

A study of Thromboprophylaxis in the Australian and New Zealand Fontan population

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Abstract

Background: The Fontan procedure was developed approximately 50 years ago and has become the definitive treatment for children born with single ventricle physiology.

Surgical refinement of the Fontan technique and improvements to critical care have led to a dramatic reduction in mortality, with the vast majority of patients expected to live well into adulthood. The reduction in mortality has led to a shift in research efforts in the Fontan population towards defining the long-term expectations and identifying modifiable factors that can reduce Fontan-associated morbidity.

Thrombosis and stroke represent important post-Fontan complications.

As such, thromboprophylaxis with warfarin or aspirin is accepted as standard of care post-Fontan. However, there is a notable lack of evidence to inform best practice in the setting of thromboprophylaxis for Fontan patients hence, selection of thromboprophylaxis has been largely dependent on institute practice and clinician preference.

Objectives: This thesis explores the long-term outcomes and the impact of current thromboprophylaxis practice in the Australian and New Zealand (ANZ) Fontan population. This study compared cerebrovascular injury, bleeding, bone mineral density (BMD) and quality of life (QoL) in Fontan patients receiving warfarin as compared to aspirin.

Methods: This was a cross-sectional, multicentre study of the Australian and New-Zealand Fontan (ANZ) population. Participants underwent cerebral magnetic resonance imaging (MRI) to detect the presence of cerebrovascular injury, dual-energy x-ray absorptiometry (DXA) to assess bone mineral density (BMD). Bleeding and quality of life were assessed using validated questionnaires (WA-BAT and PedsQL, respectively).

Results: 84 participants underwent brain MRI. Stroke was detected in 33 (39%) participants, with only 5 (15%) of those being clinically symptomatic. There was no association between stroke and Fontan type (p value 0.38, CI 0.39- 1.43) or thromboprophylaxis type. Micro- haemorrhage and white matter injury were detected in almost all participants (96% and 86% respectively), regardless of thromboprophylaxis type.

BMD was reduced in our cohort (n=120) compared to the general population however, this reduction was greater in participants receiving warfarin than those on aspirin.

High rates of bleeding were reported in both warfarin and aspirin groups however, participants on warfarin experienced more epistaxis and ceased anticoagulation more commonly prior to a medical procedure than those on aspirin.

QoL was similar between warfarin and aspirin groups; however, higher QoL scores were reported in patients on warfarin therapy with access to home-INR monitoring compared to those on warfarin therapy without home-INR monitoring.

Conclusions: This study adds significantly to the current evidence with regard to the potential consequences of warfarin and aspirin therapy, post-Fontan surgery. Specifically, cerebrovascular injury are frequent occurrences in the ANZ Fontan population, regardless of thromboprophylaxis type.

No benefit of long-term warfarin prophylaxis could be demonstrated over aspirin; however, this finding needs to be considered in relation to important clinical features such as cardiac function and lung function. Furthermore, the association of reduced bone health in children on warfarin warrants further mechanistic studies.

Declaration

This is to certify that:

1. the thesis comprises only my original work towards the PhD except where indicated in the Preface,
2. due acknowledgement has been made in the text to all other material used,
3. the thesis is fewer than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices.



Chantal Attard

August 2019

Preface

This thesis contains published work that was completed in collaboration with multiple authors. However, I (Chantal Attard) was ultimately responsible for the data collection and analysis, manuscript draft preparation, final submission as well as the corrections of the proofs for each publication. Participant recruitment was assisted by the Australian and Zealand Fontan registry team, under my detailed and specific guidance.

This study was funded by a grant from the National Health and Medical Research Council: “Functional outcomes after Fontan surgery” (APP1065794)

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Advisory committee members: Dr. Marc Seal and Prof. Fiona Newall – I am grateful for your encouragement, support and enthusiasm over my PhD journey. Thank-you for your invaluable and practical advice and for your commitment to the successful completion of my PhD.

Haematology Research team – Thank-you for your constant support, encouragement and comic relief over the years. To the team members who were there from the very start - Vicky, Sophie, Fiona and Vera. Thank-you for setting the example and high standards of how to raise children and be successful in your career. You are all a constant inspiration to me, and I really appreciate the words of encouragement and laughs we have had over the years. You made my place of work a real joy to be a part of.

Australia and New Zealand Fontan registry team: Karin, Janina, Carley, Rebecca and Namani – Your hard work and dedication to the those living with a Fontan and their families is a testament to the you and your team. This study was helped greatly by the rapport you have built over the years with the families, clinicians and researcher involved with the Fontan registry. Thank-you for making me feel like one of your own and for introducing me to the Fontan community.

I would also like to acknowledge my funding sources, the NHMRC and Murdoch Children's Research Institute. Without their support this project would not have been possible.

My family: Mum and Dad – words cannot express how grateful I am for your enthusiasm, support and assistance over the course of my entire life. Your belief in me as a Daughter, Mother and Researcher has never waned over the years. I thank-you from the bottom of my heart for the love and attention you

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To my daughters, Allegra and Francesca – I started this PhD with the hope of making a difference to the people living with a Fontan. Having you has given me a deeper appreciation of the importance of research into childhood illnesses, and how the outcomes of our research has implications for both the child and their family. Children are a blessing and it is our collective responsibility to ensure they are given the best chance at a full and happy life. Thank-you, Allegra and Francesca, for reminding me this Every. Single. Day.

Allegra, my clever, kind and funny girl, thank-you for your patience and understanding for what must have seemed like a very long time (your whole life!). Francesca, thank-you for being such a patient baby. You have provided our little family a constant stream of joy and love. I love you both so very much and I am so proud to be your Mum.

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Abbreviations

AP	Atriopulmonary
ACCP	American College of Chest Physicians
ANZ	Australian and New Zealand
BMD	Bone Mineral Density
CHD	Congenital Heart Disease
COW	Circle of Willis
CPB	Cardio-Pulmonary bypass
CVLs	Central Venous Lines
DOACs	Direct oral anticoagulants
DXA	Dual-energy X-ray absorptiometry
ECC	Extracardiac Conduit
ICA	Internal Carotid Artery
INR	International Normalized Ratio
IRR	intrarater reliability
LT	Lateral Tunnel
MCRI	Murdoch Children's Research Institute
MRA	Magnetic Resonance Angiography
MRI	Magnetic Resonance Imaging
NHMRC	National Health Medical Research Council
PE	Pulmonary Emboli
PedsQL	The Paediatric Quality of Life
QoL	Quality of life
REDCap	Research Electronic Data Capture
TOE	transoesophageal echocardiography
TTE	Transthoracic echocardiography
VA	Vertebral artery
VTE	Venous Thromboembolism
WA-BAT	Warfarin/Aspirin-bleeding assessment tool

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1. ***Attard C**, Huang J, Newall F, Monagle P, d'Udekem Y, Ignjatovic V.
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***Attard. C**, Monagle. P, d'Udekem. Y, Mackay. M.T, Briody. J, Mandelstam. S, Bani Hassan. E, Simm. P, Rice. K, Munns. C, Gentles. T, Winlaw. D, Cordina. R, Grigg. L, Ignjatovic. V. Comparison of the long-term outcomes and experience with warfarin and aspirin in people living with a Fontan circulation. Submitted to: Journal of the American College of Cardiology

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Presentations during my PhD Candidature

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1. 2019 International Society on Thrombosis and Haemostasis (ISTH)
Congress: Comparison of the incidence of stroke in Fontan patients
receiving warfarin or aspirin (Melbourne)
2. 2019 Paediatric ISTH Satellite Symposium: Fontan Registry and
Outcomes (Melbourne) *Invited*
3. 2018 PhD Completion seminar
4. 2017 Fontan Education Day: Warfarin and Bone Health (Brisbane)
Invited
5. 2017 Three Minute Thesis (Department of Paediatrics, University of
Melbourne)
6. 2016 Fontan Education Day: Preventing blood clots in patients with a
Fontan (Auckland, New Zealand) *Invited*

POSTERS

1. 2017 International Society on Thrombosis and Haemostasis Congress
(3)
2. 2016 Melbourne Children's Student Symposium (1) *best poster award*
3. 2016 PhD Confirmation seminar (Department of Paediatrics,
University of Melbourne)

Postgraduate scholarships and Awards

- 2014-2018 National Health and Medical Research Council - Public Health and Health Services Postgraduate Research Scholarship (APP1074457)
- 2014-2018 Murdoch Children's Research Institute postgraduate "top-up" scholarship
- 2017 Ackman Travelling Scholarship, University of Melbourne
- 2017 Murdoch Children's Research Institute Travel Scholarship
- 2016 Awarded Best Poster, Melbourne Children's Student Symposium

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CHAPTER 1 Literature review

1.1 Introduction

The Fontan procedure was developed approximately 50 years ago as treatment for the fatal cardiac condition, tricuspid atresia ¹. Since then, the Fontan procedure has become the definitive treatment for children born with the most severe type of congenital heart disease and is offered as palliation for a broad range of cardiac defects, including the Hypoplastic Left Heart Syndrome.

Surgical refinement of the Fontan technique and improvements to critical care have led to a dramatic reduction in mortality, with the vast majority of patients expected to live well into adulthood. The broad application of the Fontan technique for heterogenous cardiac conditions, has significantly shifted the landscape of the overall congenital cardiac population, with the Fontan group representing an ever-growing and important proportion of that population.

The reduction in mortality has led to a significant shift in research efforts in the Fontan population towards defining the long-term expectations and identifying modifiable factors that can reduce Fontan-associated morbidity.

Thrombosis and stroke represent important post-Fontan complications. As such, thromboprophylaxis with warfarin or aspirin is accepted as standard of care post-Fontan. However, there is a notable lack of evidence to inform best practice in the setting of thromboprophylaxis for Fontan patients.

This thesis explores the **long-term outcomes** and the **impact** of current thromboprophylaxis practice in the Australian and New Zealand (ANZ) Fontan population.

This thesis:

- (1) Describes the long-term outcomes of current thromboprophylaxis use in the Fontan population in terms of stroke, bone mineral density, bleeding and quality of life;
- (2) Compares the long-term outcomes of warfarin and aspirin use in the Fontan population in terms of stroke, bone mineral density, bleeding and quality of life.

The first chapter of the thesis provides a description of the Fontan population and a review of the literature describing the epidemiology, pathophysiology and outcomes of thrombosis post-Fontan surgery. This chapter also compares the drugs warfarin and aspirin which are used as thromboprophylaxis in this population.

1.2 Literature Review

This literature review was compiled in 2014 and as such includes published evidence available at that time.

A modified version of the presented literature review was accepted and subsequently published and is included in this thesis:

Attard, C., Huang, J., Monagle, P. & Ignjatovic, V. Pathophysiology of thrombosis and anticoagulation post Fontan surgery. *Thrombosis Research*, 2018;172, 204-213.



Full Length Article

Pathophysiology of thrombosis and anticoagulation post Fontan surgery

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ABSTRACT

The Fontan procedure has transformed the lives of children born with single-ventricle physiology, previously deemed inoperable. Worldwide, there are an increasing number of children with Fontan circulation, with the potential for survival into adulthood. Due to the abnormal circulation, Fontan patients have an increased risk of thromboembolic (TE) events, with up to 25% of events leading to death. Despite the importance of preventing TE events in this patient population, there is currently no clinical consensus on the optimal monitoring, thromboprophylaxis therapies, and treatment of these events. This paper reviews the available literature regarding anticoagulation in the pediatric and adult Fontan population, including the mechanisms for thrombosis and current antithrombotic therapies.

1. Introduction

The Fontan procedure has enabled children with congenital defects previously deemed inoperable to survive to adulthood. A common complication of the Fontan circulation is thromboembolism (TE), with patients usually being treated with antithrombotics, as prophylaxis. This review provides a description of the pediatric and adult Fontan population and compares the outcomes of different thromboprophylactic regimes commonly used in this population today.

2. The Fontan procedure

The Fontan procedure was initially developed by Fontan and Baudet [1] as a formal treatment for tricuspid atresia. Since its original conception, the Fontan procedure has evolved and is now used to treat patients with an array of congenital heart diseases and a single functional chamber (Table 1). In these patients, a series of palliative procedures are performed, culminating in the Fontan procedure. Once completed, the Fontan circulation directly connects the systemic venous return to the pulmonary circulation, bypassing the absent or inadequately formed structures of the heart. Although there is no ideal age for the operation, the Fontan procedure is typically performed in childhood, as procedures delayed to adolescence and adulthood have been associated with poorer outcomes [2]. For improved morbidity and mortality post-procedure, patients considered for Fontan should have reasonable ventricular function, well-sized pulmonary arteries and

near-normal pulmonary vascular resistance [3].

2.1. Surgical modifications

The original classical “Atriopulmonary” (AP) procedure has two modifications, which are commonly utilized today: the Lateral Tunnel (LT) and the contemporary Extracardiac Conduit (ECC) (see Fig. 1).

The AP connection was the original Fontan technique used to treat tricuspid atresia, where the right atrial chamber was isolated by the closure of the atrial septal defect and the hypoplastic tricuspid valve (Fig. 1a). The right atrial auricle was then anastomosed to the right pulmonary artery, bypassing the absent ventricle and restoring systemic flow. A complication of this technique was that the right atrium was susceptible to dilatation, which leads to pulmonary venous obstruction, thrombus formation and atrial arrhythmias in a significant proportion of the population.

Further modifications of the procedure led to the understanding that streamlining the systemic venous blood flow straight to the lungs improved hemodynamics and avoided right atrial dilatation. This realization led to the development of the lateral tunnel technique (LT) (Fig. 1b). In this variation of the Fontan surgery, a Goretex patch is used as an intra-atrial baffle, therefore reducing the atrial diameter and improving flow through the atrium. Additionally, the superior vena cava is anastomosed directly to the right pulmonary artery (known as a Bidirectional Glenn shunt). A benefit of this specific Fontan technique is that the procedure can be staged: the Glenn shunt procedure can be

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E-mail address: chantal.attard@mcri.edu.au (C. Attard).

Table 1
Examples of congenital heart diseases necessitating Fontan procedure.

- Tricuspid atresia
- Hypoplastic left heart syndrome
- Double outlet right ventricle
- Complete atrioventricular canal
- Congenitally correct transposition of the great arteries
- Transposition of the great arteries
- Double inlet left ventricle
- Pulmonary atresia with intact ventricular septum

performed at an earlier age, relieving the volume load of the ventricle.

The ECC procedure utilizes a Goretex tube to graft between the inferior vena cava and pulmonary artery (Fig. 2C), in replacement of the intra-atrial routing of the venous blood. This bypass of the entire right atrium is thought to improve venous flow and hemodynamics [5]. Internationally, the lateral tunnel and ECC are now the most common Fontan procedures performed.

Both the LT and ECC techniques allow for the addition of a fenestration in the baffle, which creates a communication between the systemic venous conduit and pulmonary venous circulation. The fenestration functions as a right-to-left shunt and helps to limit caval pressure, increase preload to the systemic ventricle, increase cardiac output and reduces pulmonary vascular resistance. Fontan fenestration has been credited at reducing post-operative morbidity [6]. However, these potential benefits have been accompanied by the risk of developing paradoxical emboli, where the embolization of a venous thrombus travels through the right-to-left shunt into the arterial system [7,8].

3. Thromboembolism post-Fontan surgery

Thromboembolism (TE) is a significant complication after the Fontan procedure and remains an important cause of morbidity and mortality in both the short- and long-term following surgery. This review discusses the pathophysiology, incidence, diagnosis and treatment of thrombosis post-Fontan surgery.

3.1. Pathophysiology of thromboembolism in Fontan

Thrombosis commonly presents in Fontan patients as intracardiac or intravascular thrombosis, ischemic stroke, or other embolic phenomena [9]. Thromboembolic events in the Fontan patient can be categorized into two forms. Firstly, thrombosis forming in the systemic venous system, such as in the Fontan conduit, may cause local obstruction, which can extend or embolize into the pulmonary arteries, potentially

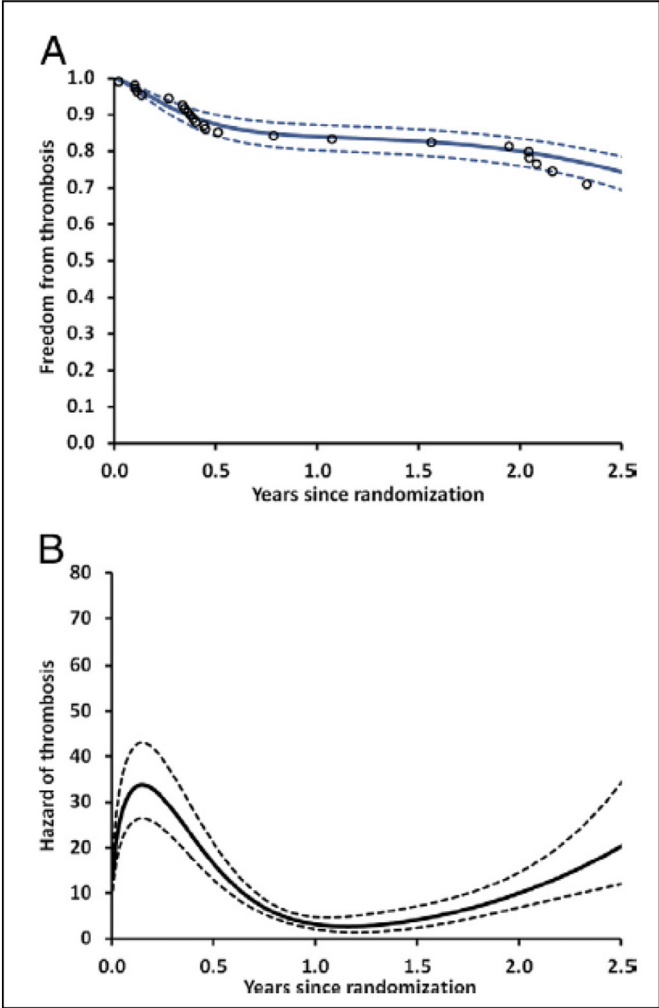


Fig. 2. Freedom from thrombosis over time, stratified by thromboprophylaxis choice.
(Used with permission McCrindle et al. [39].)

leading to Fontan failure [10–14]. Paradoxical emboli may also occur through fenestrations or right-to-left-shunts [8]. Secondly, thrombus occurring on the left of the atrial baffle may embolize to the arterial system resulting in tissue infarction or stroke [15].

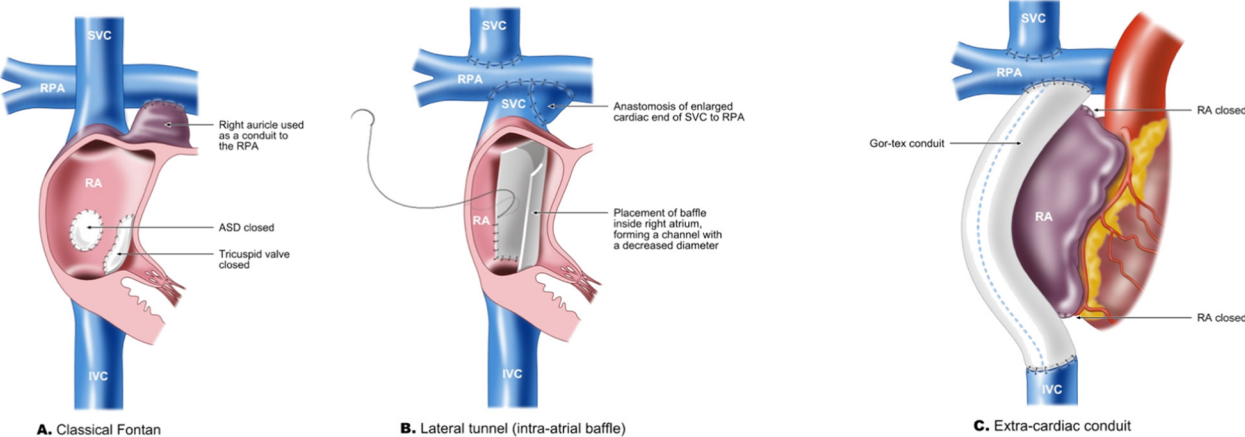


Fig. 1. Evolution of the Fontan procedure. (A) Atriopulmonary connection, (B) Lateral tunnel, (C) extracardiac conduit techniques (used with permission from d'Udekem et al., Circulation 2007) [4].

Table 2
Elements of Virchow's triad contributing to thrombosis formation in Fontan patients.

	Thrombogenic contributors in the Fontan patient	Impact on coagulation
Endothelial dysfunction	● Central venous line use ● Cardiopulmonary bypass ● Thrombogenic foreign material (the Fontan conduit) ● Surgical sites ● Inherent endothelial dysfunction	● Injury to vessel wall ● Exposure of blood to large artificial surfaces ● Activation of the coagulation system
Abnormal blood flow	● Cardiopulmonary bypass ● Surgery type: the Atriopulmonary procedure involves turbulent blood flow from a large right atrium that delivers blood directly to the pulmonary artery ● Ventricular dysfunction ● Arrhythmias ● Flow dead ends – e.g. pulmonary artery stump ● Lower cardiac output ● Higher venous pressures ● Nonpulsatile systemic venous flow ● Chamber dilatation (e.g. dilated atrium) ● Conduit stenosis ● Right to left intracardiac communication (e.g. fenestration)	● Abnormal flow rates and shear stress ● Turbulent blood flow ● Activation of the coagulation system
Hypercoagulability	● Cardiopulmonary bypass ● Thrombophilia factors ● Liver dysfunction ● Pre- and post-Fontan coagulation factor abnormalities ● Protein losing enteropathy (loss of clotting factors to gut) ● Increased platelet reactivity	● Hypercoagulable state

3.1.1. Impact of Fontan on hemostasis

The Fontan circulation represents an ideal substrate for thrombus formation, with all three factors of Virchow's triad being affected. Firstly, endothelial damage occurs with the changes in systemic pressures and the presence of surgically manipulated endothelium or prosthetic material creates an abnormal endothelium. Blood flow is affected, as there is typically low flow due to the absence of a functional right ventricle. Lastly, blood components are affected by intrinsic plasma protein changes, probably related to subtle changes in liver synthetic function and certainly accentuated in patients with protein-losing enteropathy. These three elements are present to some extent in all Fontan patients (Table 2).

3.1.2. Endothelial dysfunction

The vascular endothelium is vital in the regulation of pulmonary vascular tone, vascular smooth muscle cells and the control of coagulation, fibrinolysis, and inflammation. Cardiac surgery can cause endothelial injury which activates the vascular endothelium leading to endothelial dysfunction [16]. The endothelial dysfunction is characterized by the loss of vasodilators, such as nitric oxide, and anti-thrombotic factors such as antithrombin, Protein C and S, as well as an increase in vasoconstrictor and pro-thrombotic products [17]. The contribution of factors involved in endothelial dysfunction leads to a pro-thrombotic state [18]. Endothelial dysfunction has been observed even prior to Fontan surgery in patients with single ventricular physiology [19]. A specific hypothesis is that this inherent endothelial dysfunction occurs in the presence of chronic hypoxia, a complication observed in Fontan patients. Interestingly, in some patients, endothelial dysfunction persists post-operatively despite the absence of hypoxemia [18].

3.1.3. Abnormal blood flow

Another physiological change post-Fontan surgery is the disturbance of blood flow, which may involve a reduction of blood flow, stasis or turbulent blood flow. These disturbances in blood flow lead to localized activation of the coagulation system, resulting in the accumulation of circulating platelets and coagulation factors which are conducive to thrombus formation [20]. The Fontan surgery encompasses a broad range of procedures that may all have different impacts on blood flow. Common to all variations of the Fontan patient is that pulmonary artery flow is driven by venous pressure and so a low

flow state ensues. A reduced flow rate propagates initiation of the coagulation system, increasing the likelihood of thrombi development.

Other factors that may impact on vascular flow in Fontan patients include flow dead ends such as the pulmonary artery stump [21], low cardiac output and higher venous pressures. Over the long term, patients may develop serious complications such as chamber dilatation (particularly observed in the superseded AP connection Fontan) and conduit stenosis, both of which have serious implications for the patient including a greatly increased risk of thrombosis and Fontan failure [22,23].

Atrial arrhythmias in Fontan patients are an additional risk factor for TE complications, with several retrospective studies reporting increased incidence of thrombosis in patients with previous arrhythmias [24–26]. Egbe et al. published a recent study where atrial arrhythmias were a risk factor for systemic (hazard ratio 2.28, $P = 0.001$) and non-systemic thrombotic and embolic complications [26]. Notably, majority of the cohort (74%) had AP Fontan connection, which was also noted to be a risk factor for thromboembolism (hazard ratio 1.98, $P = 0.02$). As mentioned previously, the AP technique is more susceptible for arrhythmia development, and hence anticoagulation in these patients with a history of atrial arrhythmias is a pertinent consideration. The American Heart Association Scientific guideline for management of patients with a palliated single ventricle suggests the initiation of anti-thrombotic therapy for patients with arrhythmia [27]. Patients with both AP Fontan and a history of atrial arrhythmias are at higher risk for thrombosis, and further evaluation for the management of these patients is required.

3.1.4. Hypercoagulability

Inherent abnormalities in coagulation have been reported both pre- and post- Fontan surgery [28–34]. However, few studies have utilized age-appropriate reference ranges for the coagulation tests reported and the findings are therefore difficult to interpret. Odegard et al. described a prospective, case-controlled study of 20 post-Fontan patients that included healthy age-matched controls, to evaluate coagulation factor abnormalities [33]. Fontan patients had decreased levels of the naturally occurring anticoagulants protein C, plasminogen and antithrombin as well as coagulation factors II, V, VII and X when compared to controls. Moreover, FVIII was found to be elevated in 6 patients (35%), two of whom had had a thromboembolic event and protein-losing enteropathy. The authors speculated that in Fontan patients, the liver

might produce increased levels of factor VIII in response to elevated hepatic and central venous pressure. However, this study did not demonstrate a correlation between hepatic synthetic dysfunction or hepatocellular dysfunction and coagulation abnormalities.

Coagulation abnormalities leading to thrombotic tendency may also be due to protein-losing enteropathy, which is an enigmatic condition that results in the loss of serum proteins into the gastrointestinal tract. This condition may present weeks to years following the Fontan procedure and is associated with increased morbidity and mortality [35–37]. The substantial loss of serum proteins can have far-reaching clinical implications, including the impairment of the clotting cascade, thus creating a hemostatic imbalance [35].

Another postulated contributor to increased thrombotic risk in this population is increased platelet reactivity. Studies have shown that platelet reactivity in Fontan patients is increased both in the short- and long-term prior and post-surgery, when compared to healthy controls [38]. This finding is not surprising, as platelets become activated during turbulent blood flow and in the presence of endothelial dysfunction. Increased platelet reactivity is still observed in patients receiving aspirin as thromboprophylaxis, despite the antiplatelet properties of this drug [38].

3.1.5. Other risk factors for thromboembolic events post-Fontan

Risk of thrombosis in children post-cardiac surgery is highest in the immediate post-operative period, suggesting that surgery has an immediate effect on the hemostatic system [39]. Children undergoing cardiac surgery are an especially high-risk group for thrombosis compared to other pediatric surgical populations. In a retrospective study by Manliot et al. [40] an incidence of 7.9% symptomatic VTE was observed in children post cardiac-surgery, as compared to only 0.34–0.58% VTE previously reported by Raffini et al. [41] for all pediatric admissions over a five year period. Many factors are likely to contribute to the increased post-operative thrombosis risk including the use of central venous lines [40,42,43], cardio-pulmonary bypass (CPB) [44] and the type of surgery itself.

Presence of a right-to-left shunt, such as that present in the fenestrated Fontan, also carries a potentially increased risk of stroke. This perceived risk is attributed to paradoxical embolism. However, no study has yet proven that fenestrated Fontan patients are at increased risk of stroke compared to non-fenestrated patients.

In summary, Fontan patients clearly have an elevated risk of thrombosis due to the multitude of factors that lead to endothelial dysfunction, the disturbance of blood flow and coagulopathy. Identification of major etiological factors in individual patients remains to be determined.

