis and a lack of consumption may account for of the benefits observed.[345] There was no evidence of impaired fibrinolysis in the present studies and no change in LI30 following transfusions.

Cryopreserved platelets

Cryopreserved platelets were evaluated in the ex vivo study and compared with platelet transfusion and cryoprecipitate. It is clear from this study and previous work,[292,297,298,341] that the primary mode of action is likely to be through interaction with the coagulation system, as demonstrated by a shortened CT, rather than increasing the clot strength through enhanced platelet-fibrin networks. This study did not explore the reason for shortening of the CT. Potential reasons include exposure of more anionic phospholipid or release of VWF and FVIII from platelets during the freeze and thaw processes. Recent work by Johnson et al indicated low levels of FVIII in thawed and reconstituted cryopreserved platelets, suggesting increased phospholipid availability for enhancing coagulation as the most likely explanation.[346]

The best way to measure the effect of cryopreserved platelets, and their clinical impact, is unclear. One prior study has reported improved viscoelastic amplitudes post infusion, but only significantly so when three bags were given, much higher than platelet doses used clinically.[296] Most reports, like our findings, show minimal to no impact on amplitudes. Further studies are underway to determine whether there is efficacy in stopping bleeding,[302] but more work is needed on the best way to measure the effect. These studies suggest that at the very low platelet counts and anaemia seen in these patients that shear induced platelet thrombus formation is unlikely to be informative under the current experimental conditions. On the basis of these studies, cryopreserved platelets appeared *inferior* to alternatives, and cryoprecipitate, which may be stored at higher temperatures (-40^{o}C or -20^{o}C for shorter periods v -80^{o}C), is easy to thaw and requires no post-thaw manipulation, could be considered preferable in emergency settings.

Natem useful for clot strength but not clot initiation

The lack of difference in the NATEM results with cryoprecipitate was unexpected. NATEM was used as the clotting times are longer, and it was anticipated that this would exaggerate the differences with changes dependent on clot initiation. However, the NATEM CT has been found to be unstable in the past, with times decreasing during storage.[347] MCF was stable in that study. While the potential for pre-activation during storage may explain the lack of benefit on CT, it is unclear why there was no improvement in amplitudes.

Fibrinolysis

Significant fibrinolysis was not seen in any samples in this study. Subtle fibrinolysis has been shown to be prevalent in chemotherapy patients with thrombocytopenia when using assays with tissue plasminogen activator added, compared with controls.[348] Platelet transfusion may function to reduce fibrinolysis,[349] as may cryoprecipitate.[345] It is possible that mildly increased fibrinolysis has not been detected by thromboelastography used in this study.

APTT waveform

The waveform analysis from the APTT suggests the possibility of measuring the impact of cryoprecipitate effect better with this technique. As it contains FVIII and fibrinogen, both of which are likely to impact of the APTT, the test is well-placed to measure their combined effect on haemostasis. APTT waveforms are known to differentiate bleeding phenotypes in haemophilia[320] and are also able to identify patients at risk of thrombosis[350] so there is potential for it, as a more holistic assay, to determine people with thrombocytopenia who are more likely to bleed. Despite the APTT not increasing, the increased amplitude of the change in light transmission suggests a larger final clot. The amplitude is known to correlate with fibrinogen in the PT waveform analysis.[339]

While the increased rate of thrombus formation may be associated with increasing fibrinogen following cryoprecipitate, this was not maintained after platelet transfusion, when the fibrinogen concentration remained stable. It suggests that short acting coagulation factors from cryoprecipitate

may have a role in determining the maximum rate of clot formation, presumably through increased thrombin formation. This is presumably FVIII and suggests a role in increasing haemostatic capacity in this population. It is noteworthy though that in vitro, FVIII supplementation had no impact on viscoelastic test results.

Limitations

These were small, exploratory studies designed to determine whether supplementary fibrinogen may improve measured surrogate haemostatic parameters, and whether other available frozen products could be equivalent or superior to platelet transfusion. In this regard, the study achieved its aims. However, although it suggests a potential benefit, this is not proven. The ex vivo study used a single cryoprecipitate source, found to have unusually high fibrinogen levels, but not FVIII levels. While efforts have been made to distinguish a dosage effect from a superior action of cryoprecipitate over fibrinogen, the former cannot be completely excluded as a cause for effects, especially the shortened CT, not seen with fibrinogen.