3.2. Incidence of thromboembolism post-Fontan

Although thrombosis post-Fontan surgery is thought to be highly prevalent, the true incidence of thrombosis is unknown due to the lack of prospective studies within this population [9,15,45]. The studies utilized in this review are summarized in Table 3. Cohort studies have reported from incidences of venous thrombosis ranging from 4% to 19% [24,46–49] and the incidence of stroke ranging from 3% to 19% in children on different thromboprophylaxis regimes [24,25,47,50,51]. An Australian national registry including approximately 1006 survivors of the Fontan operation demonstrated an overall freedom from TE of 82% at 25 years post-Fontan (95% CI, 74%–87%), with events occurring at a median of 7.6 years. In subgroup analysis, the freedom from TE was 91% at 12 years in ECC patients [52], and LT Fontan patients had a TE incidence of 16% [53].

Regarding intracardiac thrombosis, cross-sectional studies using transoesophageal echocardiography (TOE) have reported a prevalence of 17% to 33% [77–79]. The most commonly observed sites for intracardiac thrombi are the systemic venous atrium (48%) and pulmonary venous chamber (44%) [35]. Discrepancies in the reported

incidence between studies may be explained by the varied duration of the follow up (e.g., semi-annually versus bi-annually), as well as the imaging techniques utilized to diagnose thrombosis (e.g., echocardiogram versus magnetic resonance imaging).

Monagle et al. performed a prospective, multicentered, randomized controlled trial assessing thromboprophylaxis post-Fontan surgery [9]. All observed thrombotic events in the study were venous with no incidence of arterial thrombosis. An overall thrombosis incidence of 22% was observed in these patients within the first two years post-Fontan surgery. Interestingly, Monagle et al. determined that the symptomatic thrombosis rate was only 7%, suggesting that silent thrombosis is highly prevalent in this population [9]. Whether silent thrombosis is clinically important is unknown at present and requires further research.

In a *post-hoc* analysis of Monagle et al.'s study, McCrindle et al. identified that at 2.5 years post-randomization, there was a time-related freedom from thrombosis of 69% [39]. Time-related risk of thrombosis was highest during the immediate post-operative period up until six months post-surgery, followed by a lower yet steadily increasing risk of thrombosis in the subsequent two years. These results are supported by other studies that also observed this pattern of the immediate high risk of thrombosis in the initial one-year period post-Fontan [51,78,80–82], with thrombosis risk plateauing at 3.5 years and being followed by a second peak after 10 years post-surgery [47,83].

All patients were included from study randomization up to first thrombotic event, study withdrawal, or end of the study, no censoring for drug discontinuation. Controlled warfarin was defined as > 30% of international normalized ratio (INR) within target over the study period. Patients on warfarin who consistently achieved target INR levels > 30% of the time or those on acetylsalicylic acid had a 3.5-fold decrease in risk of thrombosis compared patients who often failed to meet target INR level (hazard ratio: 3.53, 95% confidence interval: 1.35 to 9.20, $P = 0.01$), and difference in risk between controlled warfarin and ASA was not statistically significant (hazard ratio: 0.34, 95% confidence interval: 0.10 to 1.13, $P = 0.08$).

Despite the fact that thrombosis is a common and significant problem following Fontan surgery, optimal thromboprophylactic strategies, as well as outcome data after thrombosis are unknown.

3.3. Diagnosis of thrombosis post-Fontan

Transthoracic echocardiography (TTE) is the most commonly used imaging technique for patient follow-up post-Fontan procedure. Benefits of TTE include low cost, widespread availability, the easy-to-use and non-invasive nature of the test. Despite these benefits, an intracardiac thrombus is often poorly detected in the Fontan patient due to the presence of prosthetic material and the distance between the chest wall and the pulmonary arteries and veins. Moreover, in the post-surgical patient, chest tubes, edema and dressings further inhibit the ability of a TTE to detect thrombi.

Numerous studies have demonstrated the superiority of transoesophageal echocardiography (TOE) over TTE at detecting thrombi in Fontan patients [77–79]. TOE imaging sensitivity can be attributed to the close proximity of the probe to cardiac structures, facilitating accurate and effective detection of thrombi. However, due to the invasive nature of TOE, sedation for most patients is generally recommended. In a study by Balling et al. [77] in post-Fontan patients, TOE was able to detect intra-cardiac thrombi in 33% of the patients screened as compared to just 2% when using TTE in the same patients.

Recently, magnetic resonance imaging (MRI) has emerged as a valuable, non-invasive cardiovascular diagnostic tool for Fontan patients [84]. MRI has demonstrated accurate assessments of the complex anatomy including extracardiac structures and usually occurs without sedation or exposure to ionizing radiation. No studies exist to date evaluating the accuracy of MRI in detecting intracardiac thrombi in pediatric Fontan patients.

Table 3
Summary of review's findings.

Study design			Outcome incidence						
Author & year	n	Procedure	Prophylaxis	Follow-up	Detection method	Thrombosis, n	Embolism, n	Early mortality, n	Late mortality, n
Alphonso [54]	122	LT	Preop: C (n = 4); A (n = 10). Additional at postop: C (n = 16); A (n = 46). Additional at follow-up: C (n = 9); A (n = 19)	54 m	Transthoracic	4	4	6	7
Boshoff [55]	68	EC (n = 63); LT (n = 5)	N/A	58.8 ± 28.8 m	Transesoph ^a	0	0	N/A	N/A
Brown [56]	220	LT	L (3–5 d); C (3 d postop)	80.4 ± 46.8 m	N/A	N/A	6	1	10
Chaloupecky [57]	102	TCPA	A	60 m (12–96 m)	Transthoracic	0	3	N/A	N/A
Cheung [58]	102	APA (n = 64), LT (n = 21), EC (n = 17)	C (long-t; n = 46); A (n = B); none (n = 31)	79.2 ± 45.6 m	Transthoracic ^b	N/A	4	11	6
Chowdhury [59]	65	EC	Cap (1 d postop); C (for PTFE cond; 6 m), A	77 ± 2 m (8–79 m)	N/A	3	N/A	2	0
Dodge-Khatami [60]	9	APA	N/A	39.6 ± 12.9 m	Transthoracic	N/A	N/A	0	0
D'Udekem [61]	1006	APA (n = 203), LT (n = 271), EC (n = 532)	N/A	25 y	N/A	56	N/A	N/A	55
Fiore [62]	162	EC (n = 49), LT (n = 113)	H (postop); C (discharge till fenest. closure); A (after closure). Nonfenestrated patients: C (1 y), A (life-t).	EC: 36.0 ± 27.6 m; LT: 60.0 ± 37.2 m	Transthoracic	0	8	3	7
Giannico [23]	221	EC	Yes (unspecified)	63 m	N/A	1	1	6	22
Hasaniya [63]	160	EC	A	78.0 ± 44.4 m (1.2–180.0 m)	N/A	2	1	2	6
Hirsch [64]	100	LT	A (n = 80); C (n = 4); not followed (n = 16)	34 m	N/A	4	0	3	4
Ikai [65]	72	EC	T	29.2 m (1.7–79.7 m); 42.1 m (2.8–94.2 m)	N/A	N/A	0	2	2
Iyengar [52]	475	EC	C (n = 301); A (n = 157); none (n = 17)	7 y (IQR: 4.7–9.7)	Transthoracic	A (n = 6); C (n = 12)	N/A	N/A	N/A
Kaulitz [66]	142	TCPA	A (n = 86); C (n = 11); none (n = 45). Long-t: C (n = 22).	91.1 ± 43.9 m	Transthoracic ^b	8	10	0	0
Kavarana	54	EC	C (1 y); A (after 1 y)	33.6 ± 24.0 m	Transesoph	1	(3.6%)	1	0
Khairy [67]	101	EC (n = 3), LT (n = 98)	N/A	146 m	N/A	N/A	N/A	N/A	76
Kim [68]	200	EC	A (n = 170); C (n = 17)	52 m	N/A	13	13	6	7
Lemler [69]	96	EC	A	N/A	Transthoracic	2	N/A	4	0
McCrindle [39]	111	EC (n = 95), LT (n = 16)	C (n = 54) & ASA (n = 57)	24 m	Both	25 (2.5 y)	90	3	3
Monagle [9]	111	N/A	H + C (n = 54); A (n = 57)	≤24 m	Both	12/13 (A/H + C)	0	1	N/A
Nakano [70]	126	EC	C (n = 124)	60 m	N/A	0	1	1	6
Odegard [34]	37	N/A	A (n = 37)	96 m	Both	N/A	N/A	4	1
Petrosian [71]	285	EC	N/A	44.4 m	Transthoracic	9	N/A	3	8
Pihkala [72]	152	N/A	H (n = 152)	54 m	Transthoracic	N/A	N/A	0	0
Pizarro [71]	107	LT	N/A	64.8 m	N/A	3	N/A	5	N/A
Robbers-Visser [73]	209	EC (n = 107), LT (n = 102)	Yes (unspecified)	51.6 m (IQR: 18.0–88.8 m)	N/A	3	13	9	18
Sathanandam [74]	52	EC	A + C (n = 52)	79–87 m	Transthoracic	7	2	0	1
Stamm [75]	220	LT	No policy; C (n = 27); A (n = 118)	122.4 m	N/A	6	6	12	7
Wilson [53]	301	LT	Long-t follow-up: C (n = 108); A (n = 89); L (n = 2); combination (n = 4) Others mixed or unknown.	18.6 y (IQR: 15.1–21.1)	N/A	45		13	21

(continued on next page)

Table 3 (continued)

Study design			Outcome incidence						
Author & year	n	Procedure	Prophylaxis	Follow-up	Detection method	Thrombosis, n	Embolism, n	Early mortality, n	Late mortality, n
Woods [76]	58	EC	C (2 d postop)	23 m	N/A	4	0	3	0

Abbreviations: EC, extracardiac; LT, lateral tunnel; APA, atriopulmonary anastomosis; TCPA, total cavopulmonary anastomosis; ASA, Aspirin (Acetylsalicylic Acid); Med, median; L, Lovenox (subcutaneous); C, Coumadin/Warfarin; A, Aspirin; H, Heparin; Cap, Capitol; Cond, conduit; P, Phenprocoumon; T, Ticlopidine; Transesoph, transesophageal echocardiography; T, Ticlopidine; Transesoph, transesophageal echocardiography. Both: transthoracic and transesophageal echocardiography; PTFE, poly-tetrafluoroethylene; N/A, not available; postop, postoperative; preop, preoperative; m, months; d, days; y, years; Long-t, long term; life-t, life term; IQR, interquartile range. Thrombosis incidence following Fontan surgery (adapted from Firdouse et al. 2014).

^a Part of the sample only (not entire).

^b Subset of the sample also underwent the alternative diagnostic procedure. Note for “Diagnostic method” only transthoracic and transesophageal echocardiographies were evaluated.

3.4. Treatment of thrombosis

Treatment of thrombosis in Fontan patients includes embolectomy, thrombolytic treatment, and anticoagulant therapy. The choice of treatment is dependant on the location and size of the thrombi and other underlying potentiating factors.

As patients undergo follow-up as part of routine clinical care or for research purposes, the diagnosis of asymptomatic thrombosis may occur. The significance of asymptomatic thrombosis post-Fontan surgery is yet to be determined. No evidence links asymptomatic thrombosis with poorer outcomes in the Fontan population. Hence, no specific guidelines exist as to whether or not treatment is recommended when asymptomatic thrombi are discovered. Presently, the treating physician must determine best treatment options by taking into consideration the size, location, obstructive nature of the clot, as well as patient factors including age and Fontan function. Future studies should focus on developing guidelines through evidence-based practice.

3.4.1. Outcomes of thrombosis

There is very limited data pertaining to the management and outcome of thrombosis in the Fontan population. In general, management options include escalation of anticoagulant therapy, either by increasing the target therapeutic range of warfarin, or by adding a second agent (e.g. warfarin and aspirin). Acute therapies such as thrombectomy or thrombolysis tend to be used when the circuit is compromised in terms of reduced cardiac output. However, at this stage each case needs to be treated on individual grounds. Previous reports have ascertained that the total resolution of thrombosis occurs in only 48% of cases [85]. Moreover, thromboembolic events are associated with 25% mortality in pediatric patients [67,85] and 38% in adult patients [86], despite aggressive treatment of thrombi. Other outcomes of thrombosis include pulmonary embolism (which may lead to stroke or Fontan failure); collateral development (which may complicate future chest surgery); post-phlebotic syndrome; and thrombosis reoccurrence [87].

3.5. Thromboprophylaxis in the Fontan population

The current guidelines and consensus is that thromboprophylaxis post-Fontan surgery is appropriate, given the high risks of thrombosis and the significant morbidity and mortality associated with TE. The Scientific Statement from the American Heart Association (AHA) and American College of Chest Physician (ACCP) Guidelines both state that the current evidence base for the optimal type or duration of antithrombotic therapy is limited [27,88]. Current recommendations from AHA are:

1. The clinical assessment for anatomic and hemodynamic risk factors should be continually assessed. Risk factors for thrombus should ameliorated if possible (including arrhythmias, ventricular dysfunction & prolonged immobilization) whenever possible.
2. Long-term antiplatelet therapy is reasonable for prevention of thrombosis after Fontan procedure.
3. Initiation of antithrombotic therapy or increase in magnitude of therapy for prophylaxis is probably reasonable if anatomic or hemodynamic risk factors are present at any stage.
4. Long-term prophylactic antiplatelet therapy may be reasonable in infants and children after a superior cavopulmonary anastomosis.
5. Prophylaxis with warfarin or low molecular weight heparin may be reasonable in infants and children for 3–12 months after Fontan procedure.
6. Long-term therapy with warfarin may be reasonable after Fontan procedure for patients with anatomic or hemodynamic risk factors.
7. Initiation of antithrombotic therapy or an increase in the magnitude of antithrombotic therapy for prophylaxis after the Fontan procedure may be reasonable in adolescence or adulthood.
8. If a patient has an atrial arrhythmia (i.e. atrial fibrillation and

Table 4

Comparison of considerations when prescribing warfarin, aspirin and low molecular weight heparin.

	Warfarin	Aspirin	Low molecular weight heparin
Mechanism of action	● Vitamin K inhibitor that prevents production of vitamin K dependant clotting factors	● Inhibits platelet aggregation	● Binds to antithrombin for inhibitory effect on coagulation protein activity (mainly Factor Xa)
Considerations	● Affected by patient related factors (e.g. medications, diet) ● Increased risk of bleeding ● Can be reversed by giving vitamin K (important if emergency surgery required) ● Quality of Life may be decreased ● Potential reduction in bone density	● Increased risk of bleeding ● Unable to be reversed ● Aspirin resistance	● Increased risk of bleeding ● Known reduction in bone density ● Not reversible ● No outcome data to substantiate efficacy in Fontan
Monitoring Requirements	● Regular venous monitoring with INR (narrow therapeutic range)	● Not monitored	● Regular venous monitoring with anti-fXa activity
Convenience	● Oral tablet ● Regular monitoring required	● Oral tablet	● Daily or twice daily injections – more invasive administration ● Regular monitoring required

flutter) AHA recommends use of Warfarin INR target 2.0–3.0 for anticoagulation.

The recommendation from the CHEST Guidelines is that children after Fontan surgery should have aspirin (1–5 mg/kg/d) or therapeutic UFH followed by VKAs to achieve a target INR of 2.5 (range 2.0–3.0). The optimal duration of therapy is not currently known.

As per the recommendations in the guidelines, the most frequently prescribed, long-term antithrombotic agents in this population are aspirin and warfarin. These two agents have been shown in a meta-analysis to significantly lower the incidence of TE following Fontan [89], with the overall TE incidence of 18.6% in Fontan patients with no prophylaxis compared to 8.6–9% in patients taking aspirin or warfarin. Low molecular weight heparin has been suggested by the AHA guidelines, although there is no current outcome data to substantiate its use in Fontan patients. As outlined in Table 4, consideration must be made for differences in mode of action, drug interactions and monitoring requirement/availability, when determining drug suitability for individuals.

With regards to the use of more modern antithrombotic medications, such as New Oral Anticoagulant, there is currently insufficient safety and efficacy data for use post-Fontan surgery. These medications are not currently being used for daily practice in the pediatric Fontan population.

3.5.1. Aspirin

Aspirin is an oral, antiplatelet drug that irreversibly inhibits platelets by inhibiting cyclooxygenase-1 and cyclooxygenase-2 activity [90]. Cyclooxygenases are key enzymes in thromboxane A2 generation, which in turn potentiates platelet activation and aggregation. Aspirin acts by inhibiting the generation of thromboxane A2 and hence inhibits platelet activation and aggregation [90]. In institutions where aspirin is the preference for long-term antithrombotic therapy, patients are typically started on warfarin for 3–6 months and then transitioned to aspirin [23,52].

For thromboprophylaxis, a low dose of 1–5 mg/kg per day up to a maximum 150 mg per day is commonly prescribed [27]. As the aspirin effect on platelets is irreversible, aspirin should be discontinued 10 days before surgery to allow full regeneration of platelets. Monitoring of aspirin is not typically performed, and there are no studies measuring the effect of the drug in the Fontan population. While aspirin resistance is often cited as a concern, and hence a rationale for considering monitoring, there is very little evidence that true aspirin resistance frequently occurs in children [91]. In fact, no pediatric studies examine the relationship between laboratory and clinical aspirin resistance.

3.5.2. Warfarin

Warfarin is an oral anticoagulant that acts by specifically inhibiting

the γ -carboxylation of vitamin K dependent coagulation factors II, VII, IX, X, protein C, S and Z. For Fontan patients, a loading dose of 0.1 mg/kg per day is recommended followed by an individualized maintenance dose titrated to an INR range of 2–3. INR monitoring should be performed daily until therapeutic. The frequency of testing can be reduced to a minimum of once a month and should occur more frequently with illness, medication or diet change [27].

All thromboprophylactic agents increase the risk of bleeding; however, patients receiving warfarin are at a greater risk of bleeding than those receiving aspirin. The Australian and New Zealand Fontan bleeding incidence was 3%, with the majority of events (91%) associated with warfarin use and 36% of events occurring when the INR was > 3 [52]. Nonetheless, the safe management of warfarin in children is achievable. Australian data suggest that pediatric-specific anticoagulation clinics can reduce the risk of major bleeding to just 0.05% per patient-year [92].

In addition to bleeding risk, there is a growing concern that the long-term use of warfarin is associated with low bone mineral density and increased fracture risk [93]. As a vitamin K antagonist, warfarin reduces the carboxylation of vitamin K-dependent proteins including osteocalcin, an important protein involved in bone mineralization. Undercarboxylated osteocalcin is an independent predictor of osteoporosis and hip fracture in elderly women [94]. Suboptimal bone mineral acquisition due to the long-term use of warfarin in children is of particular concern as the majority of bone mineral acquisition occurs during childhood and adolescence. Inadequate bone mass accumulation may have implications for increased fracture risk and osteoporosis in later life. It is important to monitor bone mineral density longitudinally in pediatric patients receiving long-term warfarin [93]. A number of interventions are available to improve bone mineral density should suboptimal bone-density be discovered; these include increasing weight-bearing exercise, vitamin D and calcium supplementation and bisphosphonate therapy [93].

Furthermore, good dental hygiene is of particular importance in cardiac patients as poor dental hygiene is known to increase the risk of endocarditis and its associated sequelae, in at-risk patients. Dental issues are of increased concern in patients receiving warfarin as cessation of warfarin may be indicated in patients requiring a dental procedure that may cause bleeding. Any period below therapeutic INR range will confer an increased risk of thrombosis. The requirement for warfarin cessation needs to be carefully considered taking into account the perceived risk and degree of bleeding associated with a dental procedure as well as the individual's perceived risk if warfarin is discontinued. For minor dental procedures such as single and multiple tooth extractions and root canal procedures, the ACCP guidelines recommend the continuation warfarin therapy along with an oral prohemostatic agent or stopping warfarin two to three days before the procedure [95].

3.5.3. Other therapeutic considerations for thromboprophylaxis

When considering the administration of aspirin or warfarin drugs many patient-related factors must be considered, these include perceived thrombotic risk, therapy expense and burden of therapy. Consideration of patient-related factors is essential to determine if the benefits outweigh the potential risks of therapy. Additionally, during high-risk periods, such as the year following surgery or if a patient's condition deteriorates (i.e., ventricular dysfunction, arrhythmia, prolonged immobilization, protein-losing enteropathy, previous thrombosis) the patient may require more aggressive thromboprophylaxis.

The debate continues as to which thromboprophylactic regime is superior. Monagle et al. have conducted the only prospective, multicenter, randomized trial comparing aspirin to warfarin in a population of 111 Fontan patients [9]. Initial results demonstrated no difference in thrombotic outcomes between the aspirin and warfarin groups [9]. However, a secondary, *post-hoc* analysis of the same data revealed that poorly controlled warfarin therapy (< 30% of INR within target range) was associated with 3.5-fold increase in the risk of thrombosis compared with patients with well-controlled warfarin or those on aspirin [39]. Although this study was unable to conclusively determine the superiority of aspirin or warfarin at preventing thrombosis in Fontan patients, it has highlighted the importance of achieving minimum therapeutic targets ($\geq 30\%$ of INR measurements) when using warfarin for thromboprophylaxis. Furthermore, conclusions drawn from the aforementioned studies are limited to the two years post-Fontan surgery, and longer-term outcomes were not assessed.

A recent retrospective review of 475 patients with the ECC modification from the Australian and New Zealand Fontan registry found no difference between the use of aspirin and warfarin regarding the rate of late TE events (following one-year post-surgery) [52]. Similarly, a meta-analysis determined that there was no difference in TE events in the early (within first six months postoperatively) and late (beyond six months postoperatively) period post-surgery between Fontan patients on aspirin and warfarin [89]. Given that the risk of TE is highest with the first few months post-Fontan surgery, consideration to an earlier transition to aspirin may be a potential area for further investigation.

One argument for the use of warfarin as thromboprophylaxis following Fontan-surgery is the concern over the potential development of microvascular thrombi in the lungs. It is postulated that over the long-term, the accumulation of microvascular thrombi may lead to increased pulmonary pressure and eventual Fontan failure. Evidence to support the use of warfarin in preventing pulmonary emboli comes from a study by Varma et al. [96]. This study determined that 5 out of the 30 Fontan patients enrolled had asymptomatic PE; however, none of the patients receiving warfarin had asymptomatic PE. Due to the limited sample size, conclusions specific to anticoagulation therapy and long-term Fontan functional outcomes cannot be determined. Long-term, prospective data may facilitate answering such questions.

Quality of life (QoL) is an important measure when considering antithrombotic therapy. Even if there is a perceived benefit to thrombotic outcomes, the degree of this benefit must be weighed up against the effect on the QoL in the patient. The burden of warfarin treatment compared to aspirin is unquestionable. In addition to increased bleeding risk and association with reduced bone density, warfarin therapy requires rigorous monitoring and regular dosing adjustments for the duration of therapy to achieve safe and effective levels of the drug, particularly in pediatric patients [97]. There have been no studies specifically comparing the effect aspirin and warfarin on QoL in a pediatric congenital cardiovascular disease population. However, studies have been conducted in pediatric cohorts receiving warfarin and its effect on QoL. Jones et al. compared the commencement of in-home, point of care INR monitoring, to traditional anticoagulation clinic INR monitoring [98]. The study found that parents reported a significant improvement in their child's QoL, their own QoL and their families' functioning following commencement of in-home INR monitoring. This study suggests that if warfarin therapy is indicated, improvements to

QoL can be achieved by instituting support services such as home INR monitoring, where practical. The importance of using QoL to complement safety and efficacy outcomes to sensitively measure the impact of antithrombotic therapy in populations receiving anticoagulant therapy is also highlighted in the study by Jones et al. [98].

Lastly, regarding costs, a recent analysis in an Australian context estimated that warfarin therapy in comparison to aspirin has additional costs of AUS\$825 per annum, the majority which is borne by the patient or family [99]. The additional costs included the cost of consultations, medications, monitoring, patient time and did not include the costs of adverse events. The study proposes that a trial that assesses the difference between anticoagulation would save AUS\$5.8 million over 15 years and result in a much-needed clinical consensus regarding thromboprophylaxis in Fontan patients.

Currently, available thromboprophylactic options for Fontan patients are less than ideal due to their suboptimal ability to reduce thrombosis, side effects and the ongoing burden of therapy. Novel anticoagulants with reduced bleeding rates and without the requirement of monitoring appear promising; however, their utility in children is yet to be determined. Furthermore, recent data have suggested that some novel anticoagulants do not provide adequate anticoagulation in the presence of prosthetic materials such as prosthetic valves [100]. Since the Fontan circulation fundamentally requires the presence of prosthetic material (i.e., Goretex conduit, patches), it is possible that some novel anticoagulants may not be appropriate in this population.

4. Conclusions

Thrombosis is a significant complication following the Fontan procedure and remains an important cause of morbidity and mortality in both the short and long-term following surgery. Many questions remain regarding the development of thrombosis, its prevention and treatment in this population. There is a clear need for long-term studies with a focus on determining the factors potentiating the pathogenesis of thrombosis, the utility of specific thromboprophylactic drugs, treatment of thrombosis and long-term outcomes of both symptomatic and asymptomatic thrombosis.

Conflicts of interest

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1.3 The Fontan procedure

For babies born with the most severe congenital heart defects, surgical intervention may not achieve biventricular circulation. In these patients a series of “palliative” surgeries are performed, with the final surgery being the Fontan procedure. Once completed, Fontan circulation directly connects systemic venous return to the pulmonary circulation, bypassing the absent or inadequately formed structures of the heart. **In Fontan patients, deoxygenated blood enters the lungs passively via two mechanisms:**

1. The increased pressure in the veins due to high systemic pressure.
2. As the patient breathes in, the size of the chest is increased and consequently blood is drawn into the lungs. Subsequently, as the patient breathes out, the opposite occurs (Figure 1).

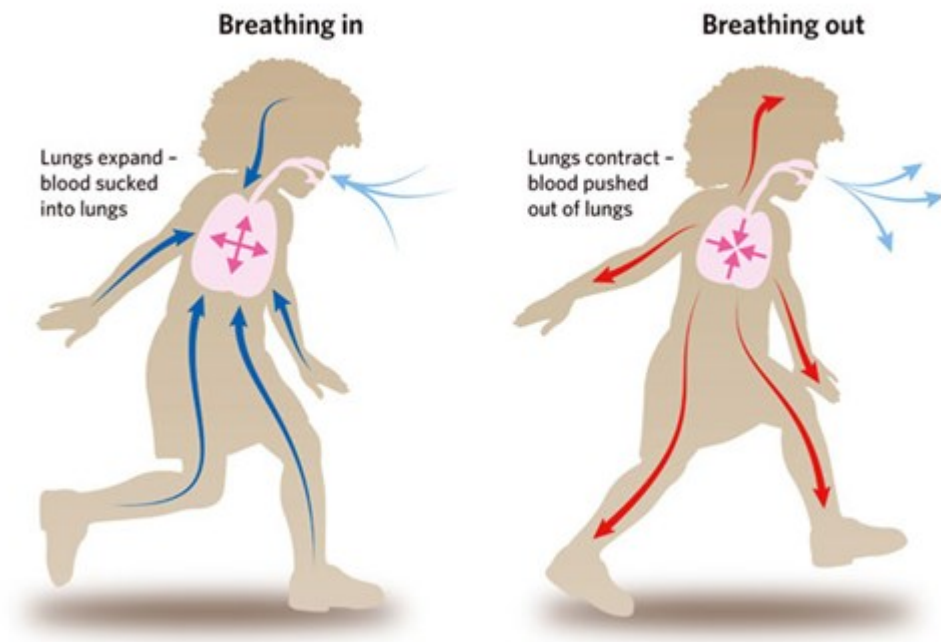


Figure 1. Passive circulation of blood in the Fontan patient (adapted from The ANZ Fontan registry website)²

The Fontan procedure was initially developed by Fontan and Baudet ¹ as a treatment for tricuspid atresia. Since its original conception, the Fontan procedure has been used to treat an array of congenital heart conditions (Table 1). The Fontan procedure has since evolved and has refined into **three main variations of the technique** (Figure 2): *The classical - “Atriopulmonary” (AP) connection; Lateral Tunnel (LT) and the contemporary Extracardiac conduit (ECC).*

The AP connection was the initial Fontan variant, where the right atrial chamber was isolated by the closure of the atrial septal defect and the hypoplastic tricuspid valve (Figure 2a). The right atrial appendage was then anastomosed to the right pulmonary artery. Although the AP connection proved to be

successful at bypassing the absent ventricle and restoring systemic flow, the right atrium was susceptible to dilation, and subsequently venous obstruction, thrombus formation and atrial arrhythmias. It was later understood that superior streaming of the blood flow in the systemic venous pathway to the lungs improved hemodynamics and avoided potential complications related to progressive atrial dilatation. This realisation led to the development of the LT technique (Figure 2b), where a Goretex patch is used to baffle the right atrium, therefore reducing the diameter and improving flow through the atrium. In addition, the superior vena cava is anastomosed directly to the right pulmonary artery. As an added benefit, this stage of the procedure was able to be performed at an earlier age (known as the Bidirectional Glenn procedure), allowing for expedited relief of volume loading of the ventricle.

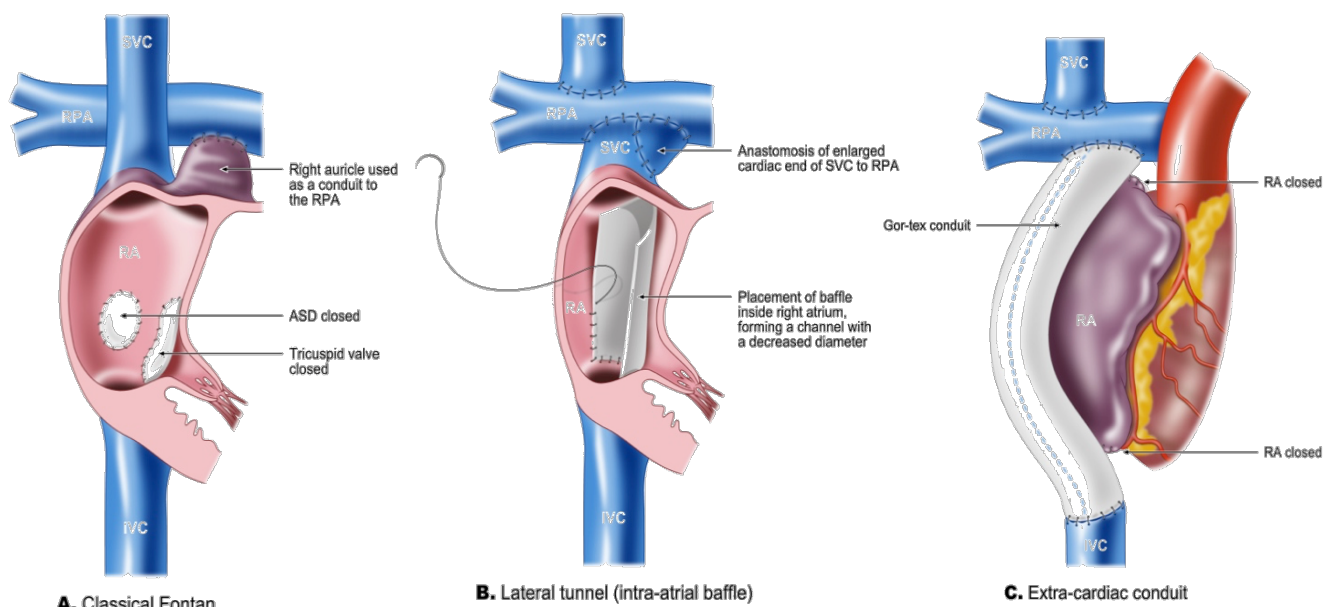


Figure 2. Evolution of the Fontan procedure. (A) Atriopulmonary connection, (B) Lateral tunnel, (C) Extracardiac conduit techniques (Adapted from d'Udekem et al, Circulation 2007) ³

The most recent modification of the Fontan technique consists of the replacement of the intra-atrial routing of the venous blood by the insertion of an ECC between the inferior vena cava and the right pulmonary artery (Figure 2c).

Across Australia and New Zealand, the AP and LT procedures have been superseded by the contemporary ECC procedure. The ECC procedure utilises a Goretex tube to bypass the right atrium completely (Figure 3), and is thought to improve venous flow and haemodynamics ⁴.

Both the LT and ECC techniques allow for the addition of a fenestration or 'hole' in the baffle. This creates a communication between the systemic venous conduit and pulmonary venous circulation. The fenestration essentially acts as a right-to-left shunt and reduces elevated pulmonary vascular resistance, such as seen in the early post-operative period. Fontan fenestration has been credited with reducing post-operative morbidity ⁵. However, these potential benefits have been accompanied by the theoretical risk of developing paradoxical emboli ^{6, 7}.

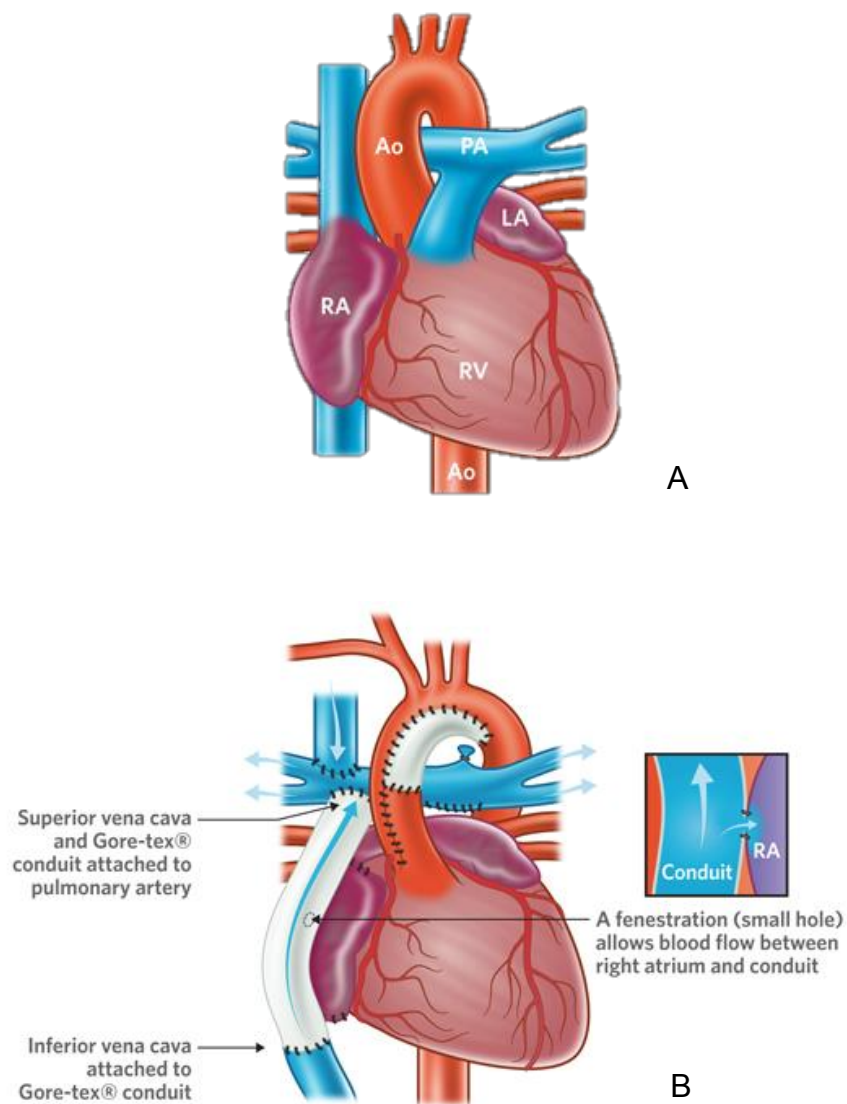


Figure 3. Normal heart (a) compared to a heart that has undergone the extracardiac Fontan procedure (b) (adapted from The Royal Children's Hospital website) ⁸.

1.3.1 The Australia and New Zealand Fontan Population

The Murdoch Children's Research Institute (MCRI) lead, National Health and Medical Research Council (NHMRC) funded, Australia and New Zealand (ANZ) Fontan Registry has identified approximately 1500 survivors of the Fontan operation that currently live in ANZ ^{9, 10}. Fifty Fontan operations are performed in this region every year, with the 20-year survival of Fontan patients expected to be over 90%, making this population one of the fastest growing group of patients carrying such a severe burden of disease ^{3, 11}.

1.3.2 Complications post Fontan surgery

Following Fontan surgery, approximately half of patients will experience an adverse event as a result of undergoing the Fontan procedure ^{12, 13}. Clinical complications may include: increased pressure causing liver cirrhosis, liver failure, and portal hypertension; increased risk for liver carcinoma; ventricular failure causing exercise intolerance, ischemia, and infarction; protein-losing enteropathy; plastic bronchitis; pericardial and pleural effusions; chylothorax; thrombosis and bleeding.

1.4 Thrombosis post Fontan surgery

Thrombosis is a significant complication after the Fontan procedure and remains an important cause of morbidity and mortality in both the short and long-term following surgery. Specifically, the prevalence of thrombosis up to 33% after the Fontan procedure has been reported ¹⁴. Thrombosis development in Fontan patients is not surprising considering that the Fontan circulation is the ideal substrate for thrombus formation. **The three factors of Virchow's triad**, namely the presence of surgically manipulated endothelium or prosthetic material, low flow due to the absence of a functional right ventricle and intrinsic plasma protein changes, probably related to subtle changes in liver synthetic function and certainly accentuated in patients with protein losing enteropathy, **are present to some extent in all Fontan patients.**

Table 1 Elements of Virchow's triad contributing to thrombosis formation in Fontan patients

	Thrombogenic contributors in the Fontan patient	Impact on coagulation
Endothelial dysfunction	• Central venous line use • Cardiopulmonary bypass • Thrombogenic foreign material (the Fontan conduit) • Surgical sites • Inherent endothelial dysfunction	• Injury to vessel wall • Exposure of blood to large artificial surfaces • Activation of the coagulation system
Abnormal blood flow	• Cardiopulmonary bypass • Surgery type: the Atriopulmonary procedure involves turbulent blood flow from a large right atrium that delivers blood directly to the pulmonary artery • Ventricular dysfunction • Arrhythmias • Flow dead ends – e.g. pulmonary artery stump	• Abnormal flow rates and shear stress • Turbulent blood flow • Activation of the coagulation system

	• Lower cardiac output • Higher venous pressures • Nonpulsatile systemic venous flow • Chamber dilatation (e.g. Dilated atrium) • Conduit stenosis • Right to left intracardiac communication (e.g. fenestration)	
Hypercoagulability	• Cardiopulmonary bypass • Thrombophilia factors • Liver dysfunction • Pre- and post-fontan coagulation factor abnormalities • Protein losing enteropathy (loss of clotting factors to gut) • Increased platelet reactivity	• Hypercoagulable state

Thrombosis commonly presents in Fontan patients as intracardiac or intravascular thrombosis, ischaemic stroke, or other embolic phenomena ¹⁵.

Thromboembolic events in the Fontan patient can be categorised in two forms:

- (1) Thromboemboli occurring via thrombosis forming in systemic venous system, such as the Fontan conduit itself. Thrombosis in this locale may cause local obstruction, which can extend or embolize into the pulmonary arteries and may lead to Fontan failure. Furthermore, thrombosis in the systemic venous system may also embolize to the arterial circulation through fenestrations or right-to-left-shunts ⁷.
- (2) Thrombus occurring on the left of the atrial baffle may embolize to the arterial system and may travel to the central nervous system and result in infarction or stroke ¹⁶.

Despite the fact that thrombosis is a common and significant problem following Fontan surgery, optimal thromboprophylactic strategies, as well as outcome data after thrombosis are unknown.

1.4.1 Epidemiology of thrombosis post Fontan surgery

Although thrombosis post Fontan surgery is thought to be highly prevalent, **the true incidence of thrombosis is unknown** due to the lack of prospective studies within this population ¹⁵⁻¹⁷. Cohort studies have reported incidences of venous thrombosis ranging from 4% to 19% ¹⁸⁻²² and the incidence of stroke

ranging from 3% to 19% ^{19, 21, 23-25}. Cross-sectional studies using transoesophageal echocardiography (TOE) have reported a prevalence of intracardiac thrombosis of 17% to 33% ^{14, 26, 27}. The most commonly observed sites for intracardiac thrombi are the systemic venous atrium (48%) and pulmonary venous chamber (44%) ²⁸. Discrepancies in the reported incidence between studies may be explained by the varied duration of the follow up (e.g. semi-annually verses bi-annually), as well as the imaging techniques utilised to diagnosis thrombosis (echocardiogram versus magnetic resonance imaging). Monagle et al performed a prospective, multicentered, randomised controlled trial assessing thromboprophylaxis post-Fontan surgery ¹⁵. In that study, all observed thrombotic events were venous with no arterial thrombosis detected. An overall thrombosis rate of 22.5% was observed in these patients within the first two years post-Fontan surgery. Furthermore, an intracardiac thrombosis rate of 19% was detected with 80% of all patients presenting with thromboses present within the Fontan circuit. Interestingly, Monagle et al also determined that the symptomatic thrombosis rate was only 7%, suggesting that asymptomatic thrombosis is highly prevalent within this population ¹⁵. Whether silent thrombosis is clinically important is unknown at present and can only be determined by future research. In a *post-hoc* analysis of the aforementioned study, McCrindle et al identified that at 2.5 years post randomisation, there was a time-related freedom from thrombosis of 69% ²⁹. Time related risk of thrombosis was highest within the immediate post-operative period up until six months post-surgery, followed by a lower yet steadily increasing risk of thrombosis in the subsequent two years. These results are supported by other studies that also observed this pattern of immediate high risk of thrombosis in

the initial one-year period post-Fontan^{25, 26, 30-32}, with thrombosis risk plateauing at 3.5 years and thereafter followed by a second peak 10 years post-surgery¹⁹.

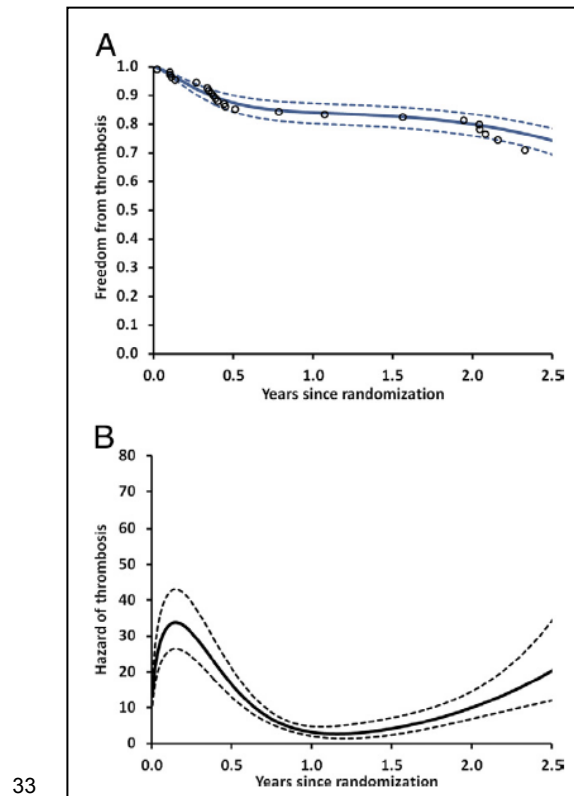


Figure 4 Freedom from thrombosis over time, stratified by thromboprophylaxis choice (adapted from McCrindle et al 2013). All patients included from study randomization up to first thrombotic event, study withdrawal, or end of study. Controlled warfarin was defined as >30% of international normalized ratio (INR) within target over the study period. Patients on warfarin who consistently achieved target INR levels >30% of the time or those on aspirin had a 3.5-fold decrease in risk of thrombosis compared patients who often failed to meet target INR level, and difference in risk between controlled warfarin and aspirin was not statistically significant.

1.4.2 Pathophysiology of thrombosis post-Fontan surgery

Children undergoing cardiac surgery are an especially high-risk group for thrombosis compared to other paediatric surgical populations. In a retrospective study by Manlhiot et al ³⁴ an incidence of 7.9% symptomatic venous thromboembolism (VTE) was observed in children post cardiac-surgery, as compared to only 0.34-0.58% VTE previously reported by Raffini et al ³⁵ for all paediatric admissions over a five year period. Risk of thrombosis in children post-cardiac surgery is highest in the immediate post-operative period ²⁹. Many factors are likely to contribute to the immediate increased thrombosis risk for these patients, including the use of central venous lines (CVLs), cardio-pulmonary bypass (CPB) and the surgery itself.

Use of CVLs is one of the most important risk factors for thrombosis in paediatric patients, with 20% of patients with a CVL developing line related thrombi ³⁶. Manlhiot et al noted that CVLs were present in 16% of the thrombosed vessels, and that the majority of thrombi were occlusive in nature (66%) and symptomatic (60%) ³⁴. In children, line related thrombi are of particular significance as related consequences include line failure and requirement for repeated CVL replacement, treatment with anticoagulation or thrombolytic therapy, pulmonary embolism and post thrombotic syndrome ³⁷.

Exposure of blood to large synthetic surfaces during CPB leads to disturbances in the haemostatic system that include: the activation of platelets, activation of coagulation factors, activation of the fibrinolytic system and a decrease in

natural anticoagulants. These changes occur primarily through haemodilution and consumption of coagulation factors ³⁸.

As previously discussed, factors that contribute to thrombosis specifically in Fontan circuits include disturbances of vascular endothelium, blood flow and coagulation systems. Important contributors of thrombosis risk the Fontan patients are listed in Table 1. Thrombus formation within the Fontan circuit may cause local obstruction of flow that can extend or embolise to the pulmonary arteries ³⁹⁻⁴³. In addition, embolisation from the systemic venous pathway to the systemic arterial circulation may also occur through right to left shunting via incidental or surgically created fenestrations, and possibly lead to emboli in the central nervous system resulting in infarction or stroke ⁷.

The vascular endothelium is vital in the regulation of pulmonary vascular tone, vascular smooth muscle cells and the control of coagulation, fibrinolysis and inflammation. Endothelial injury caused by cardiac surgery causes the vascular endothelium to become activated ⁴⁴. Endothelial dysfunction occurs as a result of endothelial injury immediately post-surgery, and is characterised by the loss of vasodilators, such as nitric oxide, and anti-thrombotic factors such as antithrombin, Protein C and S, as well as an increase in vasoconstrictor and pro-thrombotic products ⁴⁵. The contribution of factors involved in endothelial dysfunction leads to a pro-thrombotic state ⁴⁶. Endothelial dysfunction has been observed even prior to Fontan surgery in patients with single ventricular physiology ⁴⁷. It is hypothesised that this inherent endothelial dysfunction occurs in the presence of chronic hypoxia as is frequently observed in these

patients. Interestingly, in some patients, endothelial dysfunction persists post-operatively despite the absence of hypoxemia ⁴⁶.

Other physiological changes post Fontan surgery include reduction of blood flow, stasis or turbulent blood flow. These disturbances to the blood flow lead to localized activation of the coagulation system, leading to the accumulation of circulating platelets and coagulation factors all of which are conducive to thrombus formation ⁴⁸. Fontan encompasses a broad range of procedures that may all have different impact on blood flow. Common to all variations of the Fontan is that pulmonary artery flow is driven by venous pressure and so a low flow state ensues. **A reduced flow rate propagates initiation of the coagulation system, increasing the likelihood of thrombi development.**

Other factors that may impact on vascular flow in Fontan patients include flow dead ends such as the pulmonary artery stump ⁴⁹, low cardiac output and higher venous pressures. Over the long term, patients may develop serious complications such as chamber dilatation (particularly observed in the superseded AP connection Fontan) and conduit stenosis, both of which have serious implications for the patient including greatly increased risk of thrombosis as well as Fontan failure ^{50, 51}.

Presence of a right-to-left shunt, such as that present in the fenestrated Fontan, also carries a theoretically increased risk of stroke. This perceived risk is attributed to paradoxical embolism, that is, the embolization of a thrombus generated in the venous system travelling to the left heart through a right-to-left

communication and then to the brain. However, no study has yet proven that fenestrated Fontan patients are at a greater risk of stroke compared to non-fenestrated patients.

Inherent abnormalities in coagulation have been reported both pre and post Fontan surgery ⁵²⁻⁵⁸. However, few studies have utilised age-appropriate reference ranges for the coagulation tests reported, hence it is unknown if differences in the coagulation protein levels observed are simply due to normal developmental variations. Odegard et al, described a prospective, case-controlled study of 20 post-Fontan patients which included healthy age-matched controls, to evaluate coagulation factor abnormalities ⁵⁷. Decreased levels of the naturally occurring anticoagulants protein C, plasminogen and antithrombin as well as coagulation factors II, V, VII and X were demonstrated in Fontan patients as compared to controls. Moreover, FVIII was found to be elevated in 6 patients (35%), two of whom had had a thromboembolic event and protein-losing enteropathy. The authors speculated that increased levels of factor VIII may be produced by the liver in response to elevated hepatic and central venous pressure, in Fontan patients. However, this study did not demonstrate a correlation was found between hepatic synthetic dysfunction or hepatocellular dysfunction and coagulation abnormalities.

Coagulation abnormalities leading to thrombotic tendency may also be a result of protein-losing enteropathy which is an enigmatic condition that results in the loss of serum proteins into the gastrointestinal tract. This condition may present in the weeks or even years following the Fontan procedure, and is associated

with increased morbidity and mortality ^{28, 59, 60}. The substantial loss of serum proteins can have far reaching clinical implications, one of which includes the impairment of the clotting cascade via the loss of clotting factors to the gut creating a haemostatic imbalance ²⁸.

Another postulated contributor to increased thrombotic risk in this population is increased platelet reactivity. Studies have shown that platelet reactivity in Fontan patients is increased both in the short and long term prior and post-surgery, when compared to healthy controls ⁶¹. This is not surprising, as platelets become activated during turbulent blood flow and in the presence of endothelial dysfunction. Increased platelet reactivity is still observed in patients receiving aspirin as thromboprophylaxis, despite the antiplatelet properties of this drug ⁶¹. Despite evidence suggesting that platelets play a major role in the increased thrombotic risk observed in Fontan patients, there have not been any investigations of the direct effect of platelets on the overall thrombotic potential in this population. This is of particular importance as many drugs prescribed to Fontan patients target platelets as the main mechanism for thromboprophylaxis. Therefore, efficacy studies should be undertaken to assess the impact of such drugs on thrombotic potential.

In summary, Fontan patients clearly have an elevated risk of thrombosis due to the multitude of factors that lead to endothelial dysfunction, the disturbance of blood flow and coagulopathy. Identification of major aetiological factors in individual patients remains to be determined.

1.4.3 Diagnosis of thrombosis post-Fontan

Transthoracic echocardiography (TTE) is the most commonly used imaging technique for patient follow up post the Fontan procedure. Benefits of TTE include low cost, widespread availability, the easy-to-use and the non-invasive nature of the test. Improvements to temporal-spatial resolution of TTE have reduced the need for diagnostic cardiac catheterization and angiography in many patients. Despite advancements in TTE technology, intracardiac thrombus is often poorly detected in the Fontan patient due to the presence of prosthetic material and the distance between the chest wall and pulmonary arteries and veins. Moreover, in the post-surgical patients' thrombi detection by TTE is further inhibited by chest tubes, edema and dressings.

Numerous studies have demonstrated the superiority of TOE over TTE at detecting thrombi in Fontan patients^{14, 26, 27}. However, due to the invasive nature of TOE, sedation for most patients is generally recommended. In a study by Balling et al ¹⁴ in post-Fontan patients, TOE was able to detect intra-cardiac thrombi in 33% of the patients screened as compared to just 2% when using TTE in the same patients. TOE imaging sensitivity can be attributed to the close proximity of the probe to cardiac structures, facilitating accurate and effective detection of thrombi. Similar findings were obtained by Stumper et al ²⁷ and Fyfe et al ²⁶ where TTE failed to detect thrombi in two of three and five of six patients respectively.

Of late, magnetic resonance imaging (MRI) has emerged as a valuable, non-invasive cardiovascular diagnostic tool. MRI has demonstrated accurate assessments of complex anatomy including extracardiac structures. In addition

to excellent imagery, cardiac MRI usually occurs without sedation in most patients and unlike computed tomography, exposure to ionizing radiation does not occur.

1.4.4 Treatment options for thrombosis in the Fontan population

Treatment of thrombosis in Fontan patients includes embolectomy, thrombolytic treatment and anticoagulant therapy. The choice of treatment utilised is entirely dependent on the location and size of the thrombi, as well as other underlying potentiating factors.

As patients undergo follow up as part of routine clinical care (or for research purposes) the diagnosis of asymptomatic thrombosis may occur. The significance of asymptomatic thrombosis post-Fontan surgery is yet to be determined. **There is no evidence that links asymptomatic thrombosis with outcomes in the Fontan population and as such no specific guidelines exists as to whether or not treatment is recommended when asymptomatic thrombi are discovered.** Presently, the treating physician must determine best treatment options by taking into consideration the size, location, obstructive nature of the clot, as well as patient factors such as age and Fontan functioning. This subjective approach is clearly suboptimal. Ideally standardised treatment options should be determined based on robust scientific evidence, however as this evidence does not currently exist, future studies should focus on developing guidelines through evidence-based practise.

1.4.5 Outcomes of thrombosis in the Fontan population

There is very limited data pertaining to the management and outcome of thrombosis in the Fontan population. Previous reports have ascertained that total resolution of thrombosis occurs in only 48% of cases ⁶². Moreover, thromboembolic events are associated with 25% mortality in paediatric patients ⁶² and 38% in adult patients ⁶³, despite aggressive treatment of thrombi. Other outcomes of thrombosis in Fontan patients include⁶⁴:

- Pulmonary embolism – which may lead to stroke or Fontan failure
- Collateral development – which is of particular concern in cardiac patients as collateral development may complicate future chest surgery
- Post-phlebotic syndrome
- Thrombosis reoccurrence

As little data is available on outcomes of thrombosis specifically in the Fontan population, it is therefore valuable to consider thrombosis and stroke outcomes in the broader paediatric congenital heart disease (CHD) population.

The largest prospective data set of paediatric venous TE is derived from the Canadian Childhood Thrombophilia Registry, where seventy-five of the 405 children (19%) presenting with VTE had CHD as an underlying condition ⁶⁵. VTE in the CHD subgroup had an associated mortality of 7%, with morbidity including post-thrombotic syndrome and TE recurrence being 23% ⁶⁴. The Canadian Stroke Registry identified CHD as the most common identifiable cause of arterial ischaemic stroke representing 36% of all causes. Of all children presenting with stroke, only 22% of children were identified as making a full recovery, whereas 66% had residual neurological deficits and/or seizures at a mean follow up period of 2.1 years⁶⁶. These statistics highlight the

significance of paediatric stroke, where the devastating consequences can have a life-long impact.

1.5 Thromboprophylaxis in the Fontan population

Given the high risk of thrombosis following the Fontan procedure and the significant morbidity and mortality associated with thrombosis in this population, the prophylactic use of antithrombotic agents is warranted. Antiplatelet and anticoagulant agents are commonly prescribed for thromboprophylaxis in the Fontan population, however there is currently no consensus on which agents are optimal ⁶⁷. The most frequently prescribed, long-term antithrombotic agents in this population are aspirin and warfarin. Table 2 compares considerations of treatment with aspirin or warfarin that should be considered at least in the first instance.