The major limitation to these studies is the surrogate endpoint and whether this is an appropriate surrogate for the risk of bleeding. The study recruited only patients without clinically significant bleeding and platelets were transfused immediately after cryoprecipitate, so an effect of cryoprecipitate transfusion on bleeding outcomes could not be ascertained. There are mixed reports on the value of thromboelastogram in predicting bleeding. Benefit for predicting bleeding has been seen in liver surgery,[309] trauma[351] and in thrombocytopenia due to haematological malignancy treatment,[312,313,352] although others have provided data contesting its value in the latter indication.[314] While these clinical studies pave the way for larger interventional studies with a bleeding endpoint, consideration should also be given to improving the way coagulopathy can be best measured to indicate the risk of bleeding.

Potential for new approaches to haemostatic testing in thrombocytopenia

It is reasonable to assume that the total haemostatic potential of blood is more relevant than individual parts. The concept that improving one aspect of haemostatic chemistry can reduce bleeding

without necessarily replacing the known depleted product is not novel. Bypassing agents to overcome haemophilia with inhibitors are an example.[353] Thromboelastometry allows examination of large components of the haemostatic balance. It does not measure primary platelet adhesion and aggregation, although through the activation of coagulation, thrombin mediated activation is expected to play a role. The concept of a whole blood assay, incorporating shear to detect a more physiological platelet contribution to thrombus initiation and growth to detect bleeding tendency has been considered by others and explored in these studies.[327]

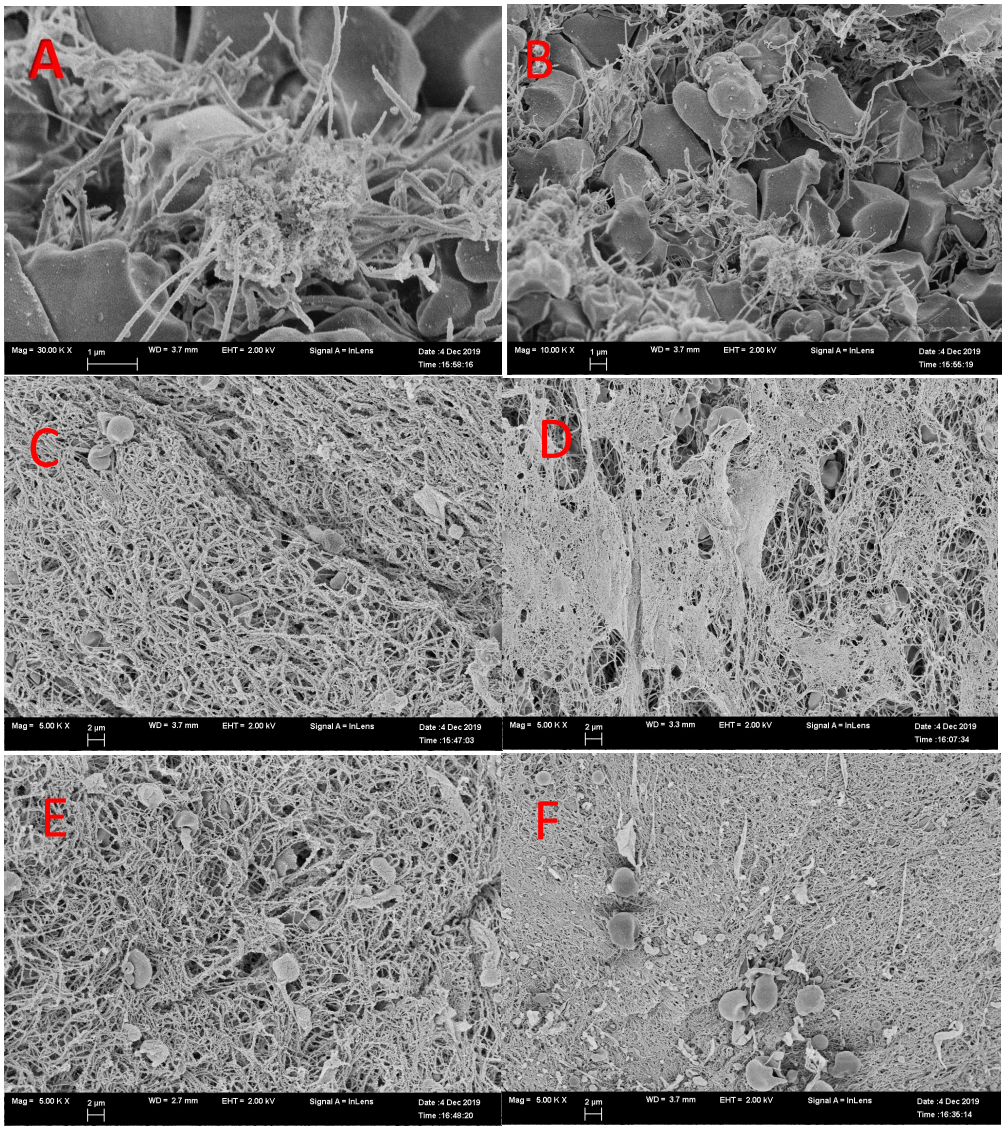
It is known that severe thrombocytopenia is an impediment to platelet thrombus formation on collagen under shear, an investigation usually used to assess platelet function in research laboratories.[330] In these experiments, to avoid coagulation activation, inhibitors of thrombin are often used. In this study I endeavoured to measure whether enhanced platelet deposition would result due to the interaction between fibrin formation and platelet aggregation. It was anticipated that when maintained under shear, re-calcification of citrated whole blood would allow increased fibrin-platelet interactions while not completely obstructing the channel with fibrin-based thrombus. However, the investigations performed here suggest that the approach is unlikely to be clinically useful under the tested conditions.