Table 2 Comparison of considerations when prescribing warfarin and aspirin

Warfarin <i>Vitamin K inhibitor that prevents production of vitamin K dependant clotting factors</i>	Aspirin <i>Inhibits platelet aggregation</i>
○ Increased risk of bleeding	○ Increased risk of bleeding
○ Regular venous monitoring (Narrow therapeutic range)	○ Not monitored
○ Affected by patient related factors (e.g. medications, diet)	○ Aspirin resistance
○ Reversed by administering vitamin K (important if emergency surgery required)	○ Unable to be reversed
○ QoL may be decreased	
○ Potential reduction in bone density (increased risk of bone fractures)	

1.5.1 Aspirin

Aspirin is an oral, antiplatelet drug that irreversibly inhibits platelets by inhibiting cyclooxygenase-1 and cyclooxygenase-2 activity ⁶⁸. Cyclooxygenases are key enzymes in thromboxane A2 generation, which in turn potentiates platelet activation and aggregation. Aspirin acts by inhibiting generation of thromboxane A2, and hence inhibits platelet activation and aggregation ⁶⁸. For thromboprophylaxis, a low dose of 1-5 mg/kg per day up to a maximum 150 mg per day is commonly prescribed ⁶⁷. Monitoring of aspirin is not typically performed and there are no studies measuring the effect of this drug on platelets or the haemostatic system. While aspirin resistance is often cited as a concern, and hence a rationale for considering monitoring, there is very little evidence that true aspirin resistance occurs frequently in children ⁶⁹. In fact, there are no paediatric studies examining the relationship between laboratory and clinical aspirin resistance. As aspirin effect on platelets is irreversible, aspirin should be discontinued 10 days prior to surgery to allow full regeneration of platelets.

1.5.2 Warfarin

Warfarin is an oral anticoagulant that acts by specifically inhibiting the γ -carboxylation of vitamin K dependant coagulation factors II, VII, IX, X, protein C, protein S and protein Z. For Fontan patients, a loading dose of 0.1mg/kg per day is recommended followed by an individualised maintenance dose titrated to an international normalised ratio (INR) range of 2-3⁶⁷. INR monitoring should be performed daily until therapeutic. Frequency of testing can be reduced to a

minimum of once a month. However, INR testing should occur with illness, medication or diet change ⁶⁷.

All thromboprophylactic agents increase the risk of bleeding, however patients receiving warfarin are at a greater risk of bleeding than those receiving aspirin. Nonetheless safe management of warfarin in children is achievable. Australian data suggests that paediatric specific anticoagulation clinics can reduce the risk of major bleeding to just 0.05% per patient year ⁷⁰.

In addition to the bleeding risk, there is a growing concern that the long-term use of warfarin is associated with low bone mineral density and increased fracture risk ⁷¹. As a vitamin K antagonist, warfarin reduces the carboxylation of vitamin K–dependent proteins including osteocalcin, an important protein involved in bone mineralization. Undercarboxylated osteocalcin is an independent predictor of osteoporosis and hip fracture in elderly women ⁷². Suboptimal bone mineral acquisition due to the long-term use of warfarin in children is of particular concern as the majority of bone mineral acquisition occurs during childhood and adolescence. Inadequate bone mass accumulation may have implications for increased fracture risk and osteoporosis in later life. It is important to monitor bone mineral density longitudinally in paediatric patients receiving long-term warfarin ⁷¹. A number of interventions are available to improve bone mineral density should suboptimal bone-density be discovered; these include increasing weight bearing exercise, vitamin D and calcium supplementation and bisphosphonate therapy ⁷¹.

Furthermore, good dental hygiene is of particular importance in cardiac patients as poor dental hygiene is known to increase the risk of endocarditis and its

associated sequelae, in at-risk patients. Dental issues are of increased concern in patients receiving warfarin as cessation of warfarin may be indicated in patients requiring a dental procedure that may cause bleeding. Any period below therapeutic INR range will confer an increased risk of thrombosis. The requirement for warfarin cessation needs to be carefully considered considering the perceived risk and degree of bleeding associated with dental procedure as well as the individual's perceived risk if warfarin is discontinued. For minor dental procedures such as single and multiple tooth extractions and root canal procedures, the American College of Chest Physician (ACCP) guidelines recommend the continuation warfarin therapy, along with an oral prohaemostatic agent or cessation of warfarin two to three days prior to the procedure ⁷³.

1.5.3 Other therapeutic considerations for thromboprophylaxis

When considering the administration of aspirin or warfarin drugs many patient related factors such as perceived thrombotic risk, therapy expense and burden of therapy must be considered. This is essential in order to determine if the benefits outweigh the potential risks of therapy. Additionally, during high-risk periods, such as the year following surgery or if a patient's condition deteriorates (e.g. ventricular dysfunction, arrhythmia, prolonged immobilization, protein-losing enteropathy, previous thrombosis) the patient may require more aggressive thromboprophylaxis.

The debate continues as to which thromboprophylactic regime is superior for Fontan patients. As previously stated, Monagle et al has conducted the only prospective, multicentre, randomised trial comparing aspirin to warfarin in a population of 111 Fontan patients ¹⁵. Initial results demonstrated no difference in thrombotic outcomes between the aspirin and warfarin groups ¹⁵. However, a *post-hoc* analysis of the same data revealed that poorly controlled warfarin therapy (<30% of INR within target range) was associated with 3.5-fold increase in risk of thrombosis compared with patients with well controlled warfarin or those on aspirin ²⁹. Although this study was unable to conclusively determine superiority of aspirin or warfarin at preventing thrombosis in Fontan patients, it has highlighted the importance of achieving minimum therapeutic targets ($\geq 30\%$ of INR measurements >2) when using warfarin for thromboprophylaxis. Furthermore, conclusions drawn from the aforementioned studies are limited to the two years post Fontan surgery, and longer-term outcomes were not assessed.

One argument for the thromboprophylactic use of warfarin after Fontan-surgery is the concern over the potential development of microvascular thrombi in the lungs (microvascular pulmonary emboli (PE)). It is postulated that over the long-term, the accumulation of microvascular thrombi may lead to increased pulmonary pressure and eventual Fontan failure. Evidence to support the use of warfarin at preventing pulmonary emboli comes from a study by Varma et al ⁷⁴. This study determined that 5 out of the 30 Fontan patients enrolled had asymptomatic PE, however none of the patients receiving warfarin had asymptomatic PE. Due to the limited sample size, conclusions around

anticoagulation therapy and long-term Fontan functional outcomes cannot be determined. Long-term, prospective data, such as that coming from the ANZ Fontan registry may facilitate answering such questions. The ANZ Fontan registry is a recently established databank that prospectively collects clinical data (including thrombosis diagnosis) from over 1500 Fontan patients across Australian and New-Zealand (www.fontanregistry.com).

Quality of life (QoL) is an important measure when considering antithrombotic therapy. Even if there is a perceived benefit to thrombotic outcomes, the degree of this benefit must be weighed up against the effect on the QoL in the patient. The burden of warfarin treatment compared to aspirin is unquestionable. In addition to increased bleeding risk and association with reduced bone density, warfarin therapy requires rigorous monitoring and regular dosing adjustments for the duration of therapy to achieve safe and effective levels of the drug, particularly in paediatric patients ⁷⁵. There have been no studies specifically comparing the effect aspirin and warfarin on QoL in a paediatric congenital cardiovascular disease population. However, studies have been conducted in paediatric cohorts receiving warfarin and its effect on QoL. Jones et al compared the commencement of in-home, point of care INR monitoring, to traditional anticoagulation clinic INR monitoring ⁷⁶. The study found that parents reported a significant improvement in their child's QoL, their own QoL and their families' functioning following commencement of in-home INR monitoring. This study suggests that if warfarin therapy is indicated, improvements to QoL can be achieved by instituting support services such as home INR monitoring, where practical. This study also highlights the importance of using QoL to

complement safety and efficacy outcomes to sensitively measure the impact of antithrombotic therapy in populations receiving anticoagulant therapy.

In summary, currently available thromboprophylactic options for Fontan patients are less than ideal due to their suboptimal ability to reduce thrombosis, side effects and the ongoing burden of therapy. Novel anticoagulants with reduced bleeding rates and without the requirement of monitoring appear promising, however their utility in children is yet to be determined. Furthermore, recent data have suggested that some novel anticoagulants do not provide adequate anticoagulation in the presence of prosthetic materials such as prosthetic valves ⁷⁷. Hence, it is possible that the use of some novel anticoagulants may not be appropriate in Fontan patients given that the Fontan circulation fundamentally requires the presence of prosthetic material (i.e. goretex conduit, patches or baffles).

1.6 Conclusions

Thrombosis is a significant complication following the Fontan procedure and remains an important cause of morbidity and mortality in both the short and long-term following surgery. Many questions remain regarding the development of thrombosis, its prevention and treatment in this population. There is a clear need for long-term studies with a focus on determining the factors potentiating the pathogenesis of thrombosis, the utility of specific thromboprophylactic drugs, treatment of thrombosis and long-term outcomes of both symptomatic and asymptomatic thrombosis.

1.7 Research Questions

1. What is the incidence of stroke in the Fontan population?
2. Is aspirin or warfarin more effective at preventing stroke in the Fontan population?
3. Do Fontan patients receiving warfarin have an increased rate of bleeding compared those receiving aspirin?
4. Do Fontan patients receiving warfarin have reduced bone density as compared to those receiving aspirin?
5. Is QoL reduced in Fontan patients receiving warfarin as compared to those receiving aspirin?

1.8 Hypothesis

There are differences in clinical outcomes between Fontan patients who receive aspirin compared to those patients that receive warfarin in terms of stroke rate, bone mineral density, bleeding rates and QoL.

CHAPTER 2 Methods

2.1 Study purpose and scope

This was a cross-sectional, multicentred study of the ANZ Fontan population which aimed to:

- a)** Assess stroke, bone density, bleeding and QoL outcomes across the Fontan population.
- b)** Compare the outcomes between those participants receiving aspirin to those receiving warfarin.

The overall study comprised of three sub-studies (outlined in detail on page 67 to page 77)

Study 1: Assessment of stroke

Study 2: Assessment bone mineral density

Study 3: Online questionnaires

- a)** Bleeding assessment
- b)** QoL assessment.

2.2 Ethics

Protocols in this study were approved by Human Research and Ethics Committees of Royal Children's Hospital Melbourne, Western Health Sunshine, the Sydney Children's Hospital Network, The Royal Alfred Hospital Sydney and the Auckland District Health Board (Appendix). Written, informed consent was obtained for all participants, except those who only completed online

questionnaires for whom consent was implied when they completed the questionnaire.

2.3 Recruitment and study sites

The Australia and New Zealand Fontan Registry

The ANZ Fontan Registry collects longitudinal clinical data on patients who have had a Fontan across the ANZ region ¹⁰. Data collection is facilitated through two mechanisms: 1) Retrospective audit of medical records; 2) Prospective review of cardiologists' clinical letters. These processes facilitate the inclusion of the vast majority of Fontan patients in the ANZ Fontan registry.

The ANZ Fontan registry functions on an opt-out framework, where participants are automatically enrolled once they have undergone the Fontan procedure. Participants are able to opt-out of participating in the registry at any time. Furthermore, participants are able to consent to be contacted regarding specific Fontan research. The electronic nature of the registry allows the study researchers the ability to efficiently identify eligible participants.

Participant recruitment

Participants of the ANZ Fontan registry, who met the eligibility criteria for each study component, and had consented to be approached for research purposes, were invited to participate in study 1, study 2 and study 3 of this project.

Participants were able to enrol in some or all components of the study i.e. some participants underwent multiple study components, whilst other completed

individual components. Figure 5 outlines the recruitment approach for each study component of the overall study.

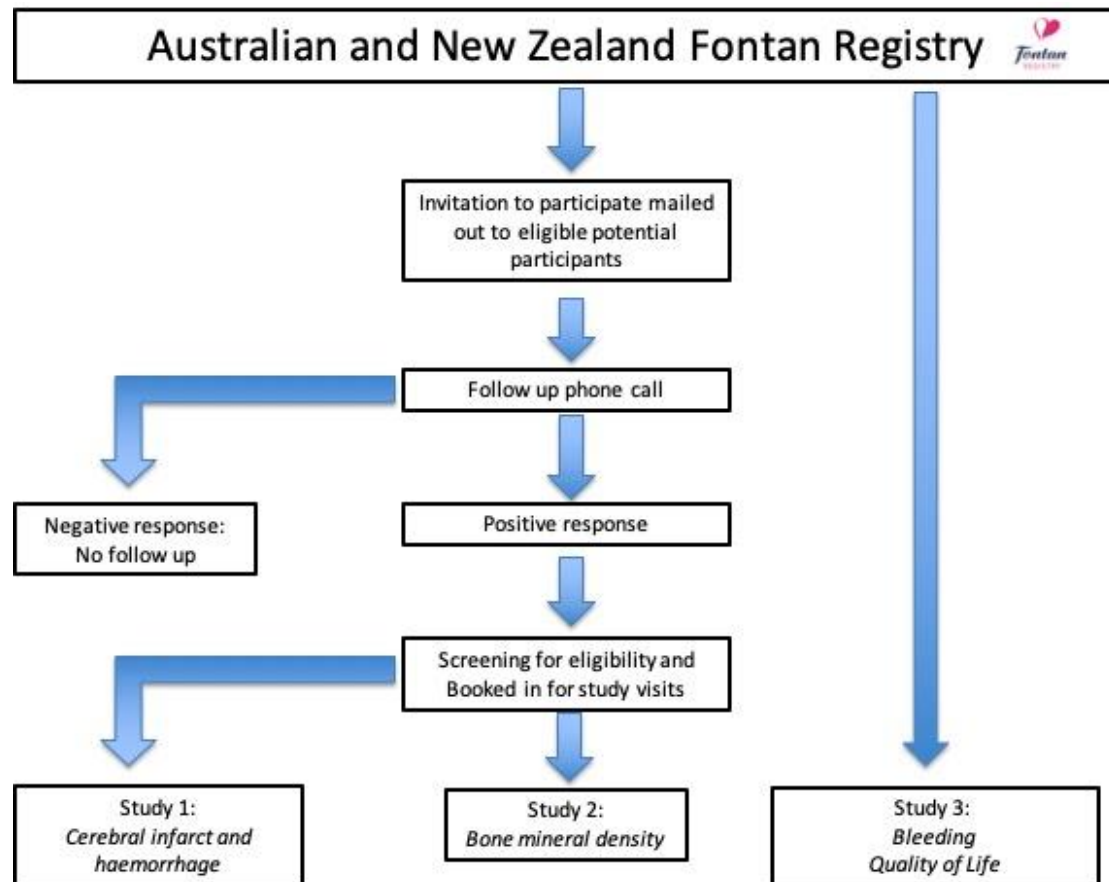


Figure 5 Study recruitment overview

2.4 Inclusion and exclusion criteria

Potential participants of the Fontan Registry were eligible for recruitment if they:

1. Underwent a Fontan procedure 5 or more years prior to participation.

The stipulation of 5 years or more post-surgery was to ensure that the findings observed are due to long-term effects of the Fontan circulation and not effects of the surgery itself.

2. Were aged 13 years or older, to ensure they were mature enough to provide cooperation in the MRI and Bone density screening, as well as comprehend the questionnaires.
3. Received aspirin or warfarin exclusively for a minimum of 5 years immediately prior to study participation.

2.5 Medical history review

Relevant medical histories were evaluated using the ANZ Fontan registry database. Additionally, a self-administered questionnaire addressing current and past thromboprophylaxis regime, surgery, thrombosis, bleeding and bone fracture history was also utilised (Appendix).

2.6 STUDY 1: Detection of cerebral infarct and haemorrhage

2.6.1 Brain Magnetic Resonance Imaging

MRI was performed in order to quantify the presence of ischemic or haemorrhagic insult in study participants. Consented participants completed an MRI safety questionnaire prior to being scanned. Brain MRI was conducted on the 1.5 Tesla Siemens equipment in Victoria and New Zealand, and on Philips equipment in New South Wales, with equivalent sequences being utilised.

All scans were reported by consensus by a Consultant Paediatric Neurologist (with subspecialty in paediatric stroke) and a Senior Paediatric Radiologist who were blinded to all clinical information. Differences in classification were

reviewed and either revised or averaged when differences persisted. Table 3 outlines the classification of radiological findings. Classification of brain injury followed the criteria utilised by Beca et al who described MRI findings in a cohort of children with congenital heart disease ⁷⁸. Stroke was classified using a standardised coding system ^{79, 80}.

Table 3 Classification of MRI radiological findings

Infarct size	<i>Small</i>	Perforator vessel: Middle cerebral artery (≥M3) or pial
	<i>Moderate</i>	Branch vessel: Middle cerebral artery (M2)/ Anterior cerebral artery (A2)/ Posterior cerebral artery (P2) branch territory
	<i>Severe</i>	Large vessel: Internal carotid artery / Middle cerebral artery (M1)/ Anterior cerebral artery (A1)/ Posterior cerebral artery (P1) territory
Haemorrhages		
Microhaemorrhages^	<i>Mild</i>	≤3 foci and all ≤2 mm
	<i>Moderate</i>	>3 and ≤10 foci or any >2 mm
	<i>Severe</i>	>10 foci
White Matter Injury	Mild	≤3 foci and all ≤2 mm
	Moderate	>3 and ≤10 foci or any >2 mm
	Severe	>10 foci
Volume Loss Score		
Ventricular dilation	Grade 1	Normal with no evidence of ventricular dilatation

	Grade 2	Mild dilatation affecting the frontal horns only
	Grade 3	Moderate enlargement resulting in rounding of the frontal horns, enlargement of the temporal horns and moderate enlargement of the occipital horns
	Grade 4	Global enlargement of a moderate to severe nature including significant enlargement of the frontal, temporal and occipital horns
Subarachnoid space	Grade 1	Small subarachnoid space which was barely visible normal sulcal spaces
	Grade 2	Mildly enlarged CSF space only with normal sulcal spaces
	Grade 3	Mild-moderate enlargement of the CSF space and visible enlargement of the space between the major sulci and the interhemispheric space in addition to the extracerebral space
	Grade 4	Substantially enlarged global subarachnoid space with visible cerebrospinal fluid between many gyri as well as interhemispheric and extracerebral
White Matter volume	Grade 1	Normal periventricular white matter volume usually associated with small ventricles
	Grade 2	Mild reduction in white matter volume with mild to moderate increased ventricular size

	Grade 3	Moderate reduction in white matter volume often occurring with marked increase in the size of the ventricle
	Grade 4	Global reduction in white matter volume always with marked increase in the size of the ventricle and/or extra-axial space

2.7 STUDY 2: Assessment of Bone density

2.7.1 Dietary Calcium intake

Dietary calcium intake was evaluated using a validated food frequency questionnaire ⁸¹ (Appendix). This questionnaire is utilised clinically as standard practice by the endocrinology department at The Royal Children's hospital to estimate dietary calcium intake of their patients.

2.7.2 Bone age and tanner stage determination

It was important for us to determine pubertal stage for our adolescent participants as peak bone acquisition occurs during the pubertal years.

For participants 18 years or below, bone age was obtained using left wrist-x-ray was determined by the Greulich and Pyle ⁸² skeletal age determination method.

Pubertal stage was assessed for participants 18 years or below using a self-reported tanner stage assessment ⁸³ (Appendix).

2.7.3 Serum Vitamin D

Serum vitamin D levels were measured using Liaison XL 25OH Vitamin D assay (DiaSorin S.p.A, Vercelli, Italy), as per manufacturer's instructions.

2.7.4 Dual-energy X-ray absorptiometry

Dual-energy X-ray absorptiometry (DXA) was utilised to assess bone mineral density (BMD) of participants. The following sites were assessed:

- Total body
- Anterior-posterior spine
- Left femoral neck
- Right femoral neck

Data was converted to age adjusted Z-scores for adults and age -height adjusted z-scores in adults, to allow for grouped comparisons. DXA was performed at each study site using available instrumentation as shown in Table 4.

Table 4 Details of site specific DXA variables

	Victoria	New South Wales			Auckland
Location	Sunshine Hospital	Children's Hospital at Westmead (Up to March 2017)	Children's Hospital at Westmead (From March 2017)	Royal Prince Albert Hospital	The Liggin's Institute
Machine	Hologic Discovery W	GE-Lunar Prodigy	GE-Lunar iDXA	GE- Lunar Prodigy	GE- Lunar Prodigy
Software	Hologic Apex 4.0.2	enCORE v13.6	enCORE v16	enCORE v13.6	enCORE v13.6
Scan Types	AP spine, hips, whole body	AP spine, hips, whole body	AP spine, hips, whole body	AP spine, hips, whole body	AP spine, hips, whole body
Scan Mode	"Array". The "whole body" scan has option for scan mode, so the default scan mode was utilised.	As appropriate for patient tissue thickness (manufacturer recommended, calculated by enCore software from height and weight).	As appropriate for patient tissue thickness (manufacturer recommended, calculated by enCore software from height and weight).	As appropriate for patient tissue thickness (manufacturer recommended, calculated by enCore software from height and weight).	As appropriate for patient tissue thickness (manufacturer recommended, calculated by enCore software from height and weight).
Quality Control	Daily Machine Quality Assurance performed prior to scanning the first patient of the day prior to scanning, using Hologic Anthropomorphic QC Spine Phantom.	Daily Machine Quality Assurance performed prior to scanning the first patient of the day. Minimum of 4 days a week Quality Control using water bath and aluminium spine phantom for Prodigy machine	Daily Machine Quality Assurance performed prior to scanning the first patient of the day. Minimum of 4 days a week Quality Control using perspex encased aluminum spine phantom for iDXA machine.	Daily Machine Quality Assurance performed prior to scanning the first patient of the day. using the Lunar DPX Series QC	Daily Machine Quality Assurance performed prior to scanning the first patient of the day. using the Lunar DPX Series QC

2.8 STUDY 3: a) Assessment of bleeding

2.8.1 a) Assessment of bleeding

Development of a bleeding assessment tool

An online Warfarin/Aspirin-bleeding assessment tool (WA-BAT) was created based on the “Molecular & Clinical Markers for the Diagnosis & Management of Type 1 von Willebrand disease” BAT ⁸⁴ (Appendix). Items were refined and language adapted to be appropriate for participants above 10 years. Items were removed (e.g. umbilical stump bleeding) and added as appropriate (e.g. bleeding post blood test).

The newly created WA-BAT underwent two methods of validity testing:

1. **Content validity** of the instrument was achieved by presenting the instrument to a panel of experts in paediatric anticoagulation. Experts included two Consultant Paediatric Haematologists and two Consultant Anticoagulation Nurses. The instrument was reviewed by the panel for appropriateness and completeness in the wording of items.
2. **Test-retest reliability** was measured by administering the instrument to a group of participants receiving anticoagulation, but whom did not have a Fontan circulation. Eligible participants were identified from our anticoagulation clinic database (Royal Children’s Hospital, Melbourne) as patients aged above 10 years and receiving long-term aspirin or warfarin. For test-retest validation, the WA-BAT was administered twice

to participants and Cohen's kappa analysis was used to determine intrarater reliability (IRR).

Potential participants for the WA-BAT validity study were phoned by the research team and invited to participate and those interested in participating were emailed the instrument. Consent was implied if the questionnaire was completed. Participants who returned the completed questionnaire were resent the questionnaire three weeks later for test-retest reliability purposes.

2.8.2 Administration of the WA-BAT

Following validity and reliability testing, an HTML link to the resulting WA-BAT questionnaire was emailed out to all Fontan registry members who:

- a) met the eligibility criteria;
- b) had previously consented to being contacted for research purposes and
- c) for whom an email address was known.

Consent was implied if participants completed the questionnaire. If the questionnaire was not completed within two weeks, a reminder email was sent.

2.9 STUDY 3: b) Assessment of Quality of life (QoL)

The Paediatric Quality of Life (PedsQL; 23 items, version 4.0, 1998)⁸⁵ inventory core scales were used to assess QoL in our participants. The PedsQL™ Measurement Model is a modular approach to measuring health-related quality of life in healthy children, adolescents and adults and those with acute and chronic health conditions. It is used extensively in health research and

distinguishes between healthy individuals and individuals with acute and chronic illness. It is responsive to clinical change over time. The PedsQL takes less than 4 minutes to complete and allows comparison with community-based norms as well as other medical cohorts. Age appropriate versions of the PedsQL were utilised in this study (Adolescent, 13-18 years of age; Young Adult, 18-25 years of age; Adults, >26 years of age).

An HTML link to the PedsQL was emailed out to all Fontan registry members who:

- a) had previously consented to being contacted for research purposes
- b) met the eligibility criteria and
- c) for whom an email address was known.

Consent was implied if participants completed the questionnaire. If the questionnaire was not completed within two weeks, a reminder email was sent.

2.10 Participant follow up

Participants were not informed of their individual results if the results were within what is considered the “normal range” for patients with a Fontan circulation. For participants of Studies 1 and 2, a summary letter of results was forwarded to the participant’s General Practitioner and Cardiologist.

All abnormal findings were fed back to the patient’s treating doctor and the patient was then informed directly. These included evidence of subclinical stroke, osteoporosis or osteopenia. For participants who had abnormalities identified through the brain MRI, a follow up consultation was arranged with a consultant neurologist.

2.11 Data storage

Study data were collected and managed using the REDCap electronic data capture tools hosted at Murdoch Children’s Research institute ⁸⁶. REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources.

2.12 Statistical analysis

All statistical analyses were performed in STATA version 14 (StataCorp 4905 Lakeway Dr, College Station, TX). Values are reported as number (%) for proportions, mean (standard deviation) for normally distributed data and median (interquartile range) for non-normally distributed data. Patient

characteristics and outcome measures were compared between groups using chi-squared tests, t-tests and ranksum tests, where appropriate.

CHAPTER 3 “Development and Validation of the Warfarin Aspirin Bleeding Assessment Tool (WA-BAT) in Children”

3.1 Introduction

Major bleeding poses a critical and potentially life-threatening complication of the thromboprophylaxis itself. Hence the impact and incidence of major bleeding has been extensively studied. Conversely, minor bleeding associated with thromboprophylaxis use have been minimally studied. Complications of minor bleeding associated with thromboprophylaxis use may include anaemia, menorrhagia or become an indication of major bleeding risk. Complications are of particular concern in the paediatric setting and when thromboprophylaxis use is long-term. Furthermore, understanding the “normal” level of bleeding associated with specific thromboprophylactic agents serves as an important comparative baseline for both current and emerging antithrombotics such as the direct oral inhibitors (e.g. rivaroxaban, apixaban), which have commenced clinical trials in children ^{87, 88}.

Bleeding assessment tools have typically been developed as a pre-diagnostic screening tool of inherited bleeding disorders such as von Willebrand disease⁸⁹. The aim of such screening tools is to reduce subjectivity and utilise discriminating bleeding symptoms to identify potential pathology prior to proceeding with laboratory diagnostic testing.

The following manuscript describes our development and subsequent validation of a warfarin and aspirin bleeding assessment tool (WA-BAT) in a cohort of children on long-term thromboprophylaxis. We aimed to develop a

tool that would allow us to quantitate bleeding as a side effect of thromboprophylaxis use with warfarin or aspirin. Furthermore, this tool was able to identify changes to thromboprophylaxis regime as a direct result of bleeding symptoms.

Development and Validation of the Warfarin-Aspirin Bleeding Assessment Tool (WA-BAT) in Children

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Summary: Bleeding assessment tools (BATs) aim to screen and estimate bleeding risk in patients with inherited bleeding disorders. However, the use of BAT as a standardized measure for comparing bleeding in patients on long-term thromboprophylaxis has not yet been validated. We developed a self-administrable BAT to assess bleeding in patients undergoing long-term thromboprophylaxis with aspirin or warfarin. Eligible participants were invited to complete the warfarin-aspirin -BAT (WA-BAT) online. The WA-BAT was readministered a number of weeks later to determine intrarater reliability. The WA-BAT showed substantial intrarater reliability and assesses major and minor bleeding associated with long-term warfarin or aspirin use.

Key Words: bleeding disorder, bleeding assessment tool, antithrombotic therapy, anticoagulants, risk assessment

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The evaluation of bleeding symptoms is an ongoing challenge given the subjectivity in the reporting and interpretation of symptoms. Bleeding assessment tools (BAT) have been developed to screen and estimate bleeding risk in patients with bleeding disorders, standardizing the symptoms recorded in a bleeding history. BATs aim to reduce subjectivity and utilize discriminating hemorrhage symptoms to distinguish pathology from normal. In both adults and children, BATs have been utilized for identifying abnormal bleeding patterns of inherited bleeding disorders (eg, von Willebrand disease).¹

One potentially useful application of BAT would be as a standardized measure to compare rates of bleeding in patients on long-term thromboprophylaxis (TP) with either antiplatelet therapy or anticoagulation. Bleeding symptoms are a significant adverse effect of TP in pediatric patients, especially with vitamin K antagonists.² The use of BAT to evaluate bleeding in patients on TP would assist in predicting bleeding risk of the individual patient, reducing unnecessary laboratory

investigations, evaluating symptom severity, and the adjustment of TP therapy. In particular, pediatric patients where TP therapy recommendations (including dose ranges, monitoring requirements) have weak evidence, BATs would help to monitor hemorrhagic side effects and provide a reproducible clinical measure for future research.² To date, BATs have not been validated as a measure of bleeding rates in pediatric patients on long-term TP.

Thus, the aim of this study was to develop and validate a self-administrable BAT to assess bleeding in patients undergoing long-term TP with aspirin or warfarin.

MATERIALS AND METHODS

Ethics Approval

This study was approved by The Royal Children's Hospital Human Research Ethics Committee (HREC number 33274).

Study and BAT Design

An online warfarin-aspirin-BAT (WA-BAT) was created based on the "Molecular & Clinical Markers for the Diagnosis & Management of Type 1 von Willebrand Disease" BAT³ (Supplemental Digital Content 1, <http://links.lww.com/JPHO/A317>). Items were refined, and language adapted to be appropriate for participants older than 10 years of age. Items were removed (eg, umbilical stump bleeding) and added as appropriate (eg, bleeding post blood test). The WA-BAT underwent validation by 4 experts (2 pediatric hematologists and 2 anticoagulant nurse consultants).

Study data were collected and managed using REDCap (Research Electronic Data Capture) electronic data capture tools hosted by Murdoch Children's Research Institute. REDCap is a secure, web-based application designed to support data capture for research studies.⁴

Patients

Eligible participants were identified from our anticoagulation clinic and stroke clinic database (Royal Children's Hospital, Melbourne) as patients aged 10 years or more and receiving long-term aspirin or warfarin. Exclusion criteria included severe intellectual disability or non-English speaking. Potential participants were telephoned and invited to participate via a REDCap⁴ survey.

For test-retest validation, the WA-BAT was administered a second time to participants with an invitation after a 3-week interval following the first WA-BAT administration.⁵

Statistical Analysis

Cohen κ analysis was used to determine intrarater reliability (IRR). All analyses were performed with Stata

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TABLE 1. Participant Characteristics

Age (y)	16.2 (± 0.85)*
Male (%)	50
Warfarin (%)	80

*Median $\pm$ SD.

14.0 statistical software package (StataCorp LLC, College Station, TX).

RESULTS AND DISCUSSION

Seven of the 10 invited participants completed the WA-BAT. Of these, 4 completed the WA-BAT for the second time (Table 1). Table 2 demonstrates the agreement between subsequent WA-BAT administrations. The overall Cohen κ IRR was 0.77 (range, 0.6 to 1) with a mean of 78 days between administrations. Substantial IRR agreement was determined.

TP in the form of antiplatelets or anticoagulants is being increasingly utilized in pediatric patients, although the medication use carries an adverse risk of bleeding. BATs have been developed and utilized to help diagnose bleeding disorders and evaluate bleeding risk. One potential use of BATs is as a measure of bleeding in patients on long-term TP, and to our knowledge, BATs have not been validated in this setting previously. The results of this study sought to validate a self-administrable BAT to assess bleeding in patients undergoing long-term TP with aspirin or warfarin.

The WA-BAT is the first self-administrable tool developed to assess bleeding in patients receiving aspirin or warfarin as TP. Our validation of the WA-BAT showed substantial IRR. Hence, this BAT may be utilized to assess major and minor bleeding associated with long-term warfarin or aspirin use. In addition, the WA-BAT standardized questions make it suitable for longitudinal evaluation of long-term TP. Further, a potential use for the WA-BAT may be for comparing bleeding rates or types of hemorrhagic symptoms within populations on aspirin or warfarin. The evaluation of TP bleeding rates would be extremely useful in determining baseline adverse event rates for antiplatelets and anticoagulants in the pediatric population, where the data is scarce and long-term randomized controlled trials are often not feasible.^{2,6} In addition, with the rising popularity of direct oral anticoagulants in adults, such as oral factor Xa inhibitors, future studies evaluating direct oral anticoagulants in children would benefit from a standardized BAT for comparison with the current most commonly utilized TP agents.

Being self-administrable, the WA-BAT can be completed without assistance, removing the need for expert administration. Similarly, Deforest et al's⁷ self-administrable BAT for von Willebrand disease bleeding scores was comparable with the expert-administered International Society on Thrombosis and Haemostasis BAT. Hence, self-administrable BATs may be a suitable and equally effective option for ongoing monitoring of bleeding symptoms.

TABLE 2. Intrarater Reliability Coefficients

Participants	Agreement Between WA-BAT		Days Between WA-BAT Completion
	Administrations (%)	Cohen κ	
1	82	0.65	91
2	100	1	41
3	82	0.6	135
4	94	0.85	43
Overall	90	0.77	78*

*Mean days.

WA-BAT indicates warfarin-aspirin-bleeding assessment tool.

Our study is limited by a small sample size. This validation was a single site, and future researchers should seek out a larger sample size to further validate the ability of the WA-BAT to assess bleeding events. In addition, the WA-BAT is limited by the patient and family comprehension of the BAT language and health literacy. Our WA-BAT was validated in children older than 10 years, and as such, younger pediatric population will still require evaluation of parental or expert administration of this tool.

The WA-BAT could improve long-term monitoring of TP adverse effects and improve communication between patients and clinicians. In the future, we would like to further evaluate its clinical effectiveness in determining bleeding risk and comparison of bleeding rates.

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CHAPTER 4 “Comparison of the long-term outcomes with warfarin and aspirin in people living with a Fontan circulation”

4.1 Introduction

Due to the risk of thrombosis, it is universally accepted that thromboprophylaxis use post-Fontan surgery is a reasonable precaution. Due to the lack of good clinical evidence, selection of thromboprophylaxis has been largely dependent on institute practice and clinician experience or preference. Given the typically dichotomous nature of thromboprophylaxis selection in the ANZ Fontan population, **we were uniquely positioned to directly compare the long-term outcomes of warfarin and aspirin use.**

The presented manuscript describes and compares the long-term outcomes of warfarin and aspirin use in the ANZ Fontan population and includes:

- *Cerebral infarct and haemorrhage*
- *Bone mineral density*
- *Bleeding*
- *Quality of life*

This manuscript is currently under review at *Journal of the American College of Cardiology* (submitted 03/08/2019).

Comparison of the long-term outcomes with warfarin and aspirin in people living with Fontan circulation

Running title: long-term outcomes with warfarin and aspirin in people living with Fontan circulation

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ABSTRACT

Background: Due to the nature of the Fontan physiology, patients are at an increased risk of thromboembolic complications. As such, warfarin or aspirin are generally prescribed life-long for thromboprophylaxis.

Objectives: This study aimed to compare cerebrovascular injury, bleeding, bone mineral density (BMD) and quality of life (QoL) in Fontan patients receiving warfarin as compared to aspirin, over the long-term.

Methods: This was a cross-sectional, multicentre study of the Australian and New-Zealand Fontan (ANZ) population. Participants underwent cerebral magnetic resonance imaging (MRI) to detect the presence of cerebrovascular injury and dual-energy x-ray absorptiometry to assess bone mineral density (BMD). Bleeding and quality of life were assessed using validated questionnaires (WA-BAT and PedsQL, respectively).

Results: Stroke was detected in 33 (39%) participants, with only 5 (15%) of those being clinically symptomatic. There was no association between stroke and Fontan type (p value 0.38, CI 0.39- 1.43) or thromboprophylaxis type. Micro- hemorrhage and white matter injury were detected in almost all participants (96% and 86% respectively), regardless of thromboprophylaxis type. Bleeding rates were high in both groups however, bleeding was more frequent in the warfarin group.

BMD was reduced in our cohort compared to the general population however, this was further attenuated in the warfarin group. QoL were similar between warfarin and aspirin groups. Home INR monitoring was associated with better QoL scores in the warfarin group.

Conclusions: Cerebrovascular injury are frequent occurrences in the ANZ Fontan population regardless of thromboprophylaxis type. No benefit of long-term warfarin prophylaxis could be demonstrated over aspirin; however, consideration must be given to important clinical features such as cardiac function and lung function. Furthermore, the association of reduced bone health in children on warfarin warrants further mechanistic studies.

CONDENSED ABSTRACT

Fontan patients are at an increased risk of thromboembolic complications. Warfarin or aspirin are generally prescribed life-long for thromboprophylaxis.

Our study compared cerebrovascular injury, bleeding, bone mineral density (BMD) and quality of life (QoL) in Fontan patients receiving warfarin or aspirin, over the long-term.

Cerebrovascular injury were frequent occurrences in the Fontan cohort regardless of thromboprophylaxis type. BMD was reduced and bleeding rates were high overall however, these outcomes were poorer in the warfarin group. No benefit of long-term warfarin prophylaxis could be demonstrated over aspirin; however, consideration must be given to important clinical features.

KEY WORDS

Fontan, Single Ventricle, Warfarin, Aspirin, Stroke, Bone Density

ABBREVIATIONS LIST

BMD	Bone mineral density
QoL	Quality of life
ANZ	Australian and New Zealand
MRI	Magnetic Resonance Imaging
DWI	Diffusion weighted imaging
T1	T1-weighted image
T2	T2-weighted image
FLAIR	Fluid Attenuated Inversion Recovery
SWI	Susceptibility weighted imaging
MRA	Magnetic resonance angiography
WA-BAT	Warfarin and Aspirin Bleeding assessment tool
DXA	Dual-energy X-ray absorptiometry
HAZ	Height for age
OR	Odds ratio
PedsQL	Paediatric Quality of Life Inventory core scales
SD	Standard Deviation
INR	International Normalized Ratio
IU	International Unit
kg	Kilogram
IQR	Interquartile Range

INTRODUCTION

The Fontan procedure is the culmination of staged surgeries to treat patients with single ventricle physiology by rerouting systemic venous blood flow directly to the lungs. Although the Fontan procedure is non-curative, the majority of Fontan-palliated patients are expected to survive well into adulthood. Since mortality post-Fontan has reduced dramatically over the recent years, the focus of patient outcomes has now shifted to reducing Fontan-associated comorbidities such as arrhythmia, liver dysfunction, protein losing enteropathy and thrombosis.

Due to the nature of the Fontan physiology, patients are at an increased risk of thromboembolic complications. Abnormalities in all three aspects of Virchow's triad are present due to the presence of abnormal endothelium or prosthetic material, low flow and intrinsic plasma protein changes most likely related to subtle derangement in liver synthetic function which is more pronounced in those with protein losing enteropathy. Cohort studies have reported incidence of venous thrombosis from 4% to 19% (1-4) and stroke from 3% to 19% (1, 5-8). To mitigate the risk of thrombosis post-Fontan, patients are generally prescribed life-long warfarin or aspirin. The American Heart Association Guidelines consider the long-term prophylactic use of either drug in the Fontan population as 'reasonable', while acknowledging that the current evidence base for the optimal type or duration of antithrombotic therapy is limited (9).

This study aimed to compare stroke, bone mineral density (BMD), bleeding and quality of life (QoL) in Fontan patients receiving long-term warfarin or aspirin.

METHODS

Study participants

Study participants were recruited for this cross-sectional study using the Australian and New Zealand (ANZ) Fontan registry, with the specific registry design and enrollment procedures previously described (10).

An overview of the recruitment procedure for this specific study is outlined in **Error! Reference source not found.** Specifically, potential participants were invited to participate if they satisfied all three of the following criteria: at least 13 years of age, minimum of 5 years post-Fontan surgery and prescribed 5 consecutive years of thromboprophylaxis exclusively with warfarin or aspirin immediately prior to study enrolment.

Baseline characteristics and follow-up data were obtained from the ANZ Fontan Registry database [1]. Additionally, a self-administered questionnaire addressing thromboprophylaxis regime, surgery, thrombosis, bleeding, pubertal stage, calcium intake and bone fracture history was also utilised.

Study sites

This multi-centre study was approved by the national and local hospital Ethics Committees of each study centre. Victoria (Australia): Royal Children's Hospital; Australian Institute for Musculoskeletal Science (AIMSS) and University of Melbourne; New South Wales (Australia): Children's Hospital at Westmead and Royal Prince Alfred Hospital; Auckland (New Zealand): Starship Children's Health.

Patients were able to opt out of some aspects of the study if they did not wish to undergo all investigations.

Cerebrovascular injury

Clinical history of stroke was collected for all participants using the ANZ Fontan registry database. Brain Magnetic Resonance Imaging (MRI) was performed to quantify the presence of cerebrovascular injury on a 1.5 Tesla Siemens MRI in Victoria and Auckland, and on a Philips MRI in NSW, with equivalent sequences being utilised. Sequences included Diffusion weighted imaging (DWI), T1-weighted imaging, T2-weighted imaging, Fluid Attenuated Inversion Recovery (FLAIR), Susceptibility weighted imaging (SWI) and Magnetic resonance angiography (MRA). All scans were reported centrally and classified by consensus by a Senior Paediatric Neurologist (with subspecialty stroke expertise) and Paediatric Neuroradiologist, both of whom were blinded to all clinical information. Classification of brain injury followed the criteria utilised by Beca et al. who described MRI findings in a cohort of children with congenital heart disease (11).

Bleeding

We used a Warfarin and Aspirin Bleeding assessment tool (WA-BAT) developed and previously validated by our team to specifically assess bleeding in individuals receiving oral thromboprophylaxis (12). The WA-BAT consisted of 25 questions assessing types, rates and severity of bleeding. Following validity and reliability testing the questionnaire was administered electronically through REDCap. A link to the questionnaire was emailed out to all Fontan registry members who met the eligibility criteria, and for whom an email address was known.

Bone mineral density

Bone mineral density was measured using Dual-energy X-ray absorptiometry (DXA). Data were converted to age-adjusted Z-scores to allow for grouped comparisons. Z scores were calculated using reference population data provided by the respective DXA manufacturers, as per clinical practice. DXA was performed at each study site using available instrumentation (supplementary Table 1).

Height for age z score was calculated using Centers for Disease Control growth chart (13). Plain left-hand radiograph was used to determine bone age in participants less than 18 years. Pubertal stage was estimated in participants younger than 18 years using self-reported tanner assessment (14). Dietary calcium intake was evaluated using a validated food frequency questionnaire (15) which is utilised clinically as standard practice by the endocrinology department at The Royal Children's Hospital, Melbourne to estimate dietary calcium intake of their patients. Serum vitamin D was measured using the Liaison XL 25OH Vitamin D assay (DiaSorin S.p.A, Vercelli, Italy), as per manufacturer's instructions.

Quality of life

To assess health-related quality of life in our participants, age-appropriate versions of the Paediatric Quality of Life Inventory core scalesTM (PedsQL; 23 items, version 4.0, 1998) were administered using REDcap, concurrently with the WA-BAT. The PedsQL Measurement Model is a modular approach to measuring health-related quality of life in children, adolescents and adults (16).

Data storage and statistical analysis

Study data were collected and managed using the Research Electronic Data Capture (REDCap). Statistical analyses were performed in STATA version 14 (StataCorp 4905 Lakeway Dr, College Station, TX). Values are reported as number (%) for proportions, mean (standard deviation) for normally distributed data and median (interquartile range) for non-normally distributed data. Patient characteristics and outcome measures were compared between groups using chi-squared tests, t-tests and ranksum tests, where appropriate. Odds ratio were used to quantify strength of association between outcomes and variables.

RESULTS

Cerebrovascular injury

A total of 489 patients were invited to participate with 84 participants undergoing brain MRI. The characteristics of the cohort are presented in Table 1 and Table 2. Rates of stroke and hemorrhage are presented in Table 3.

Very high rates of focal and generalized brain abnormalities were observed, with all participants (100%) presenting with abnormal brain images. Figure demonstrates the broad spectrum of brain injury as observed in a single patient and included parietal cortical and subcortical infarct, hemorrhagic transformation and micro- hemorrhages.

Evidence of ischaemic infarction was detected in 39% of participants, only 15% of whom had a clinically recognised event. There were no differences between warfarin

and aspirin groups for stroke and hemorrhage in terms of incidence, type or severity (number of infarcts/micro-or macrohemorrhages) (Table 3). Furthermore, there was no association between Fontan type and stroke (OR 0.75, p value 0.38, CI .39- 1.43). All participants in the aspirin group and 93% of the warfarin group had evidence of micro-hemorrhage with only one participant (1%) having evidence of macro hemorrhage.

White matter injury was evident in 86% of the cohort with no differences in observed between warfarin and aspirin groups (OR 0.99, p value 0.99, CI 0.24-4.0).

Variation in practice in terms of anticoagulation and fenestration was noted however those two factors did not seem to influence the incidence of clinically symptomatic or subclinical strokes (fenestration and all stroke (OR 1.45, p value 0.45, CI. 0.56-3.79; fenestration and clinical stroke OR 1.57, p value 0.636, CI 0.25-10).

Bleeding

To assess bleeding, a total of 100 respondents completed the WA-BAT questionnaire. Analysis of the WA-BAT demonstrated differences between the warfarin and aspirin groups in terms of the cessation of anticoagulation, epistaxis and oral contraceptive use (Table 4). More participants on warfarin stopped their anticoagulant during the previous five-year period (56% vs 21%; p value 0.001) and stopped anticoagulation prior to undergoing a medical procedure (49% vs 9%; p value <0.0001). Participants taking warfarin also experienced more episodes of epistaxis compared to participants on aspirin (47% vs 24%; p value 0.023). Additionally, there was an increased number of warfarin patients taking the combined oral contraceptive pill (p value 0.01).

The rates of minor bleeding were comparable between patients on warfarin and aspirin. No patient with either treatment had a change of thromboprophylaxis type as a result of bleeding or thrombosis. Furthermore, self-estimated menstrual bleeding volumes were similar between females on both therapies. Iron deficiency anemia was reported in approximately a quarter of all respondents, regardless of thromboprophylaxis type.

Bone mineral density (BMD)

A total of 120 participants underwent a DXA scan (Table 5). Overall, participants had a lower mean BMD than age-matched reference population (z score <0), regardless of the type of thromboprophylaxis (Table 6). Participants receiving warfarin had reduced BMD than their aspirin counterparts at all measured sites, with both left and right femoral neck being statistically significantly lower. Rates of clinically significant reduced BMD (as determined by a z score of <-2), was detected most often at the lumbar spine site (17%). No differences in clinically significant reduced BMD was observed between warfarin and aspirin groups at any of the measured sites.

Height for age (HAZ), fracture rates, vitamin D and dietary calcium levels were comparable between the groups, however the aspirin group were younger (median 19 vs 24 year; p value 0.004). 45% of males and 41% for females had experienced bone fracture over their lifetime. None of the fractures reported were deemed low impact fractures.

In general, bone age matched chronological age in both groups with mean difference between age and bone age being 0.87 years (SD 0.82).

Quality of life

A total of 90 participants completed the PedsQL assessment. There was no difference between warfarin and aspirin groups for overall health-related QoL, as well as for each of the assessed dimensions (Table 7). A *post-hoc* analysis of participants undergoing warfarin home-INR monitoring demonstrated a positive impact on QoL (Table 8) with the home-INR group scoring a mean of 10 points higher (mean 70.5) compared to the warfarinized, non-home-INR group (mean 60.4; p value 0.05).

DISCUSSION

The first Fontan procedure was performed more than 50 years ago (17). An estimated 70,000 post Fontan patients are alive today, with this population expected to double within 2 decades (18, 19). Thromboprophylaxis reduces the incidence of thrombosis, however the wide practice variation evident in our data suggests that uncertainty still exists with regard to the best thromboprophylactic strategy for these patients. A single randomized controlled trial demonstrated no difference between two strategies, aspirin and warfarin, as did a retrospective analysis of data from our own registry (20). Nevertheless, recent clinical guidelines do not definitively recommend aspirin in preference to warfarin in low risk patients (19). In this regard, our study adds to current evidence with regard to the potential consequences of these two therapies.

Cerebrovascular injury

The clinically reported symptomatic stroke rate for our cohort was 6% post Fontan and is comparable with previously published rates of 3-19% (21). However, a strikingly large proportion of asymptomatic cerebral infarction was revealed on MRI

brain imaging, with 39% of our cohort having radiological evidence of stroke. A significant finding of our study was the absence of difference in rates of cerebral infarction in patients on aspirin compared to those on warfarin. Whilst similar findings have been observed previously, those studies only reported clinically diagnosed stroke and did not screen patients for asymptomatic stroke (20, 22). Some physicians may believe that particular patients may benefit from warfarin because they are more susceptible to thromboembolism. As an example, one could postulate that the shunting related to the fenestration may put their patients at a higher risk of stroke. Our cohort was remarkably uniform in the incidence of stroke. The variation of practice by region allowed us to compare the outcomes of patients with and without fenestration for both warfarin and aspirin and we could not demonstrate any differences in the brain MRI of these patients after more than 5 years consecutive treatment.

In the early stages of life, patients undergoing surgery for congenital heart defects suffer from brain injuries at a significant rate. Beca et al quoted a rate of white matter injury as high as 44% in infants post cardiac surgery (11). Furthermore, Cordina et al reported that adults with chronic cyanotic heart disease were likely to have stroke on cerebral imaging (23).

This study shows extremely high rates of white matter injury. At which stage of life or which surgical stage these events occurred remains to be determined. The need for further study in this sphere has recently been emphasized in a scientific Statement from the American Heart Association (19).

Bleeding

In this era of introduction of new anti-thrombotic agents, the characterization of the bleeding complications of a target population such as the one living with a Fontan circulation gives insights of the results of conventional practice. Overall there were high rates of heavy menstrual bleeding, bruising, bleeding from minor wounds and after tooth removal, across the cohort. Despite these incidences of bleeding, thromboprophylaxis was only stopped due to bleeding in one individual. Patients on warfarin experienced more epistaxis and ceased anticoagulation more commonly prior to a medical procedure than those on aspirin.

Interestingly, there were minimal differences in bleeding rates between warfarin and aspirin groups. The lack of difference in perceived menstrual bleed volumes between the groups was unexpected. We hypothesised that an increase in the rate of menorrhagia would be expected in the warfarin group. The lack of difference may be because therapy, such as the contraceptive pill, is used as treatment for menorrhagia and hence likely prescribed to warfarinized women who have experienced excessive menstrual bleeding.

Our study demonstrated that micro-hemorrhages on brain MRI are ubiquitous in the Fontan population. The vast majority of warfarin patients, and all aspirin patients, had evidence of micro-haemorrhage. The pervasive nature of these events suggests their aetiology is due to events common to both thromboprophylaxis groups such as chronic venous hypertension, surgical variables or cardio pulmonary bypass, where 300-500 IU/kg continuous infusion of heparin are routinely administered.

Bone mineral density

Our Fontan cohort had reduced BMD compared to the healthy reference population. Previous studies have also observed a reduction in BMD both children and adults with Fontan circulation, compared to the healthy reference population (24) (D'Ambrosio et al. *in press* Congenital Heart Disease).

The results of our study demonstrate that participants receiving warfarin have further decreased levels of BMD compared to those on aspirin. Previous studies have similarly observed an association of warfarin with reduced BMD, however these studies were limited by small numbers (24, 25). Whether reduced BMD associated with warfarin is direct drug effect or reflects differences in patient selection or surgical management requires further investigation.

Fracture rates were equivalent in the warfarin and aspirin groups (45 and 39% respectively). Lyons et al reported on a South Wales population where by 15 years, 64% of boys and 39% of girls can expect to have fractured a bone (26). Males in our cohort were older (median 20.8 years) and had a lower incidence of fracture (45%) than in the Lyons study population. We speculate that the physical restriction placed on Fontan patients, either due to their condition or prescribed due to bleeding risk, has subsequently reduced the opportunity for fractures to occur throughout childhood. The importance of peak bone mass and its corresponding relationship to osteoporosis risk and fracture rate have been well defined (27). The lower BMD observed in our cohort may well reflect higher risk of osteoporosis and its attendant morbidity as this population ages, which supports the focus on bone health outcomes in this group.

The findings of our study also justify the recommendation of regular BMD screening in the Fontan population, and particularly those on warfarin.

Quality of Life

QoL has increasingly been identified as an important clinical outcome measure of thromboprophylaxis management in children (28). We hypothesized that participants receiving warfarin would have poorer health related quality of life than those on aspirin, due to the lack of INR monitoring required and restrictions on physical activity. Interestingly, no statistical difference in QoL was observed between participants when using the PedsQL. These results may indicate that health related quality of life is more influenced by the patients underlying health condition, rather than thromboprophylaxis. This statement is supported by a study from Uzark et al that demonstrated a significant decrease in QoL scores in Fontan patients compared to controls, using the PedsQL tool (29). However, in the Uzark study, thromboprophylaxis regime was not assessed.

A *post-hoc* analysis of warfarinized patients on home versus non-home INR monitoring demonstrated reduced QoL in the non-home INR group compared to home-INR group. This is in line with a study by Jones et al. who found an improvement in parent's perceived QoL of their children when they commenced home INR monitoring after a period of non-home INR monitoring (30). We hypothesize that the increase in perceived QoL in the home-INR group may be attributed to an increase in the patient's sense of control over their health. Furthermore, as home INR monitoring is conducted under the guidance of a

comprehensive anticoagulation clinic, the clinical support received from the clinic staff may also improve QoL perception.

Conclusions and clinical implications

Our study found that cerebrovascular injury is a frequent occurrence in the ANZ Fontan population regardless of thromboprophylaxis type. Furthermore, we report lower BMD and higher bleeding rates associated with warfarin therapy.

Given the high rates of clinically asymptomatic stroke and ubiquitous findings of micro-haemorrhages and white matter injury, a study with serial MRI may be useful to determine when these changes occur and what factors are associated with their occurrence. Early detection of cerebrovascular injury may assist with the identification of children at higher risk of neurodevelopmental problems and allow early implementation of interventions to reduce the long-term burden of disease. The timing of MRI may be appropriate in the post-operative period, when risk of thromboembolic events and cerebral injury is known to be high.

Given that low BMD is clinically important and potentially treatable, an emphasis should be placed on bone health. The importance of vitamin D sufficiency, adequate calcium intake and appropriate weight bearing exercise should be communicated to patients. Furthermore, where long-term warfarin therapy has occurred, BMD screening is recommended.

Our results suggest that aspirin should be offered as primary long-term thromboprophylaxis after Fontan surgery, however, consideration must be given to important

clinical features such as cardiac function and lung function, neither of which were investigated in this study. A shift from warfarin to aspirin therapy post-Fontan would have a cost benefit to the patient and to the healthcare system (31).

Limitations

An important limitation in comparing warfarin to aspirin outcomes is the geographical distribution of patients receiving each treatment. 91% of warfarin patients came from either Region 1 or 2 and 71% of aspirin patients came from Region 3. The possibility that differences in other aspects of care (surgical, supportive) requires further examination.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: **asymptomatic stroke and ubiquitous brain injury** are frequent occurrences in the Fontan population, regardless of thromboprophylaxis type. Bone health in the Fontan population is reduced compared to the general population, particularly in those on long-term-warfarin therapy.

TRANSLATIONAL OUTLOOK: Delineating the time of cerebral injury is imperative to the identification of modifiable factors that may reduce the accumulation and extent of brain injury post-Fontan surgery. Longitudinal follow up with cerebral imaging commencing at antenatal diagnosis, with serial imaging timed with known periods of high-risk (perioperative, pre/post cardio pulmonary bypass), would be the ideal methodology of determining the natural history of cerebral events.