While there appeared to be a trend towards increasing thrombus formation under shear following platelet transfusion, this was neither consistent nor statistically significant. Addition of cryoprecipitate, which increased fibrinogen levels in this cohort, did not appear to increase the rate of thrombus formation. Indeed, perhaps due to the platelet dilutional effect of cryoprecipitate infusion, there was a reduction in thrombus formation in the two cases where thrombus was detected prior to transfusion. This could be interpreted as suggesting that the model is independent of fibrin formation, despite recalcification. These results may also be due to chance, as one of the three samples without thrombus formation pre-transfusion developed significant thrombosis post transfusion, the number of patients low and the variation within the test is high.

Within individual samples there was a significant variation in the number and size of thromboses within the channel. Attempts to improve thrombus formation by the addition of thromboplastin or VWF did not appear to improve the test performance. Variability within the assay is a significant limitation to measuring the combined effect of platelet number and function and the coagulation cascade in thrombus formation. Platelet numbers are likely to be limiting here just as they are in testing platelet function with assays used in clinical practice, including platelet aggregation and the PFA.

The end point of clot formation is a mesh of fibrin with platelets integrated into the meshwork (Figure 4.14A). Red cells are trapped within, but are not part of the structure (Figure 4.14B) and therefore do not contribute to the clot strength. By examining the clot structure, it is easy to visualise how fibrinogen may interchange for platelets within the final clot. This is illustrated using clots from Extem analysis shown in 4.14 (C-F) from a thrombocytopenic patient and addition of cryopreserved platelets, room temperature stored platelets, or following platelet transfusion. Clots under electron microscopy are heterogeneous and the images are shown for illustrative purposes only.

Figure 4.14: Scanning electron micrographs of thrombus formed in Extem (Patient 7). A: Platelet with adherent fibrinogen fibres (x30K). B: Fibrinogen and platelets, showing enclosed but not adherent red cells (x10k). C: Severe thrombocytopenia (x5K). D: Addition of cryopreserved platelets (x5K). E: Addition of D7 stored room temperature platelets (x5K). F: Post platelet transfusion (x5K). Taken with assistance of Elmira Mohammed on Zeiss UltraPlus field emission scanning electron microscope (FESEM) following coating with Platinum.



Clinical Implications

These results may have immediate clinical implications, although ideally further clinical studies are needed. Platelets stored at room temperature are in limited supply and usually kept only at major haematological oncology and trauma centres. For thrombocytopenic patients presenting to other centres, particularly in rural and regional areas, platelet transfusions are not available to assist in

obtaining haemostasis. Surgical or interventional radiological procedures to stop bleeding may be available, but are generally useful only when there is bleeding from a single site, not diffuse bleeding sometimes seen in thrombocytopenia. Surgical interventions may also have additional risks in the thrombocytopenic patient.