Furthermore, BMD and its associated with thromboprophylaxis, surgical management and other patient related factors requires further mechanistic investigation.

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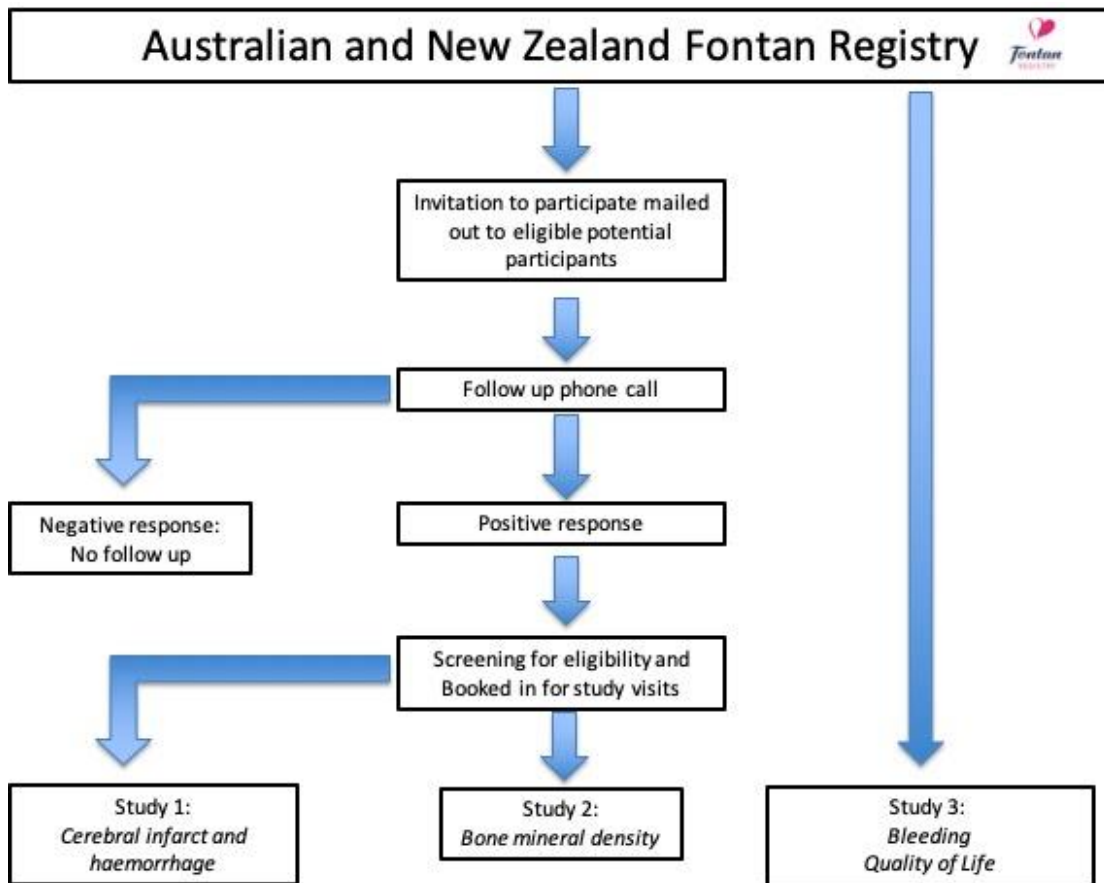


Figure 1 Patient Recruitment strategy

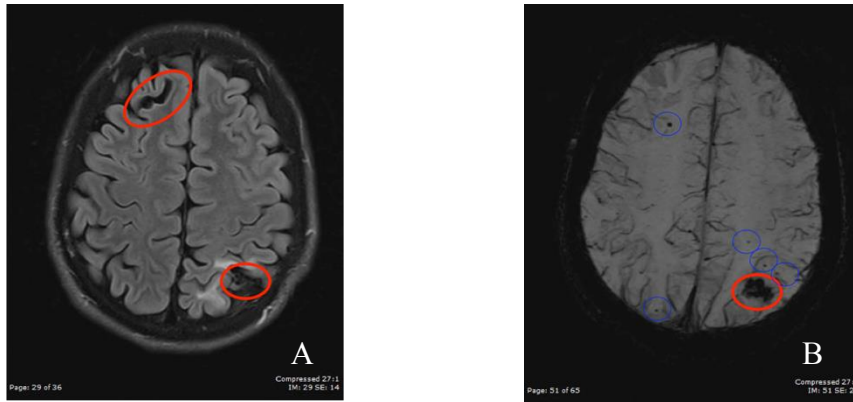


Figure 2 Spectrum of brain injury as observed in one participant

- A) Axial Fluid Attenuated Inversion Recovery (FLAIR) sequence: Frontal Infarct; Parietal cortical and subcortical infarct
- B) Susceptibility weighted imaging (SWI) sequence: Hemorrhagic transformation; Micro-hemorrhages

Table 1 MRI study participant characteristics based on thromboprophylaxis type

	Total (n=84)	Warfarin (n=46)	Aspirin (n=38)	p value
Male (%)	49 (58)	27 (59)	22 (58)	0.94
Age, median (IQR)	20.7 (15.9-27.4)	22.6 (17.4-28.7)	17.9 (14.9-24.9)	0.03
Age at Fontan, median (IQR)	4.7 (3.5-6.9)	4.8 (3.5-7.9)	4.6 (3.4-6.2)	0.32
Isomerism/ Heterotaxy (%)^	9 (11)	5 (11)	4 (10)	0.36
Dominant ventricular morphology (%)~				0.47
<i>Left</i>	48 (57)	27 (59)	21 (55)	
<i>Right</i>	25 (30)	14 (30)	11 (29)	
<i>Biventricular</i>	5 (6)	3 (7)	2 (5)	
<i>Indeterminate</i>	5 (6)	1 (2)	4 (11)	
Fontan type^				0.135
<i>ECC</i>	53 (63)	25 (54)	28 (74)	
<i>LT</i>	20 (24)	13 (28)	7 (18)	
<i>AP</i>	9 (11)	7 (15)	2 (5)	
Fenestrated Fontan (%)^	25 (30)	21 (47)	4 (11)	<0.001

MRI magnetic resonance Imaging; IQR inter quartile range; ECC extra cardiac conduit; LT lateral tunnel; AP Atriopulmonary Connection; ^missing 2; ~missing 1

Table 2 Thromboprophylaxis and fenestration by Fontan surgery location

	<i>Region 1</i>	<i>Region 2</i>	<i>Region 3</i>	<i>Region 4</i>	<i>Total</i>	<i>P value</i>
<i>Thromboprophylaxis</i>						<0.001
<i>Warfarin (%)</i>	27 (87)	12 (75)	5 (16)	1 (25)	45 (54)	
<i>Aspirin (%)</i>	4 (13)	4 (25)	27 (84)	3 (75)	38 (46)	
<i>Fenestration</i>						0.013
<i>No (%)</i>	17 (55)	9 (56)	29 (94)	2 (50)	58 (70)	
<i>Yes (%)</i>	14 (45)	7 (44)	2 (6)	2 (50)	25 (30)	

Table 3 Clinical stroke and radiological findings by thromboprophylaxis type

	Total (n=84)	Warfarin (n=46)	Aspirin (n=38)	p value^a	Odds Ratio	Adjusted p value*	95% CI
Clinical Stroke (%)	5 (6)	3 (7)	2 (5)	0.81	0.83	0.93	0.02-38.91
Radiological findings							
Infarct (%)	33 (39)	20 (44)	13 (34)	0.39	0.87	0.87	0.18-4.26
Infarct number (%) <i>n=45w; 37a</i>				0.710	1.11	0.91	0.18 -6.93
<i>Single</i>	19 (23)	11 (24)	8 (22)				
<i>Multiple</i>	14 (17)	9 (20)	5 (14)				
Macrohemorrhage (%)	1 (1)	1 (2)	0 (0)	0.357			
Microhemorrhage (%) <i>n=43w; 36a</i>	76 (96)	40 (93)	36 (100)	0.106			
Microhemorrhage severity (%) <i>n=40w; 36a</i>				0.332			
<i>Mild</i>	4 (5)	1 (3)	3 (8)				
<i>Moderate</i>	18 (24)	8 (20)	10 (28)				
<i>Severe</i>	54(71)	31(78)	23(64)				
White matter injury (%)	72 (86)	40 (87)	32 (84)	0.72	0.99	0.99	0.24-4.0

CI confidence interval, ^a χ^2 p-value of difference, *adjusted for Fontan type, sex, age, fenestration and age at Fontan; Mild = ≤ 3 foci and all ≤ 2 mm, Moderate = > 3 and ≤ 10 foci or any > 2 mm, Severe = > 10 foci

Table 4 WA-BAT measures by thromboprophylaxis type

Question	Warfarin (n=66)	Aspirin (n=34)	p value
<i>Stopped using anticoagulant for a period within the last 5 years</i>	37 (56.1%)	7 (20.6%)	0.001
<i>Stopped anticoagulant as a result of bleeding</i>	2 (3.0%)	1 (2.9%)	0.980
<i>Stopped anticoagulant due to procedure</i>	32 (48.5%)	3 (8.8%)	<0.0001
<i>Stopped anticoagulant due to other reasons</i>	5 (7.6%)	3 (8.8%)	0.828
<i>Changed anticoagulation due to doctor's recommendation</i>	8 (12.1%)	3 (8.8%)	0.618
<i>Changed anticoagulation due to bleeding</i>	1 (1.5%)	0 (0%)	0.471
<i>Changed anticoagulation due to blood clot</i>	3 (4.6%)	0 (0%)	0.207
<i>Experienced nosebleeds</i>	31 (47.0%)	8 (23.5%)	0.023
<i>Experienced bruising at time of questionnaire completion</i>	38 (57.6%)	22 (64.7%)	0.491
<i>Experienced bleeding from minor wounds</i>	49 (74.2%)	23 (67.7%)	0.487
<i>Experienced bleeding after blood tests</i>	(n=33)	(n=33)	0.570
	32 (48.5%)	18 (54.6%)	
<i>Experienced bleeding in mouth</i>	22 (33.3%)	8 (23.5%)	0.311
<i>Experienced bleeding after tooth removal</i>	(n=31)	(n=13)	0.488
	18 (58.1%)	9 (69.2%)	
<i>Experienced gastrointestinal bleeding</i>	7 (10.6%)	1 (2.94%)	0.181
Female specific Questions			
<i>Taking combined oral contraceptive pill</i>	(n=40)	(n=23)	0.010
	18 (45%)	3 (13.0%)	
<i>Experience flooding (blood running around pad or tampon)</i>	(n=32)	(n=20)	0.532
	18 (56.3%)	13 (65%)	
<i>Experience clots (visible on pad or tampon)</i>	(n=32)	(n=20)	0.274
	23 (71.8%)	17 (85%)	
<i>Diagnosed with iron deficiency anaemia</i>	(n=32)	(n=20)	0.805
	9 (28.1%)	5 (25.5%)	
<i>Experienced pregnancy</i>	(n=40)	(n=23)	0.803
	6 (15%)	4 (17.4%)	

Table 5 BMD study participant characteristics based by thromboprophylaxis type

	Total (n=120)	Warfarin (n=67)	Aspirin (n=53)	p value
Male (%)	68 (57)	39 (58)	29 (55)	0.7
Age, median (IQR)	20.8 (15.5-28.1)	24.0 (17.0-29.2)	18.6 (14.9-24.3)	0.004
Age at Fontan, median (IQR)	4.8 (3.6-6.6)	4.8 (3.8-7.3)	4.7 (3.5-6.0)	0.24
Isomerism/Heterotaxy (%)	11 (9)	7 (10)	4 (8)	0.16
Dominant ventricular morphology (%)[@]				0.15
<i>Left</i>	71 (59)	40 (61)	31 (59)	
<i>Right</i>	33 (28)	20 (30)	13 (25)	
<i>Biventricular</i>	8 (7)	5 (8)	3 (6)	
<i>Indeterminate</i>	7 (6)	1 (2)	6 (11)	
Fontan type (%)[#]				0.047
<i>ECC</i>	78 (66)	38 (58)	40 (77)	
<i>LT</i>	28 (24)	18 (27)	10 (19)	
<i>AP</i>	12 (10)	10 (15)	2 (4)	
Fenestrated Fontan (%)[#]	36 (31)	32 (49)	4 (8)	<0.001

IQR inter quartile range, *ECC* extra cardiac conduit, *LT* lateral tunnel, *AP* Atriopulmonary Connection, [@]missing n=1, [#]missing n=2,

Table 6 BMD, fracture rate, vitamin D and calcium intake based on thromboprophylaxis type

	<i>Total</i>	<i>Warfarin</i>	<i>Aspirin</i>	<i>p value</i>	<i>Adjusted p value[^]</i>	<i>95% CI</i>
Z score^{\$}						
Total Body (SD) <i>n=43w;51a</i>	-0.71 (1.18)	-0.94 (1.15)	-0.52 (1.17)	0.09	0.064	-1.18-0.03
Left femoral neck (SD) <i>n=67w;52a</i>	-0.67 (1.03)	-0.91 (0.97)	-0.36 (1.04)	0.004	0.004	-1.16 -0.23
Right femoral neck (SD) <i>n=58w;41a</i>	-0.56 (1.09)	-0.89 (1.03)	-0.1 (1.0)	0.0002	<0.0001	-1.51 -0.54
Lumbar spine (SD) <i>n=63w;50a</i>	-1.08 (1.07)	-1.23 (1.08)	-0.90 (1.03)	0.09	0.07	-1.01 - 0.04
Clinically significant reduced BMD (z score <-2)						
Total Body (SD) <i>n=43w;51a</i>	13 (13.4)	7 (16.3)	6 (11.8)	0.53		
Left femoral neck (SD) <i>n=67w;52a</i>	11 (9.2)	8 (11.9)	3 (5.8)	0.25		
Right femoral neck (SD) <i>n=58w;41a</i>	7 (7.1)	5 (8.7)	2 (4.9)	0.47		
Lumbar spine (SD) <i>n=63w;50a</i>	19 (16.8)	12 (19.1)	7 (14)	0.48		
Fractures						
Individuals who had fracture (%) <i>n=60w; 46a</i>	45 (43)	27 (45)	18 (39)	0.55	0.28	0.64- 4.70
Total fractures <i>n=60w; 46a</i>	60	28	32	0.06	0.87	0.39-3.0
Number of fractures (%) <i>n=60w; 46a</i>						
1	26 (25)	18 (30)	8 (17)		-	
2	11 (10)	5 (8)	6 (13)		-	
3	4 (4)	0 (0)	4 (8)		-	
Height for age z score (SD)	-0.44 (1.07)	-0.51 (1.03)	-0.33 (1.13)	0.36	-	-0.63 – 0.57

<i>Vitamin D (SD)</i> <i>n=35w; 39a</i>	61.75 (24.28)	59.38 (25.73)	63.86 (23.04)	0.43	-	-6.81- 15.79
<i>Calcium intake mg/day (SD)</i> <i>n=58w; 46a</i>	1160 (649)	1153 (700)	1168 (586)	0.91	-	-240- 271

*^{\$}adjusted for age and gender, [^]adjusted for vitamin d and calcium intake; SD
standard deviation; mg milligrams*

Table 7 Comparison of QOL outcomes by thromboprophylaxis type

<i>PedsQL Dimensions</i>	<i>Warfarin (n=59) Mean (SD)</i>	<i>Aspirin (n=31) Mean (SD)</i>	<i>p value</i>
<i>Total Scale Score</i>	63.5 (18.5)	65.8 (16.7)	0.564
<i>Physical Functioning</i>	60.7 (24.6)	65.7 (19.9)	0.327
<i>Emotional Functioning</i>	61.6 (19.7)	61.1 (20.0)	0.913
<i>Social Functioning</i>	70.9 (17.5)	69.7 (20.0)	0.764
<i>School/Work Functioning</i>	62.3 (23.2)	66.1 (21.9)	0.453

QoL Quality of Life; SD standard deviation

Table 8 Comparison of QOL outcomes of patients receiving warfarin participating in home INR monitoring to those undergoing community-based monitoring

<i>PedsQL Dimensions</i>	<i>Home INR monitoring (n=18) Mean (SD)</i>	<i>Non Home INR monitoring (n=41) Mean (SD)</i>	<i>p value</i>
<i>Total Scale Score</i>	70.5 (21.0)	60.4 (16.7)	0.054
<i>Physical Functioning</i>	71.5 (24.0)	56.0 (23.6)	0.025
<i>Emotional Functioning</i>	68.6 (23.6)	58.5 (17.3)	0.071
<i>Social Functioning</i>	75.6 (18.1)	68.9 (17.0)	0.178
<i>School/Work Functioning</i>	65.6 (24.8)	60.9 (22.7)	0.483

QoL Quality of Life; INR international Normalized Ratio; SD standard deviation

4.2 Addendum

The following results were not included in the submitted manuscript due to the word limit requirements of the journal.

4.2.1 Results

Table 5 demonstrates the wide range of brain injuries identified in our cohort with. Overall, 86% of participants presented with white matter injury and 25% with deep grey matter injury. Brain structural abnormalities were identified on Magnetic Resonance Angiography in 58% of participants, with the majority of abnormalities being congenital in nature.

Table 5 Neuroradiological findings identified in Fontan patients by thromboprophylaxis*

	<i>Total</i>	<i>warfarin</i>	<i>aspirin</i>
<i>White matter injury severity (%)</i>			
<i>mild</i>	11 (13.3)	4 (4.8)	7 (8.4)
<i>moderate</i>	29 (34.9)	13 (15.7)	16 (19.3)
<i>severe</i>	24 (28.9)	16 (19.3)	8 (9.6)
<i>Deep grey matter injury (%)</i>	21 (25.3)	10 (12)	11 (13.3)
<i>Volume Loss Score</i>			
<i>Ventricular dilation score (%)</i>			
<i>Grade 1</i>	28 (33.7)	15 (18.1)	13 (15.7)
<i>Grade 2</i>	19 (22.9)	12 (14.5)	7 (8.4)

<i>Grade 3</i>	28 (33.7)	15 (18.1)	13 (15.7)
<i>Grade 4</i>	8 (9.6)	3 (3.6)	5 (6)
<i>Subarachnoid score (%)</i>			
<i>Grade 1</i>	50 (60.2)	26 (31.3)	24 (28.9)
<i>Grade 2</i>	13 (15.7)	9 (10.8)	4 (4.8)
<i>Grade 3</i>	17 (20.5)	10 (12)	7 (8.4)
<i>Grade 4</i>	3 (3.6)	0 (0)	3 (3.6)
<i>White Matter Volume score (%)</i>			
<i>Grade 1</i>	41 (49.4)	23 (27.7)	18 (21.7)
<i>Grade 2</i>	30 (36.1)	18 (21.7)	12 (14.5)
<i>Grade 3</i>	10 (12)	4 (4.8)	6 (7.2)
<i>Grade 4</i>	2 (2.4)	0 (0)	2 (2.4)
<i>MRA abnormality (%)</i>	48 (57.8)	26 (31.3)	22 (26.5)
<i>abnormal congenital variant</i>	32 (38.6)	18 (21.7)	14 (16.9)
<i>tortuosity</i>	22 (26.5)	12 (14.5)	10 (12)
<i>abnormal ICA/VA</i>	13 (15.7)	8 (9.6)	5 (6)
<i>abnormal COW branches</i>	4 (4.8)	2 (2.4)	2 (2.4)

MRA Magnetic Resonance Angiography, ICA Internal carotid artery, VA
 Vertebral artery, COW Circle of Willis

CHAPTER 5 Discussion and future directions

This thesis explored the long-term outcomes of thromboprophylaxis use post-Fontan surgery. Specifically, this study directly compared the use of warfarin to aspirin on *stroke, bone mineral density, bleeding and quality of life*.

The major finding of this study is that stroke was not influenced by thromboprophylaxis selection however, bone mineral density and bleeding outcomes were poorer in patients on warfarin compared to aspirin. Our results suggest that based on these parameters alone, aspirin should be offered as primary long-term thromboprophylaxis after Fontan surgery, however clinical uncertainty still exists in patients with high risk features. Further, we have not compared other important outcomes like cardiac function, nor lung function which are also conceivable impacted by the choice of anticoagulant. These outcomes also need to be considered. In addition to less invasive and time-consuming treatment, a shift from warfarin to aspirin therapy would have a cost benefit to the patient, as well as to the healthcare system ⁹⁰.

An important outcome of this study was the identification of widespread nature of asymptomatic stroke and ubiquitous brain injury in the ANZ Fontan population. These injuries were found despite thromboprophylaxis type. In a previous study of infants with congenital heart disease who underwent cardiac surgery, rates of brain injury post-surgery were approximately 44%⁷⁸. Beca et al found that single ventricle patients had increased rates and severity of brain injury than in other congenital heart

disease groups ⁷⁸. Given that Fontan surgery is predominantly single ventricle palliation, this finding may explain the high rate of brain injury in our cohort. Furthermore, given the age of our participants, it is likely that our cohort represents an accumulation of brain injury over the life-course of the patient. This hypothesis is supported by previous studies that have demonstrated that detection of brain lesions increased as children progress through the surgical stages of bidirectional Glenn to Fontan surgery ⁹¹.

Due to the cross-sectional nature of our study, the timing of cerebral events was not able to be determined. Such data would be imperative to the identification of modifiable factors that may reduce the accumulation and extent of brain injury. Longitudinal follow up with cerebral imaging commencing at antenatal diagnosis, with serial imaging timed with known periods of high-risk (perioperative, pre/post cardio pulmonary bypass), would be the ideal methodology of determining the natural history of cerebral events.

The clinical, social, developmental and psychological implications of the specific brain injuries identified in the Fontan population were not assessed in this study, however they represent important and meaningful outcome data for this cohort. The impact of the brain injuries associated with the Fontan procedure are important to fully elucidate through comprehensive neurological, cognitive and motor assessments. Early identification of deficits is crucial for the successful implementation of interventions that can reduce the burden of disease and subsequently improve quality of life of patients.

Our study found that BMD was reduced across our cohort. Furthermore, the results of our study demonstrate that participants receiving warfarin have further decreased levels of BMD compared to those on aspirin. The association of reduced BMD with warfarin therapy have been reported previously however, these studies were limited by low participant numbers ^{71, 92}. Moreover, a recent study in a small cohort of Fontan patients reported associations between low BMD with cardiac dysfunction and lower oxygen saturation levels (D'Ambrosio et al. *in press* Congenital Heart Disease). These associations are not surprising given that interplay between cardiac functioning and oxygen saturation levels with physical activity, muscle mass and muscle strength, all of which have been independently correlated with BMD in children⁹³. As muscles increase during growth, bones adapt by increasing cortical dimensions, improving BMD. In light of these findings, other factors associated with reduced exercise capacity may also influence BMD. Fontan fenestration, to varying extents, has been associated with reduced oxygen saturations^{94, 95}. Whether reduced oxygen saturations cause decreased exercise capacity remains controversial. However, given that Fontan fenestration routinely performed in some surgical centers, associations between fenestration and duration of fenestration patency with BMD should be investigated.

Whether BMD associated with warfarin is direct drug effect or reflects differences in patient selection or surgical management requires further investigation.

Another important facet of this study is the ability for our data to act as a comparator for alternative and emerging antithrombotic therapy for use in Fontan patients. The use of direct oral anticoagulants (DOACs) such as rivaroxaban, dabigatran and apixaban have been approved for use as thromboprophylaxis and treatment for venous thrombosis in specific adult populations (e.g thromboprophylaxis after hip and knee replacement). Currently, trials are underway in paediatrics and include those focusing on Fontan patients. DOACs have promising efficacy profiles with favourable properties such as limited requirement for monitoring. However, trials of DOACs in adult cardiac patients have been prematurely closed due to an excess of thromboembolic and bleeding events among patients in the DOAC groups. In these studies, DOACs were trialled against standard of care in the setting of prosthetic valves ⁷⁷, Ventricular Assist Device ⁹⁶ and Transcatheter aortic valve implantation ⁹⁷. A common feature of the failed trials is that DOACs were used in the setting of prosthetic material. **Given that Fontan sub-types all contain differing amounts of prosthetic material (e.g. lateral tunnel, intra-atrial baffle; extracardiac conduit, gortex conduit), it is imperative that studies sufficiently represent each Fontan sub-type.** Studies such as ours provide valuable data for comparing emerging antithrombotics to standard of care such as warfarin and aspirin. Given the high rate of asymptomatic stroke in our cohort, cerebral MRI during DOAC trials in Fontan patients would give the best evidence of superiority/inferiority to warfarin and aspirin against stroke. Unfortunately, cerebral MRI has not been proposed as an outcome modality to date in DOAC trials. Furthermore, the utility of the WA-BAT may be extended to the DOACs, where bleeding

types and rates can be quantitated and compared to warfarin and/or aspirin as an outcome of clinical trials.

The Fontan population is growing rapidly, with Fontan palliation being offered for an expanded array of congenital cardiac abnormalities. Moreover, patients who underwent the first generation of Fontan surgeries are now entering their fourth decade of living with the Fontan circulation. The implications of the aging Fontan circuit and its impact on blood flow are an important consideration when managing thromboprophylaxis in the Fontan patient. As is the case for the broader population, age impacts negatively on flow dynamics by reduced vessel compliance, atherosclerosis and valve stenosis all of which potentiate turbulence of flow. Blood turbulence causes activation of the haemostatic system and hence increases the likelihood of thrombus formation. The impact of aging on flow dynamics is further exemplified in the Fontan heart where disruptions of flow inherently exist in the form of *cul de sacs*, dilated atria, low cardiac output and chronically elevated venous pressure. **We predict that high resolution modalities such as cardiac MRI, will play an ever increasingly important role in the assessment of cardiac functioning and fluid dynamics, and subsequently will inform thromboprophylaxis management of Fontan patients, particularly as they age.**

5.1 Clinical recommendations and conclusions

The presented study adds to current evidence with regard to the potential consequences of thromboprophylaxis therapy with warfarin and aspirin.

A number of recommendations can be made based on the findings of this study:

1. Where no further clinical complexities exist, it is reasonable to offer aspirin to Fontan patients as thromboprophylaxis. However, before one could be definitive about optimal thromboprophylaxis, consideration must be given to important clinical features such as cardiac and lung function, neither of which were assessed by this study.
2. We recommend routine BMD screening for all Fontan patients, particularly those on long-term warfarin therapy. As with all populations that are particularly vulnerable to osteopenia/osteoporosis, vitamin D sufficiency and adequate dietary calcium intake are important factors to ensure bone mass accrual and maintenance. Furthermore, patients should be encouraged to engage in weight bearing exercise, in line with their physical capabilities.
3. The WA-BAT is a comprehensive and validated measurement tool to assess bleeding in patients receiving thromboprophylaxis with warfarin or aspirin. The results of our study may act as a comparator to NOACs being trialled in the Fontan population. However, studies should be conducted to determine the WA-BATs validity in the setting of emerging NOACs.

4. When warfarin is indicated, a comprehensive anticoagulation service that facilitates home INR monitoring can be of great benefit to the patient in terms of both QoL and financially.

5.2 Limitations

An important limitation in comparing warfarin to aspirin outcomes in the Fontan population, is the geographical distribution of patients receiving each treatment. The majority of warfarin patients (91%) came from Region 1 and 2 whereas, the majority of aspirin patients (71%) came from Region 3. The possibility that differences in other aspects of care (surgical, supportive) requires further examination.

Furthermore, our study was cross-sectional in nature and therefore the timing of cerebral injury was not able to be determined. Longitudinal, serial follow-up with cerebral imaging is required to determine the natural history of cerebral events and to identify modifiable factors that may reduce such events.