Tranexamic acid has been used in a variety of settings to reduce bleeding. This can, and should, be utilised. This study shows that improvements in haemostasis, as measured by thromboelastometry and APTT clot waveform, are seen with cryoprecipitate. At least in this model, this effect is significantly greater in vitro than the effect of cryopreserved platelets and there may be additional benefits, such as reduced fibrinolysis and increased clot activation with higher FVIII levels over fibrinogen concentrate. It is unknown what impact this has on bleeding, however given the known association of thromboelastometry amplitudes and bleeding risk, an effect is highly plausible. Further studies should be undertaken to establish whether this in vitro effect translates into bleeding cessation. In the interim, cryoprecipitate may be considered as an aid to haemostasis when platelet transfusions are not available.

Chapter 5: Discussion

In this thesis I've explored how the way we measure the function of blood can influence the way we perceive and manage it. Patient blood management is the practice of evaluating and optimising the function of the blood to meet the current and anticipated needs of patients. Some practices, such as identifying and managing anaemia pre-operatively are just good care. However, there are limitations to our current approaches. How do we measure the physiological impact of the blood to understand the significance of a deficit? What shall our target be when prescribing therapies and how should triggers to action and targets change based on the circumstances of the patient? We need to consider how our measurement approaches focus our decision-making around reductionist values. Having more holistic testing approaches may allow us to re-frame our thinking to patient-centred outcomes.

In chapter 2, the distinction was drawn between a normal range of a test and the value at which further investigation and treatment may be warranted. In PBM, where anticipated blood loss is expected, we should move beyond aiming for normal, preferring instead to optimise the red cell mass to cope with impending blood loss and the ability of the marrow to respond to this loss. This changes the focus from correcting a deficiency to improving outcomes, an approach demonstrably working in iron management in renal and cardiac disease to improve functional outcomes.[108,143]

The results presented in chapter 2 support aiming for a value of ferritin above 30µg/L with impending blood loss. The reluctance to screen universally for ferritin[101] could be partly addressed by using the MCV with a threshold value of under 87fL to select people, in the absence of anaemia, who may benefit from specific ferritin screening. This could be applied in the perioperative setting where anticipated blood loss from high risk procedures could be mitigated by anticipatory iron optimisation.

Based on these studies too, ferritin screening for iron deficiency in pregnancy is preferred in first trimester. Others have shown improvement in haemoglobin with intravenous iron treatment in pregnancy, although it is unclear at what stage of gestation these women were treated, so whether treating non-anaemic iron deficiency later in gestation will improve haemoglobin as a PBM measure,

remains unclear. [137,140,142] At present there are concerns about increasing iron in pregnancy increasing the ferritin or haemoglobin with resultant adverse outcomes.[101] There is no physiological reason why ensuring adequate iron will lead to supranormal haemoglobin concentrations, and this has proven by others.[140] Higher ferritins in population studies are more likely to be a reflection of underlying inflammation rather than iron overload, so the association between ferritin and adverse pregnancy outcomes should not be considered one of causation.[137] While the lack of randomised clinical trials on screening is acknowledged,[101] in their absence the weight of evidence favours intervention.[102] Without universal iron supplementation, screening should be adopted in first trimester using ferritin. Where this is not in place, ferritin should be ordered on every pregnant woman with an MCV under 87fL.

Addressing the need for red cell transfusion in the chronic setting was the subject of Chapter 3. The PBM pillar 3 literature has largely been focused on the perioperative setting and despite increasing numbers of clinical trials examining the indications for transfusion, few have been performed in chronic anaemia such as in haematological outpatients.[354] This may have been appropriate. The short-term need for blood transfusion after surgery provides an opportunity to implement strategies that save blood. This reduces the demands on donors, saves money, bed days and improves overall quality of care with probable reduction in complications, such as fluid overload, pulmonary infiltrates and mortality.[46,355] Good planning can prepare people for surgery, and if anaemia can be tolerated long enough for erythropoiesis to replace lost red cell volume, then blood is spared.

The chronic transfusion field is more difficult, with red cells frequently given to improve quality of life, but often based on triggers either handed down from mentors past or derived from trials evaluating mortality and other outcomes in hospitalised patients. While quality of life assessment has the perception of being a little more nebulous than overall survival rates, it is measurable. The measurement of quality of life is the basis upon which erythropoiesis stimulating agents were approved for management of anaemia.[356] This area has received less attention too perhaps because

it is seen as unsolvable. Chronic anaemia needs transfusion; or at least that's the perception. Red cell transfusion thresholds have declined in this population over recent years, but the decline is based on no better evidence than the higher thresholds that were used previously.[72,357] It is an area in need of research.

The haemoglobin is relied upon still for transfusion decisions, despite guidelines indicating the wide variability through which red cell transfusion may be considered appropriate.[34] A patient's condition overall needs to be considered in the decision-making process, but there are many factors, and it is not clear how they should impact our decisions. In the intensive care unit, transfusion in cardiac disease leads to an increased risk of pulmonary complications.[16] Will this still hold true in the chronic setting, or will chronic anaemia increase the work of the heart and contribute to left ventricular hypertrophy? What difference will transfusion make on quality of life, perhaps the most important outcome in this cohort? The haemoglobin does not answer these questions and better approaches are needed.

These studies explored the role of measuring tissue oxygen saturation for the management of anaemia. The systematic review provided the background – transfusion mostly improves tissues oxygen saturation, but there are many confounders that impact microvascular perfusion when used in the critical care setting. In the outpatient setting there was an association with tissue oxygenation levels, but the variation between individuals was too high to set meaningful targets. Changes in tissue oxygenation during exercise with anaemia were explored for the first time. Unfortunately, this is not *the* answer to detecting who does and does not need transfusion, for the changes are proportional to effort, reflecting the amount of muscle work rather than impairment in oxygen delivery.

While this did not fulfil the intended goal, the study has demonstrated that in most chronically anaemic patients, local supply of oxygen to exercising muscle does not appear to be the rate limiting factor in performance. Although, as others have also found, performance was affected.[164] It is possible

that with a more standardised exercise regimen, exercise may separate people likely to benefit from transfusion than not.

Another approach would be to detach red cell transfusion decisions from any particular measurement. Haemoglobin is used, but is flawed, but as yet there is no superior measure to replace it. In other areas of medicine, patient symptoms are acknowledged and patients given the opportunity to tailor their therapy to meet their needs, such with patient controlled analgesia. It is worth examining patients themselves deciding on the need for blood transfusion, based on symptoms, using self-management approaches successful elsewhere in medicine. After all, if transfusions are primarily for symptoms, who would know them better?

Chapter 4 addressed the second PBM pillar. Just as with red cell transfusion, much of the literature in PBM revolves around the perioperative setting. From the haematologist perspective, controlling bleeding to prevent excess blood loss involves optimising haemostasis. In thrombocytopenic patients, platelet transfusion is the standard of care in some subgroups, and tranexamic acid is under investigation.[277] These studies explored both the role of alternative products to improve haemostasis when platelet transfusions are not available and investigated assays that may help to distinguish between those susceptible to bleeding or not through consideration of whole blood assays.

The results suggest that cryoprecipitate may be a suitable alternative to platelet transfusion when platelets are not available. It is not conclusive – this would require a large trial and be difficult as it would need to enrol bleeding thrombocytopenic patients and only where platelets, as the standard of care, were not available. However, there is a measurable improvement in the strength of the clot formed following cryoprecipitate transfusion. In the absence of large clinical trials, similar responses have been used to guide treatment, for example with the use of prothrombin complex concentrates in the treatment of oral direct Xa inhibitors.[358,359] This study provides a rationale for the use of cryoprecipitate and more evidence should be sought. Given that cryoprecipitate is proposed as an alternative to platelet transfusion only when the latter is not available, a randomised trial comparing

these two is not appropriate. However, a registry of cases where cryoprecipitate is used when platelets are not available to treat bleeding would be worthwhile.

Bleeding scores were collected during this study. Purposefully, serious bleeding requiring immediate platelet transfusion was excluded and the numbers are small, so the study could not determine values that predict serious bleeding. Viscoelastometry has potential utility, although there are conflicting studies.[306,312,314] Exploration of platelet adhesion under shear found that at the very low platelet counts, the tests lacked sensitivity to detect changes induced by transfusion of platelets or cryoprecipitate. More work examining APTT clotting curves is warranted.

It could be argued that this thesis has extended beyond PBM. Certainly, it deals with more than implementation science by questioning the measurement approaches used in decision making in PBM and transfusion. Research should look both upstream to develop new approaches to be tested, and downstream, to ensure that findings of our research are translated to the bedside.