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Appendix

1. Royal Children's Hospital Human Research Ethics Committee approval
2. Site Specific Ethics approval – Royal Children's Hospital
3. Site Specific Ethics approval – The Children's Hospital at Westmead
4. Site Specific Ethics approval – Royal Prince Alfred Hospital
5. Site Specific Ethics approval – Starship Children's Hospital
6. Ionising Radiation assessment, Medical Physicist's Report
7. Study recruitment invitation letter
8. Parent Guardian Information and Consent form – MRI
9. Participant Information and Consent form – MRI
10. Parent Guardian Information and Consent form – DXA
11. Participant Information and Consent form – DXA
12. Thromboprophylaxis data collection form
13. History of bone fractures data collection form
14. Food frequency questionnaires
15. Self-assessment of Tanner stage
16. Study recruitment invitation email for questionnaires (WA-BAT and QoL)
17. Warfarin and Aspirin Bleeding Assessment Tool (WA-BAT)
18. Papers published during candidature

RCH HUMAN RESEARCH ETHICS COMMITTEE APPROVAL

HREC REF. No: 33274A

PROJECT TITLE: Functional Outcomes after Fontan Surgery: A Cross-Sectional Study Examining the Impact of Anticoagulation on Quality of Life, Exercise Capacity, Cardiac Function, Neuroimaging and Bone Density

APPROVED DOCUMENTS: PIS CF v4 dated 17 June 2014
PGIS CF v4 dated 17 June 2014
Participant Recruitment Letter dated v2 dated 5 May 2014
Parent Guardian Recruitment Letter dated v2 dated 5 May 2014
Telephone script v2 dated 17 June 2014

APPROVED PROTOCOL: Research Protocol v5 dated 17 June 2014

PRINCIPAL INVESTIGATOR: Yves d'Udekem

DATE OF ORIGINAL APPROVAL: 17 June 2014

DURATION: 48 Months

DATE OF APPROVAL EXPIRY: 17 June 2018

SIGNED:



17 June 2014

COMMITTEE REPRESENTATIVE

CONDITIONS: The study must not commence until the Multi-Institutional Agreement has been executed.

APPROVED SUBJECT TO THE FOLLOWING CONDITIONS:

ALL PROJECTS

1. The study must not commence until all Research Agreements have been executed (if applicable)
2. Must comply with the [Investigator's Responsibilities in Research Procedure](#) and other Campus Research Policies and Procedures
3. Any proposed change in the protocol or approved documents or the addition of documents must be submitted to the Human Research Ethics Committee (HREC) for approval prior to implementation, including:
 - flyers, brochures, advertising material
 - Increase in recruitment target
4. The Principal Investigator must notify Research Development & Ethics of:
 - Any serious adverse effects of the study on participants and steps taken to deal with them.
 - Any unforeseen events (e.g. protocol violations or complaints).
 - Investigators withdrawing from or joining the project.
5. A progress report must be submitted annually and at the conclusion of the project.
6. RCH HREC approval must remain current for the entire duration of the project. If the project is not completed in the allocated time a renewal request must be submitted to the Research Development & Ethics. Investigators undertaking projects without current HREC approval risk their indemnity, funding and publication rights.

The Royal Children's Hospital Melbourne
50 Flemington Road
Parkville Victoria 3052 Australia
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CLINICAL TRIALS

7. Must comply with [Good Clinical Practice \(GCP\)](#)
8. Must report all internal (occurring in RCH participants) Serious Adverse Events (SAE) to the sponsor and the RCH HREC within 72 hours of occurrence.
9. Must report all Suspected Unexpected Serious Adverse Reactions (SUSARS) to the Therapeutic Goods Administration (TGA) (for sponsored studies the sponsor may take this responsibility).

Site Specific Ethics approval – The Royal Children's Hospital



The Royal Children's Hospital Melbourne
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Parkville Victoria 3052 Australia
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17 June 2014
A/Prof Yves d'Udekem
Deputy Director
Cardiac Surgery
RCH

Dear A/Prof d'Udekem

Study title: Functional Outcomes after Fontan Surgery: A Cross-Sectional Study Examining the Impact of Anticoagulation on Quality of Life, Exercise Capacity, Cardiac Function, Neuroimaging and Bone Density

HREC Reference Number: HREC/13/RCHM/26

RCH HREC Number: 33274A

Protocol version: Research Protocol v5 dated 17 June 2014

The Royal Children's Hospital Human Research Ethics Committee reviewed the above application at the meeting held on 3rd February 2014. This reviewing HREC is accredited by the Consultative Council for Human Research Ethics under the single ethical review system.

Decision of the reviewing HREC

The HREC approved the above application on the basis of the information provided in the application form, protocol and supporting documentation.

Approval

The HREC approval is from the date of this letter.

Approval is given in accordance with the research conforming to the *National Health and Medical Research Council Act 1992* and the *National Statement on Ethical Conduct in Human Research (2007)*. The HREC has ethically approved this research according to the Memorandum of Understanding between the Consultative Council and the participating organisations conducting the research.

Approval is given for this research project to be conducted at the following sites and campuses:

OTHER SITES LIST HERE

Children's Hospital at Westmead

You must comply with the conditions of ethics approval which have been listed on the RCH Approval Certificate.

The Coordinating Principal Investigator is responsible for notifying Principal Investigators. The Coordinating Principal Investigator and Principal Investigators should forward a copy of this letter to their site's Research Governance Officer.

Approved documents

For documents reviewed and approved please refer the RCH Approval Certificate.

Site-Specific Assessment (SSA)

SSA authorisation is required at all sites participating in the study. SSA must be authorised at a site before the research project can commence.

The completed Site-Specific Assessment Form and a copy of this ethics approval letter must be submitted to the Research Governance Officer for authorisation by the Chief Executive or delegate. This applies to each site participating in the research.

If you should have any queries about your project please contact Research Development & Ethics office at RCH.

The HREC wishes you and your colleagues every success in your research.

Yours sincerely



Research Development & Ethics, on behalf of the
RCH Human Research Ethics Committee

Checklist: Post-ethics approval requirements that must be met before a research project can commence at a study site.

Please ensure that as a PI (including the CPI) the following are completed at each study site.

Requirements	Yes/No/NA
Ethics approval notification The PI must send a copy to the RGO at that study site.	Yes
CTN notification The PI must sign the CTN and forward to the RGO so the authority approving the conduct of the trial, at that site, can complete and sign.	Yes
SSA authorisation notification The PI must forward the SSA form and attached documents (e.g. CTRA) to the RGO so the authority approving the conduct of the trial, at that site, can complete and sign.	Yes
Radiation If applicable, the RGO must contact the Medical Physicist to notify DHS, Radiation Safety Section to list the project on the Institute's licence.	N/A
Other Commonwealth statutory requirements Ensure compliance with the following e.g. Office of the Gene Technology Regulator, NHMRC Licensing Committee, NHMRC Cellular Therapies Advisory Committee.	N/A

The Royal Children's Hospital Human Research Ethics Committee (RCH HREC) is constituted according to the National Health and Medical Research Council's 'National Statement on Ethical Conduct in Humans Research (2007)'. The committee operates in accordance with these guidelines and is registered with the NHMRC.

17 June 2014

A/Prof Yves d'Udekem
Deputy Director
Cardiac Surgery
RCH

Dear A/Prof d'Udekem

Study title: Functional Outcomes after Fontan Surgery: A Cross-Sectional Study Examining the Impact of Anticoagulation on Quality of Life, Exercise Capacity, Cardiac Function, Neuroimaging and Bone Density

HREC Reference Number: HREC/13/RCHM/26

RCH HREC Reference: 33274A

SSA Reference Number: SSA/14/RCHM/7

Thank you for submitting a Site Specific Assessment Form for authorisation of the above project at The Royal Children's Hospital, Parkville Victoria. I can confirm that the submission was received on 20 December 2013.

I am pleased to inform you that authorisation has been granted for this project to be conducted at The Royal Children's Hospital, Melbourne. **Please note this authorisation is only valid while approval from the reviewing HREC is current.**

Documents
AU/5/3466114
Research Protocol v5 dated 17 June 2014
PIS CF v4 dated 17 June 2014
PGIS CF v4 dated 17 June 2014
Participant Recruitment Letter dated v2 dated 5 May 2014
Parent Guardian Recruitment Letter dated v2 dated 5 May 2014
Telephone script v2 dated 17 June 2014

Please note the following conditions of approval:

1. Researchers must comply with the [Investigator's Responsibilities in Research Procedure](#) and [Good Clinical Practice \(ICH GCP\)](#)
2. Any proposed change in the protocol (or approved documents) or the addition of documents must be submitted to the Reviewing Human Research Ethics Committee (RHREC) for approval prior to implementation. The PI must also submit a copy of all documents relating to approved amendments to RCH Ethics Office for governance approval.
3. The Principal Investigator must notify the 1) CPI, 2) RCH Ethics Office and sponsor (if applicable) of:
 - i. All internal (occurring in RCH participants) Serious Adverse Events (SAE) within 72 hours of occurrence.
 - ii. Any other serious adverse effects to or complaints from RCH participants and steps taken to deal with them.

- iii. RCH Investigators withdrawing from or joining the project.
NOTE: All SUSARs must be submitted to the TGA (as per the CTN guidelines).
- 4. A progress report must be submitted to RCH Ethics Office annually and at the conclusion of the project. A copy of this report should also be provided to the CPI.
- 5. RHREC approval must remain current for the entire duration of the project. Investigators undertaking projects without current RHREC approval risk their indemnity, funding and publication rights. Evidence of RHREC renewal approval will be required.
- 6. The PI must ensure that:
 - A fully executed CTN has been sent to the TGA prior to commencement and must place a copy of the TGA acknowledgment letter in the study file when received (2 weeks following submission).
 - The Clinical Trial Research Agreement and Indemnities are fully executed (i.e. signed by all parties) prior to commencement of the study.

If you have any matters that arise regarding conduct of the research at this site, please ensure you contact the Research Development & Ethics office at RCH, Melbourne.

I wish you and your colleagues every success in your research.

Yours sincerely



Research Development and Ethics

Phone : (03) 9345 5044

Email : rch.ethics@rch.org.au

Web : www.rch.org.au

Site Specific Ethics approval – The Children's Hospital at Westmead

Contact for this correspondence:

Research and Development

Ethics & Governance Administration Assistant

Phone: (02) 9845 1253

Facsimile: (02) 9845 1317

Email: ethics.schn@health.nsw.gov.au

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14 July 2015

A/Professor Yves d'Udekem
Cardiac Surgery
Royal Children's Hospital, Victoria

Dear A/Professor Yves d'Udekem,

HREC Reference: HREC/15/SCHN/249

Project title: Improving functional outcomes after Fontan surgery by a cross-sectional study of the outcomes following variation in practice in Australia and New Zealand: focus on anti-coagulation and cardiac shunting by fenestration

Sites: The Children's Hospital at Westmead

Thank you for submitting the above project for single ethical and scientific review. This project was first considered by the Executive Committee of Sydney Children's Hospitals Network Human Research Ethics Committee ("the Committee") out of session on 14 July 2015.

The HREC has been accredited by the NSW Department of Health as a lead HREC under the model for single ethical and scientific review, and by the National Health and Medical Research Council as a certified committee in the review of multi-centre clinical research projects..

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's *National Statement on Ethical Conduct in Human Research* and *CPMP/ICH Note for Guidance on Good Clinical Practice*.

I am pleased to advise that the Committee has granted ethical approval of this research project. Your approval is valid for five (5) years, effective the date of this latter.

The documents reviewed and approved by the Committee are:

Document	Version	Date
NEAF, submission Code: AU/1/4CFF18		10 July 2015
Background Information Questionnaire	1	03 October 2014
Booking confirmation email ParentGuardian BONE DENSITY v1 10072015	1	10 July 2015
Booking confirmation email ParentGuardian MRI v1 10072015	1	10 July 2015
Booking confirmation email Participant BONE DENSITY v1 10072015	1	10 July 2015
Booking confirmation email Participant MRI v1 10072015	1	10 July 2015
Booking confirmation letter ParentGuardian BONE DENSITY v1 10072015	1	10 July 2015
Booking confirmation letter ParentGuardian MRI v1 10072015	1	10 July 2015
Booking confirmation letter Participant BONE DENSITY v1 10072015	1	10 July 2015

Document	Version	Date
Booking confirmation letter Participant MRI v1 10072015	1	10 July 2015
Calcium Food Frequency Questionnaire dated 2007		2007
PGIS and CF BONE DENSITY NSW v1 01062015	1	01 June 2015
PGIS and CF MRI NSW v1 01072015	1	01 July 2015
PIS and CF BONE DENSITY NSW v1 01062015	1	01 June 2015
PIS and CF MRI NSW v101072015	1	01 July 2015
ParentGuardian Recruitment letter	1	10 July 2015
Participant Recruitment letter	1	10 July 2015
Email Invitation QoL v1 10072015	1	10 July 2015
Email Invitation ParentGuardian NSW v1 09072015	1	09 July 2015
Email Invitation Participant NSW v1 09072015	1	09 July 2015
Telephone script v3 22092014	3	22 September 2014
Telephone script Eligibility questions Phase C v1 10072015	1	10 July 2015
Telephone script Eligibility questions Phase A v1 10072015	1	10 July 2015
MRI pre-scan questionnaire	4	February 2012
GHQ12 v1 16122014	1	16 December 2014
MSQRRQ Phone Interview v1	1	16 December 2014
Tanner Stages dated 2004		2004
Signed Ionising Radiation Declaration 26032015	1	26 March 2015
Signed Radiation Dosimetry Report 26032015	1	26 March 2015
Peer Review Functional Outcomes after Fontan Surgery AP		
Health Status Scales EQ-5D-3L (adults) & CHU-9D (children)		1990 & 2008
PedsQL (Paediatric Quality of Life Inventory) v4 (includes parent proxy TEEN PedsQL; PedsQL 13-18; PedsQL Adult; PedsQL Young Adult)	4	2000
PedsQL cardiac module v3 & 4b (includes parent proxy PedsQL cardiac module; PedsQL cardiac module)	3&4	2000
PedsQL family impact module v2	2	2009

Please note the following conditions of approval:

1. The Coordinating Investigator will immediately report anything which may warrant review of ethical approval of the project in accordance with the SCHN adverse event reporting policy.
2. All proposed changes to the research protocol, including the conduct of the research, changes to site or personnel, or an extension to HREC approval, are to be provided to the HREC or its delegate for review before those changes can take effect.
3. The HREC will be notified, giving reasons, if the project is discontinued at a site before the expected date of completion.
4. The co-ordinating investigator will provide an annual report to the HREC on the anniversary of this approval letter, and a final report on completion of the study.
5. Your approval is valid for five (5) years from the date of the final approval letter. If your project extends beyond that five year period and you are still actively recruiting you will be required to resubmit your application incorporating any amendments within six (6) months of

that approval expiry date. If your project is in follow up on, or analysis, please submit and application for amendment to extend the approval period. Ethics approval can be extended for a period of twelve (12) months at a time.

6. In the event of a project **not having commenced** within 12 months of its approval, the approval will lapse and reapplication to the HREC will be required.

Should you have any queries about the HREC's consideration of your project please contact the Ethics Administration Assistant on (02) 9845 1253.

You are reminded that this letter constitutes ethical approval only. You must not commence this research project at a site until separate authorisation from the Chief Executive or delegate of that site has been obtained. A copy of this letter must be forwarded to all site investigators for submission to the relevant Research Governance Officer.

The SCHN HREC wishes you every success in your research.

Yours faithfully



**Ms Jillian Shute
Executive Officer
Sydney Children's Hospitals Network Human Research Ethics Committee**

NB: All clinical trials must now be registered on a publicly accessible registry such as the Australian New Zealand Clinical Trials Registry. For further information please go to www.anzctr.org.au. Please provide this office with a copy of your registration number for our records if you have not already done so.

Site Specific Ethics approval – Royal Prince Alfred Hospital

ADDRESS FOR ALL CORRESPONDENCE
RESEARCH ETHICS AND GOVERNANCE OFFICE
ROYAL PRINCE ALFRED HOSPITAL
CAMPERDOWN NSW 2050



Health
Sydney
Local Health District

TELEPHONE: (02) 9515 7899
EMAIL: maree.larkin@sswahs.nsw.gov.au
REFERENCE: X17-0033

27 February 2017

Dr Rachael Cordina
Department of Cardiology
Level 6, Building 75
Royal Prince Alfred Hospital

Dear Dr Cordina,

Re: Protocol No X17-0033- "Improving functional outcomes after Fontan surgery by a cross-sectional study of the outcomes following variation in practice in Australia and New Zealand: focus on anti-coagulation and cardiac shunting by fenestration"

HREC/15/SCHN/249

SSA/17/RPAH/39

Thank you for submitting a Site Specific Assessment Form (AU/2/2CAB215) for this study. I am pleased to inform you that the delegate of the Chief Executive has granted authorisation for it to be undertaken at the **Royal Prince Alfred Hospital**.

The following documents are authorised for use at the site:

- **Participant Information Statement and Consent Form (NSW Version 4, 20 October 2016)**

The following conditions apply to this research study. These are additional to those conditions imposed by the human research ethics committee (HREC) that granted ethical approval:

1. **A copy of the annual report** and any other reports to the approving HREC, accompanied by a copy of the HREC's acknowledgement letter, **must be provided to me for review**.
2. When required, the appropriate documentation must be submitted to me for authorisation before any new external researcher is authorised to be on-site for the project.
3. Proposed amendments to the research protocol or conduct of the research, which may affect the ethical acceptability of the study and which are submitted to the lead HREC for review, must be copied to me.
4. Proposed amendments to the research protocol or conduct of the research, which may affect the ongoing site acceptability of the study, must be submitted to me.

I wish you every success in your research.

Yours sincerely,

Maree Larkin
Research Governance Officer
SLHD (RPAH Zone)

RGO - Maree\CORRES\X17-0033

Sydney Local Health District
ABN 17 520 269 052
www.slhd.nsw.gov.au

Site Specific Ethics approval – Starship Children’s Hospital



Health and Disability Ethics Committees

Ministry of Health
Freyberg House
20 Aitken Street
PO Box 5013
Wellington
6011

0800 4 ETHICS
hdec@moh.govt.nz

27 January 2015

Dr Tom Gentles
Paediatric and Congenital Cardiac Service,
Starship Children's Health,
Private Bag 92024
Starship Children's Hospital 1142

Dear Dr Gentles

Re:	Ethics ref:	14/STH/176
	Study title:	Improving functional outcomes after Fontan surgery by a crosssectionalstudy of the outcomes following variation in practice in Australia and New Zealand: focus on anticoagulation and cardiac shunting by fenestration

I am pleased to advise that this application has been approved by the Southern Health and Disability Ethics Committee. This decision was made through the HDEC-Full Review pathway.

Conditions of HDEC approval

HDEC approval for this study is subject to the following conditions being met prior to the commencement of the study in New Zealand. It is your responsibility, and that of the study's sponsor, to ensure that these conditions are met. No further review by the Southern Health and Disability Ethics Committee is required.

Standard conditions:

1. Before the study commences at *any* locality in New Zealand, all relevant regulatory approvals must be obtained.
2. Before the study commences at *a given* locality in New Zealand, it must be authorised by that locality in Online Forms. Locality authorisation confirms that the locality is suitable for the safe and effective conduct of the study, and that local research governance issues have been addressed.

After HDEC review

Please refer to the *Standard Operating Procedures for Health and Disability Ethics Committees* (available on www.ethics.health.govt.nz) for HDEC requirements relating to amendments and other post-approval processes.

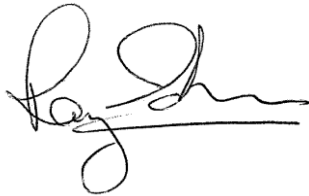
Your next progress report is due by 27 January 2016.

Participant access to ACC

The Southern Health and Disability Ethics Committee is satisfied that your study is not a clinical trial that is to be conducted principally for the benefit of the manufacturer or distributor of the medicine or item being trialled. Participants injured as a result of treatment received as part of your study may therefore be eligible for publicly-funded compensation through the Accident Compensation Corporation (ACC).

Please don't hesitate to contact the HDEC secretariat for further information. We wish you all the best for your study.

Yours sincerely,

A handwritten signature in black ink, appearing to read 'Raewyn Idoine', with a horizontal line drawn underneath the signature.

Ms Raewyn Idoine
Chairperson
Southern Health and Disability Ethics Committee

Encl: appendix A: documents submitted
 appendix B: statement of compliance and list of members

Appendix A

Documents submitted

<i>Document</i>	<i>Version</i>	<i>Date</i>
Protocol: Cross Sectional MRI Study	1	11 October 2014
Telephone Script	1	11 October 2014
Budget	1	11 October 2014
Funding	1	11 October 2014
Survey/questionnaire: PedsQLTMGenericCoreScales version 4.pdf	4	11 October 2014
Survey/questionnaire: CDI 2 Self Report Long-WATERMARKED.pdf	1	11 October 2014
Survey/questionnaire: NZ questionnaires for cross sectional study.pdf	1	11 October 2014
Survey/questionnaire: PedsQLTMCardiacmodule version 3.pdf	3	11 October 2014
Survey/questionnaire: PedsQLTMCardiacmodule version 4b TEEN.pdf	4b	11 October 2014
Survey/questionnaire: PedsQLTMFamilyImpactModule version 2.pdf	2	11 October 2014
Supporting Dept WH.pdf	1	11 October 2014
Medical Physics report 20140818.pdf	1	11 October 2014
Module 4 Version 2 UoM.pdf	2	11 October 2014
Module 4 Version 2 WH.pdf	2	11 October 2014
Supporting Dept UoM.pdf	1	11 October 2014
PIS/CF: NZ cross sectional study Assent form 12-15 year old V1_11102014.doc	2	13 January 2015
PIS/CF: CliiSandCFParentGuardian_v1_11102014BONE.doc	2	13 January 2015
PIS/CF: CliiSandCFParentGuardian_v1_11102014MRI.doc	2	13 January 2015
PIS/CF: CliiSandCFParticipant_v1_11102014BONE.doc	2	13 January 2015
PIS/CF: CliiSandCFParticipant_v1_11102014MRI.doc	2	13 January 2015
CVs for other Investigators: Kathryn's CV.doc	1	16 October 2014
CVs for other Investigators: Michelle Holmes	1	16 October 2014
CVs for other Investigators: P Hofman CV	1	16 October 2014
CV for CI: CV Tom Gentles	1	17 October 2014
Approval Certificat RCH Melbourne	1	17 October 2014
AuRED Approval	1	17 October 2014
AuRED SSA	1	17 October 2014
CVs for other Investigators: CV Tim Hornung	1	23 October 2014
Evidence of scientific review	1	29 October 2014
Application		
Outstanding Ethical Issues addressed following HDEC Committee Discussion.	1	16 January 2015
Covering Letter: Cover Letter Addressing Questions around the study involving non-consenting participants.	1	16 January 2015
Response to Request for Further Information	1	-

Appendix B

Statement of compliance and list of members

Statement of compliance

The Southern Health and Disability Ethics Committee:

- is constituted in accordance with its Terms of Reference
- operates in accordance with the *Standard Operating Procedures for Health and Disability Ethics Committees*, and with the principles of international good clinical practice (GCP)
- is approved by the Health Research Council of New Zealand's Ethics Committee for the purposes of section 25(1)(c) of the Health Research Council Act 1990
- is registered (number 00008713) with the US Department of Health and Human Services' Office for Human Research Protection (OHRP).

List of members

<i>Name</i>	<i>Category</i>	<i>Appointed</i>	<i>Term Expires</i>
Ms Raewyn Idoine	Lay (consumer/community perspectives)	01/07/2012	01/07/2015
Mrs Angelika Frank-Alexander	Lay (consumer/community perspectives)	01/07/2012	01/07/2015
Dr Sarah Gunningham	Non-lay (intervention studies)	01/07/2012	01/07/2015
Assc Prof Mira Harrison-Woolrych	Non-lay (intervention studies)	01/09/2014	01/09/2015
Dr Fiona McCrimmon	Lay (the law)	01/09/2014	01/09/2015
Dr Nicola Swain	Non-lay (observational studies)	01/07/2012	01/07/2015
Dr Devonie Waaka	Non-lay (intervention studies)	01/07/2013	01/07/2016
Dr Mathew Zacharias	Non-lay (health/disability service provision)	01/07/2012	01/07/2015

<http://www.ethics.health.govt.nz>

Ionising Radiation assessment, Medical Physicist's Report



Western Health

WESTERN HEALTH
Medical Physics
Sunshine Hospital, St. Albans
Victoria 3021
AUSTRALIA
ABN: 61 166 735 672
Phone: (03) 8345 0532
Facsimile: (03) 8345 1620
Email: leah.biffin@wh.org.au

18 August 2014

Chantal Attard BSc (Hons)
PhD Scholar, Department of Paediatrics, The University of Melbourne
Research Assistant, Haematology Research, Murdoch Childrens Research Institute

Dear Chantal

Study: Functional Outcomes after Fontan Surgery

HREC study: HREC/13/RCHM/26

Principal Investigator: A/Prof Yves d'Udekem

The Victorian Department of Health requires that as a condition of licence to conduct research involving the exposure of humans to ionising radiation that the research is carried out in accordance with ARPANSA RPS 8 'Code of Practice for Exposure of Humans to Ionising Radiation for Research Purposes'¹ (hereafter referred to as 'the Code'). For projects that require the research volunteer to undergo radiation exposure that is additional to the normal care for the condition being treated and/or investigated, the Code requires that an independent dose assessment is undertaken by an approved medical physicist.

The research protocol for the abovementioned study states that volunteers may undergo procedures involving the use of ionising radiation during the study period, as shown in Table 1.

Table 1: Proposed procedures for study

Procedure	Frequency	Standard or Additional care	Estimated radiation dose	Licensed site for radiation procedure
Wrist X-ray (left)	one only	Additional	Less than 0.01 mSv	Western Health
DXA hips (both sides)	one only	Additional	Less than 0.02 mSv	University of Melbourne
DXA spine	one only	Additional	Less than 0.01 mSv	University of Melbourne
DXA whole body	one only	Additional	Less than 0.01 mSv	University of Melbourne
pQCT of forearm and tibia	one only	Additional	Less than 0.01 mSv	University of Melbourne

As per advice received from yourself, the scans are considered to be additional to standard care procedures.² This means that if the volunteer were not enrolled in this study, they would not undergo these procedures as part of their normal care within the timeframe detailed in the research protocol.

The duration of the trial is advised to be up to one month. The volunteers have a life expectancy of greater than five years.² Participants are aged between 10 and 40 years, hence this study includes paediatric patients.

Based on typical parameters, it is estimated that the volunteers may receive radiation dose as outlined in Table 1.^{3,4} It is therefore estimated that a volunteers undergoing the trial at Western Health and University of Melbourne may receive a radiation dose of less than 0.06 mSv from the five examinations throughout the duration of the trial.

The Code specifies dose constraints, which should be met wherever possible, for radiation exposure that is additional to standard care. For research participants with a life expectancy greater than five years, the total effective dose for adults should not exceed 5 mSv in any one year or 10 mSv over a period of five years. For

children the dose constraint is 0.5 mSv in any one year with a maximum limit during childhood (up to age 18 years) of 5 mSv. Where these dose constraints are exceeded the Human Research Ethics Committee is advised to give particular attention to the justification for the radiation exposure, and if necessary, seek further independent authoritative advice before approving the proposal. Where the dose constraints are exceeded, obtain verification of the dose assessment by a second medical physicist.

The estimated radiation dose for each volunteer in this study does not exceed the dose constraint criteria in the Code for participants in research with a life expectancy less than five years. Based on the estimated dose of less than 0.06 mSv for the Western Health and University of Melbourne sites, the research project is classified by the Code as being in Category I. This classification carries a level of risk described as 'Minimal' (i.e. the risk is less than 1 in 100,000). The Code describes exposures in category I as follows:

The dose range for this project category is less than 0.2 mSv which is the dose delivered by natural background radiation in a few weeks. It is considerably less than the variations in annual dose from natural background radiation to persons living in different locations, and the risk level is considered minimal. The level of benefit needed as the basis for approval of research with doses in this category will be minor and will include those investigations expected only to increase knowledge.

Recommendations

- Participant Information and Consent Form (PICF) risk statement or plain language statement provided to volunteers
 - The following statement outlining the risks associated with this study should be included in risk statement section of the plain language statement or PICF.