PBM has been described in terms of the "three pillars", described in the introduction and throughout this paper.[41,355] Implementation strategies based on all three of these pillars have improved costs, decreased hospital length of stay and reduced mortality.[360] In 2019, Isbister suggested adding to this a "keystone" of good governance. In forming an arch one needs not only pillars, but the keystone to lock it in place. It is effective imagery to capture the need for sound practice implementation governance strategies to apply what we already know. Others have described a fourth pillar, or strategy, of patient centred care.[121] I suggest these models overlook the foundation upon which the pillars stand – the evidence upon which each is based. Without a foundation in evidence it is shaky ground indeed. As shown in chapter 1, transfusion practices have been hard to change. Good clinical evidence has been required to effect change and has been even more important than the acknowledged infectious risks our blood supply has brought with it.

There is still much to learn and to question in what we think we know in transfusion medicine. Failure to do so, as I referred to in the introduction, has led to overuse of transfusion. It would be easy to

look back now at cases where transfusion was not been indicated and the recipient, contracting a blood borne infectious disease has later suffered or even succumbed to the effect of inappropriate transfusion. It may be harder to see the harm done from failure to manage anaemia, or liberal transfusion strategies, but the data suggest these are equally real. It is difficult to support good transfusion practice when the lack of evidence creates uncertainty about what best practice is.

To the three pillars, good governance and patient centred care, we must add research to build the foundation of evidence. More needs to be learned on how to restructure how we work to implement what we know, but there is still so much we don't know. As yet there is no certainty of what a transfusion trigger should be. We do not know what physiological variables to measure to best described the impact of anaemia or to determine those patients most likely to bleed.

The PBM pillars are transformed: Optimise the blood, minimise blood loss and harness or optimise the physiological reserve of in anaemia. Hold the pillars together with a patient centred approach to care and good governance. It all needs to be embedded on a foundation of evidence from scientific research (Figure 5.1). The PBM model of care is completed, but without attention to its base it will not continue to improve patient care.

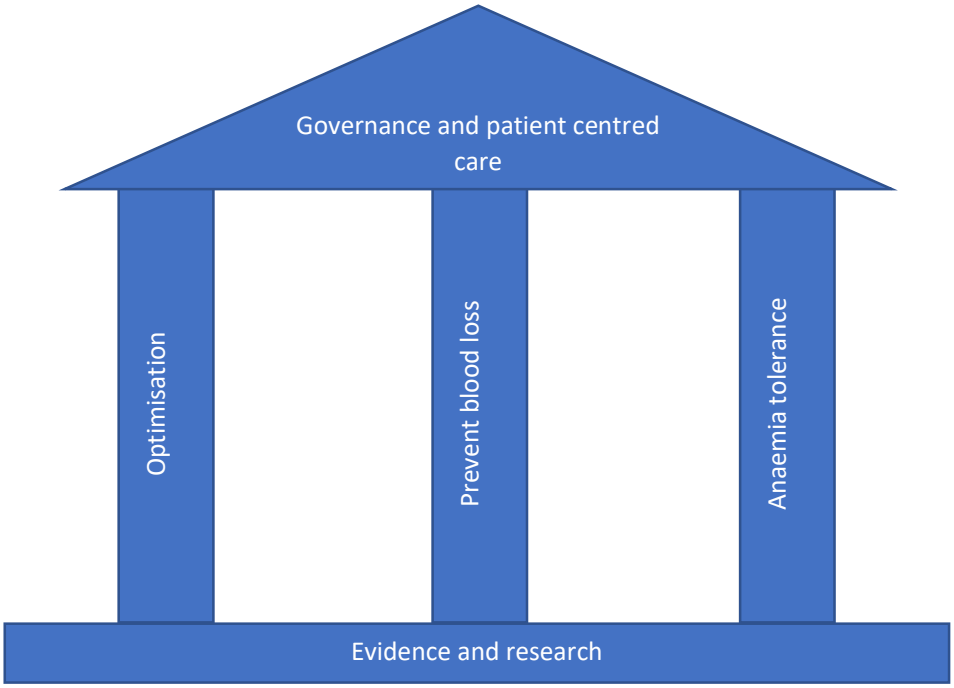


Figure 5.1:Revised Patient Blood Management Pillars

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