This research study involves exposure to a very small amount of radiation. As part of everyday living, everyone is exposed to naturally occurring background radiation and receives a dose of about 2 millisievert (mSv) each year. The effective dose from this study is less than 0.06 mSv. At this dose level, no harmful effects of radiation have been demonstrated as any effect is too small to measure. The risk is believed to be minimal.

- Notification to the Radiation Safety Section, Victorian Department of Health
 - This research project involves the exposure of human volunteers to ionising radiation that is additional to their standard clinical care. Therefore after approval is received from the Ethics Committee a notification form for the project must be submitted to the Western Health Radiation Safety Officer.
 - The notification is then passed on the Department of Health Radiation Safety Section for assessment. Commencement of imaging may take place after this form has been submitted.
 - Appropriate notification forms are available by contacted the Research Office or the Western Health Radiation Safety Officer.

Please do not hesitate to contact me if you require further information.

Kind Regards



Leah Biffin
Western Health Radiation Safety Officer / Medical Physicist
Department of Radiology
Western Health

References

1. Australian Radiation Protection and Nuclear Safety Agency (ARPANSA), Radiation Protection Series No. 8 Code of Practice for the Exposure of Humans to Ionising Radiation for Research Purposes, 2005
2. Email correspondence from Chantal Attard, 18/08/2014
3. Conference of Radiation Control Program Directors, Inc Publication E-06-5, *Technical White Paper: Bone Densitometry*, October 2006
4. Lewis M., Blake G., Fogelman I., *Patient Dose in Dual X-ray Absorptimetry*, Osteoporosis Int. (1994) 4:11-15

Study recruitment invitation letter

The Royal Children's Hospital Melbourne
50 Flemington Road
Parkville Victoria 3052 Australia
TELEPHONE +61 3 9345 5522
www.rch.org.au



Number/Street Name
Suburb
State/Postcode

Date

Dear Parent/Guardian of child's name,

Research Project Title: Functional Outcomes after Fontan Surgery

We are writing to let you know about a new research project which you and your child may be interested in participating.

Why are you being asked?

We are sending you this information because you may have agreed to be contacted about new research projects when you joined the Australia and New Zealand Fontan Registry and your child may be suitable to take part.

What is the research about?

The project involves several phases (*see page over*) and participation in any of the tests are optional. We have enclosed an **information statement and consent form** describing Phase A of the project (bone density). The form explains the research in detail, including:

- What we aim to learn from the research
- What is involved if you take part
- What the risks and benefits of participation are
- What happens to information collected and how confidentiality of information is protected

What do you need to do?

We would be grateful if you could take some time to read the information statement and consider taking part.

Please call or email us on the details below to let us know if you are interested in taking part or not. Or alternatively fill out the consent form included with this letter and post it back to us in the reply paid envelope provided. If we do not hear from you in the *next 2 weeks*, we will contact you to check you received this letter and tell you more about the project.

Kind regards,

Fontan Registry Research Team
Heart Research

SMS: 0431 659 911
T: (03) 9345 6161 (Karin) or (03) 9345 4236 (Janina)
E: info@fontanregistry.com

This Fontan research project will be conducted in 3 phases (see below).

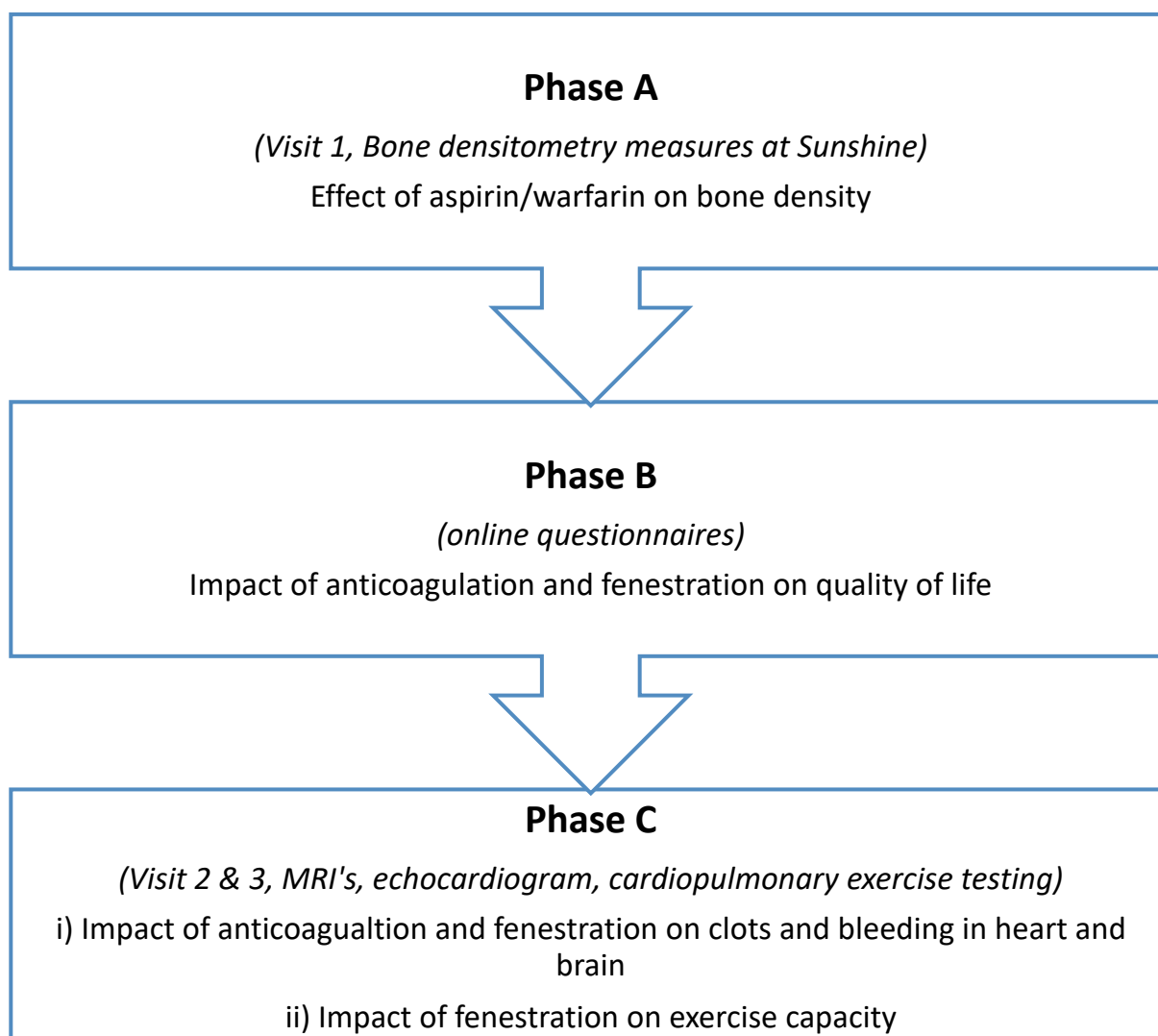
Your child is welcome to participate in any or all phases of the project.

Please contact us to let us know if they might be interested in participating:

SMS: 0431 659 911

T: (03) 9345 6161 (Karin) or (03) 9345 4236 (Janina)

E: info@fontanregistry.com



Information statement and consent form

HREC Project Number: HREC 33274

Research Project Title: Functional Outcomes after Fontan Surgery – PHASE C

Principal Researcher: A/Prof Yves d'Udekem, Consultant Cardiac Surgeon

Version Number: 11 **Version Date:** 01/08/2016

Thank you for taking the time to read this **Parent/Guardian Information Statement and Consent Form**. We would like to invite your child to participate in a research project that is explained below. This document is **6 pages long**. Please make sure you have all the pages.

If you speak a language other than English and would like this Information Statement and Consent Form in your language, please ask the person explaining this research project to you.

What is an Information Statement?

These pages tell you about the research project. It explains to you clearly and openly all the steps and procedures of the project. The information is to help you decide whether or not you would like your child to take part in the research. Please read this Information Statement carefully.

Before you decide if you want your child to take part or not, you can ask us any questions you have about the project. You may want to talk about the project with your family, friends or health care worker.

If you would like your child to take part in the research project, please sign the consent form at the end of this information statement. By signing the consent form you are telling us that you:

- understand what you have read
- had a chance to ask questions and received satisfactory answers
- consent to your child taking part in the project or its subcomponents.

We will give you a copy of this information and consent form to keep.

1. What is the research project about?

This research project will study the overall health of patients who have had a Fontan procedure. We will also look at factors that contribute to long-term health in the Fontan population including blood thinning medications and fenestration. Areas we are investigating include:

1. Comparing the blood thinning medications, warfarin and aspirin, to look at how well they work at preventing blood clots and if they cause any bleeding.
2. Comparing those patients who have a fenestration (a hole placed between the Fontan circuit and the heart to relieve the pressure) in their Fontan pathway to those that don't in regards to exercise ability and any changes in the blood vessels and neurocognitive function of the brain.

We hope 157 adolescents and young adults will take part in this research project across Australia and New Zealand.

2. Who is funding this research project?

The project is being funded by a National Health and Medical Research Council research grant, as well as a HeartKids Grants-in-Aid Grant.

3. Why is my child being asked to be in this research project?

Your child is being asked to be in this project because you have had a Fontan procedure more than 5 years ago, are taking either aspirin or warfarin, and have agreed to be contacted about research projects when you joined the Australia and New Zealand Fontan Registry.

4. What does participation in this research involve?

You will be telephoned by the recruitment coordinator to arrange an appointment for your child at the Royal Children's Hospital.

The tests for this project will take place over two hospital visits. The length of time of each visit will depend on which tests you have consented for your child to participate in. The tests will be spread over the two days and it is likely that each visit will last between 2-3 hours.

Upon your child's arrival the recruitment coordinator will greet you and explain the project to you and your child in further detail to ensure you understand what is involved when your child participates in this research study. The recruitment coordinator will guide you through the tests you choose your child to participate in.

All the tests and procedures that are part of this research project are optional. This means you may give permission to complete all, some or even only one of the following tests. You will need to complete the Optional Consent tick boxes on the attached consent form to show which tests you agree to or not.

The tests in this study will include the following over 2 visits at Royal Children's Hospital:

- A brain MRI scan- This test takes approximately 30mins

MRI is short for magnetic resonance imaging. The MRI scanner uses a very strong magnet to take clear pictures of the inside of the body. Unlike an X-ray or CT scan, an MRI scan involves no radiation. It is a safe, painless and non-invasive test.

The MRI scanner is a large machine that is shaped like a big tube and is open at both ends. It has a bed to lie on and a head rest. The bed moves into the magnetic cylinder so that the part of the body that is scanned is in the centre.

- Since the MRI machine is a big magnet, care must be taken not to take any metal into the scanner room. Before your scan, we will ask you to complete an MRI safety questionnaire with your child. On the day of the MRI scan, one of the MRI Technologists will go through the safety questionnaire again with you.
- Before the scan your child will need to remove anything made of metal, for example jewellery, hair pins, watches etc. They may also need to remove make up, hair extensions or glitter as these items interfere with MRI pictures.
- We will ask your child to lie on the bed and we will make sure they are comfortable.

- We will give your child some earphones because the scanner makes loud knocking noises while the pictures are taken. They can watch a video or listen to music during the scan.
- Your child will need to lie very still while the pictures are taken.

- A heart MRI

This test is the same as the above but will be over your child's chest instead. This test will take approximately 45 minutes, and will include insertion of a cannula in your arm to administer a contrast dye.

- An exercise MRI

This test will take approximately 45 minutes. This test is done to see how well your child's heart is working and how well the blood is flowing through the heart during exercise. This machine is similar to the above MRI machine however the table your child will lie on will have pedals attached. Your child will be instructed to cycle while they are in the MRI scanner and the difficulty will be increased slowly.

- An exercise test

This test measures your child's fitness level and also how well their heart and lungs work while they exercise. Your child will be asked to cycle on an exercise bike as the difficulty is increased. ECG (electrocardiogram) and blood pressure will be recorded before, during and after the test and he/she will be asked to breathe in and out through a mouthpiece.

Your child will also be asked to do a 6 minute walk test where we will see how far he/she can walk in 6 minutes.

The exercise tests will take approximately 40 minutes in total.

We ask that for the exercise test your child is fasted (no breakfast, just clear fluids – if your appointment is first thing in the morning, or a light breakfast and fasted for 4 hours if your appointment is in the afternoon).

- 24-hour blood pressure monitoring – completed at home

We will fit the 24-hour blood pressure monitoring system during your child's visit to RCH and give you instructions on how to use it. This will show changes in blood pressure in daily life. After wearing it for 24 hours your child can take the BP monitor off his/her arm and send it back by post to the hospital. The monitor automatically inflates and measures and records blood pressure about every 20 minutes during the day and once every half hour at night.

Your child should continue their normal daily activities, but swimming and contact sports such as wrestling and football are to be avoided for the 24 hour duration of the blood pressure monitoring.

You/your child should also keep a journal (see Figure 1) indicating times and duration of activities and events that may influence blood pressure readings, including stressful situations and light exercise, and also when he/she takes their ACE-inhibitor medication.

We will include detailed instructions on how to use the blood pressure machine plus the 24-hour journal in the pack that we give you. Upon completion of the 24 hour monitoring you can mail the blood pressure machine and journal sheet back to us in the reply-paid parcel post bag.

24-Hour Blood Pressure Monitor Journal

Name: _____ Time Asleep: _____

Date: _____ Time Awake: _____

Where are you?				What are you doing?						
What Time is it?	Home	Work/School	Other (specify)	ACE inhibitor medication	Light activity	Relaxing	Eating	Driving	Stressed	Other

- **Figure 1: Blood pressure journal** Echocardiogram

This test is done to check the structure of the heart and how well it is functioning. This test uses sound to make a picture of your heart. The echocardiogram will take about 60 minutes. An electrocardiogram (ECG) will also be done at this time and your blood pressure will be measured.

- Neurocognitive Testing

These tests are done to assess brain function and thought process. We want to understand whether anticoagulation or fenestration has an impact on cognition. The tests are done by your child on a computer and will take about 30 minutes. We will explain to your child how to do the test. We will also ask you whether your child has a friend or sibling (similar age and gender) who you will be willing to nominate to participate in the neurocognitive testing (we will still need to get their permission too). The friend/sibling match helps us to control for other factors which might influence the results (e.g., age, gender etc.). This is not simply an intelligence test and you will not be provided with a score. Reports will be highly confidential. If we find that significant abnormalities exist we will provide a report to your treating specialist so that they can arrange appropriate follow-up that might help with your child's neurocognitive development.

- Storage of Information in the Fontan Registry

Information that is collected during this study will be stored in the Fontan Registry database for future reference and for its use in future research with your consent. The information collected will be added to the current information that we have regarding your child's medical condition and will help give us more information about your child's health and your condition.

5. What are my child's alternatives to taking part?

Participation in a research project is voluntary. It is your choice to let your child take part in this research. You do not have to agree if you do not want them to.

If you give your consent and change your mind, your child can withdraw from the project at any time. You do not need to tell us the reason why you or your child wants to stop participating in the project. If your child leaves the project we will use any information already collected unless you tell us not to.

Whatever your decision, it will not affect any treatment or care your child gets, or your family's relationship with The Royal Children's Hospital.

6. What are the possible benefits for my child and other people in the future?

We are unable to say if there will be any direct benefit to your child. The tests we are doing are for research purposes but these tests might pick up early signs of problems with your child's blood vessels or bone density that may not yet have been discovered. If this happens, we will offer appropriate medical follow-up and treatment.

We hope this project will show us what are the best medications and treatments for patients after having a Fontan procedure. This sort of information may change the way doctors perform Fontan operations as well as how doctors monitor people who have had a Fontan procedure.

7. What are the possible risks, side-effects, discomforts and/or inconveniences?

MRI risks

To date, there are no known long-term health risks related with MRI scans and it is considered a safe procedure when performed at a centre with appropriate guidelines, such as The Royal Children's Hospital.

The MRI scanner has a strong magnet. The magnetic attraction for some metal objects can pose a safety risk. It is important that metal objects are removed and not taken into the scanner room. You must tell us if your child has metal implanted in their body, such as a pacemaker or metal pins after being involved in an accident.

For the heart MRI a cannula will be placed in your child's arm for the administration of gadolinium (non-radioactive contrast dye), your child will be offered a local anaesthetic (Angel) cream if required. Gadolinium poses a low risk for a minor contrast reaction (less than 0.05%) or for an anaphylactic reaction (0.03% - 0.1%). Most injections of intravenous contrast dye occur uneventfully.

While it is operating the scanner is noisy but your child will wear earphones to protect their ears. The scanner is shaped like a long tube and may cause some people to feel cramped or claustrophobic. Some people can feel anxious during the scan. If your child feels anxious we will stop the scan.

The scans we are taking are for research purposes. They are not meant to be used to help diagnose, treat or manage a particular medical condition. After the MRI scan a radiologist will look at your MRI scans for unusual features or unexpected findings. If the radiologist finds something that needs further examination, a referral to an appropriate medical doctor will be organised. The discovery of an unusual feature could have consequences for you and may affect your ability to work in certain professions or get life insurance cover. However, the discovery of an unusual feature may also help you to get treatment that may be of benefit. If anything unexpected is found on your MRI scan, we will contact you to talk about it.

Exercise test & blood pressure monitoring:

Exercise testing is a routine clinical investigation. Risks of exercise testing are very low in people with a Fontan circulation. Some of the more serious risks include abnormal heart beat, chest pain or discomfort, low blood pressure, fainting or heart attack. There is a small risk of muscle or joint injury. If your child feels unwell during the testing he/she will be allowed to stop. The staff who will be performing the testing are trained to deal with any complications. Although there are no risks associated with the 24-hour blood pressure monitoring test, it is possible that your child may find it uncomfortable and he/she may wake up at night when it takes readings.

Receiving health information:

Some people may find it distressing to receive information about their child's health.

If you or your child wish to discuss any personal issues or require supportive counselling as a result of your participation in this study, we can refer you to an independent counsellor. We are also happy to discuss any concerns you may have.

Inconvenience

The main inconvenience is the time to come to The Royal Children's Hospital on two separate occasions to complete the tests for this research study. We will try to organise visits at a time that suits you best.

Reimbursement of costs:

1. All participants will be given a voucher to cover the cost to park at The Royal Children's Hospital on study visit days if they choose to park here.
2. We have some additional, limited funding to assist with travel and accommodation, which if required should be discussed with the research team. This assistance will be a maximum of \$1800 per child participant (which includes travel and accommodation for one child participant plus one parent/guardian) and will be organised by the research team. Please note, once this funding runs out, we will not be able to provide financial assistance for this.

8. What will be done to make sure my child's information is confidential?

In this study we will collect and use personal and health information about your child for research purposes. Any information we collect that can identify your child will be treated as confidential and used only in this project unless otherwise specified. We can disclose the information only with your permission, except as required by law.

All information will be stored securely in the Heart Research department at the Murdoch Children's Research Institute.

The following people may access information collected as part of this research project:

- the research team involved with this project
- The Royal Children's Hospital Human Research Ethics Committee

The stored information will be re-identifiable. This means that we will remove identifying information such as your child's name and give the information a special code number. Only the research team can match your child's name to their code number, if it is necessary to do so.

We are required to keep information collected as part of a research project for a certain length of time. Because the participants in this project are under 18 years old, we must keep information until the youngest participant turns 25 years old. The research information may be destroyed or kept indefinitely in secure storage after this time.

In accordance with relevant Australian and/or Victorian privacy and other relevant laws, you have the right to access and correct the information we collect and store about your child. Please contact us if you would like to access this information.

At the end of the study, results may be presented at conferences or published in medical journals. This will be done in such a way that your child cannot be identified.

9. Will we be informed of the results when the research project is finished?

We will send you a summary of the overall project results. The summary will be of the whole group of research study participants, not your child's individual results.

If you would like more information about the project or if you need to speak to a member of the research team in an emergency please contact:

Name: A/Prof Yves d'Udekem

Contact telephone: (03) 9345 5200

If you have any concerns and/or complaints about the project, the way it is being conducted or your child's rights as a research participant, and would like to speak to someone independent of the project, please contact:

Director, Research Ethics & Governance, The Royal Children's Hospital Melbourne on telephone: (03) 9345 5044.

CONSENT FORM (Parent/Guardian)

HREC Project Number: HREC 33274

Research Project Title: Functional Outcomes after Fontan Surgery

Version Number: 11 **Version Date:** 01/08/2016

- I have read, or had read to me in my first language, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my child's involvement in this project.
- I voluntarily consent for my child to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by The Royal Children's Hospital Melbourne Human Research Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007).
- I understand I will receive a copy of this Information Statement and Consent Form.

☐ I do	☐ I do not	consent to my child having a brain MRI scan
☐ I do	☐ I do not	consent to my child having a cardiac MRI scan
☐ I do	☐ I do not	consent to my child having a cardiac exercise MRI scan
☐ I do	☐ I do not	consent to my child having contrast dye (i.e., gadolinium) injected for the heart MRI scan
☐ I do	☐ I do not	consent to my child doing an exercise stress test
☐ I do	☐ I do not	consent to my child participating in 24-hour blood pressure monitoring
☐ I do	☐ I do not	consent to my child having an echocardiogram
☐ I do	☐ I do not	consent to my child completing computer-based neurocognitive testing
☐ I do	☐ I do not	consent to my child's re-identifiable results/tests performed in this study to be stored in the Fontan Registry which may be used in future ethically approved research related to Fontan surgery .

Child's Name

For neurocognitive testing - Sibling or Friend nomination (similar age & gender) **Name & contact number*** (**will still need to be contacted and complete a separate consent form*)
☐ Tick if you have checked with your friend/sibling that it is OK to share their details with us

Parent/Guardian Name

Parent/Guardian Signature

Date

Parent/Guardian Mobile Phone Number

Other Phone Number

Parent/Guardian email address

Name of Witness to Parent/Guardian's Signature

Witness Signature

Date

Note: All parties signing the Consent Form must date their own signature.

Declaration by researcher: I have explained the project to the parent/guardian who has signed above, and believe that they understand the purpose, extent and possible risks of their child's involvement in this project.

Research Team Member Name

Research Team Member Signature

Date

Information statement and consent form

HREC Project Number: HREC 33274E

Research Project Title: Functional Outcomes after Fontan Surgery – PHASE C

Principal Researcher: A/Prof Yves d'Udekem, Consultant Cardiac Surgeon

Version Number: 11

Version Date: 01/08/2016

Thank you for taking the time to read this **Participant Information Statement and Consent Form**. We would like to invite you to participate in a research project that is explained below. This document is **7 pages** long. Please make sure you have all the pages.

If you speak a language other than English and would like this Information Statement and Consent Form in your language, please ask the person explaining this research project to you.

What is an Information Statement?

These pages tell you about the research project. It explains to you clearly and openly all the steps and procedures of the project. The information is to help you to decide whether or not you would like to take part in the research. Please read this Information Statement carefully.

Before you decide to take part or not, you can ask us any questions you have about the project. You may want to talk about the project with your family, friends or health care worker.

If you would like to take part in the research project, please sign the consent form at the end of this information statement. By signing the consent form you are telling us that you:

- understand what you have read
- had a chance to ask questions and received satisfactory answers
- consent to taking part in the project or its subcomponents.

We will give you a copy of this information and consent form to keep.

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**Murdoch Childrens
Research Institute**

Healthier Kids. Healthier Future.

1. What is the research project about?

This research project will study the overall health of patients who have had a Fontan procedure. We will also look at factors that contribute to long-term health in the Fontan population including blood thinning medications and fenestration. Areas we are investigating include:

1. Comparing the blood thinning medications, warfarin and aspirin, to look at how well they work at preventing blood clots and if they cause any bleeding.
2. Comparing those patients who have a fenestration (a hole placed between the Fontan circuit and the heart to relieve the pressure) in their Fontan pathway to those that don't in regards to exercise ability and any changes in the blood vessels and neurocognitive function of the brain.

We hope 157 adolescents and young adults will take part in this research project across Australia and New Zealand.

2. Who is funding this research project?

The project is being funded by a National Health and Medical Research Council research grant, as well as a HeartKids Grants-in-Aid Grant.

3. Why am I being asked to be in this research project?

You are being asked to be in this project because you have had a Fontan procedure more than 5 years ago, are taking either aspirin or warfarin, and have agreed to be contacted about research projects when you joined the Australia and New Zealand Fontan Registry.

4. What does participation in this research involve?

You will be telephoned by the recruitment coordinator to arrange an appointment at The Royal Children's Hospital.

The tests for this project will take place over two hospital visits. The length of time of each visit will depend on which tests you have consented to participate in. The tests will be spread over the two days and it is likely that each visit will last between 2-3 hours.

Upon your arrival the recruitment coordinator will greet you and explain the project to you in further detail to ensure you understand what is involved when participating in this research study. The recruitment coordinator will guide you through the tests you choose to participate in.

All the tests and procedures that are part of this research project are optional. This means you may give permission to complete all, some or even one of the offered tests. You will need to complete the Optional Consent tick boxes on the attached consent form to show which tests you agree to or not.

The tests in this study will include the following over 2 visits at Royal Children's Hospital:

- A brain MRI scan- This test takes approximately 30mins

MRI is short for magnetic resonance imaging. The MRI scanner uses a very strong magnet to take clear pictures of the inside of the body. Unlike an X-ray or CT scan, an MRI scan involves no radiation. It is a safe, painless and non-invasive test.

The MRI scanner is a large machine that is shaped like a big tube and is open at both ends. It has a bed to lie on and a head rest. The bed moves into the magnetic cylinder so that the part of the body that is scanned is in the centre.

- Since the MRI machine is a big magnet, care must be taken not to take any metal into the scanner room. Before your scan, we will ask you to complete an MRI safety questionnaire. On the day of the MRI scan, one of the MRI Technologists will go through the safety questionnaire again with you.
- Before the scan you will need to remove anything made of metal, for example jewellery, hair pins, watches etc. You may also need to remove make up, hair extensions or glitter as these items interfere with MRI pictures.
- We will ask you to lie on the bed and we will make sure you are comfortable.

- We will give you some earphones because the scanner makes loud knocking noises while the pictures are taken. You can watch a video or listen to music during the scan.
- You need to lie very still while the pictures are taken.

- A heart MRI

This test is the same as the above but will be over your chest instead. This test will take approximately 45 minutes, and will include insertion of a cannula in your arm to administer a contrast dye.

- An exercise MRI

This test will take approximately 45 minutes. This test is done to see how well the heart is working and how well the blood is flowing through the heart during exercise. This machine is similar to the above MRI machine however the table you lie on will have pedals attached. You will be instructed to cycle while you are in the MRI scanner and the difficulty will be increased slowly.

- An exercise test

This test measures your fitness level and also how well your heart and lungs work while you exercise. You will be asked to cycle on an exercise bike as the difficulty is increased. ECG (electrocardiogram) and blood pressure will be recorded before, during and after the test and you will be asked to breath in and out through a mouthpiece.

You will also be asked to do a 6 minute walk test where we will see how far you can walk in 6 minutes.

The exercise tests will take approximately 40 minutes in total.

We ask that for the exercise test you are fasted (no breakfast, just clear fluids – if your appointment is first thing in the morning, or a light breakfast and fasted for 4 hours if your appointment is in the afternoon).

- 24-hour blood pressure monitoring – completed at home

We will fit the 24-hour blood pressure monitoring system during your visit to RCH and give you instructions on how to use it. This will show changes in blood pressure in daily life. After wearing it for 24 hours you can take the BP monitor off your arm and send it back by post to the hospital. The monitor automatically inflates and measures and records your blood pressure hourly.

You should continue their normal daily activities, but swimming and contact sports such as wrestling and football are to be avoided for the 24 hour duration of the blood pressure monitoring.

You should also keep a journal (see Figure 1) indicating times and duration of activities and events that may influence blood pressure readings, including stressful situations and light exercise, and also when you take their ACE-inhibitor medication.

We will include detailed instructions on how to use the blood pressure machine plus the 24- hour journal in the pack that we give you. Upon completion of the 24 hour monitoring you can mail the blood pressure machine and journal sheet back to us in the reply-paid parcel post bag.

24-Hour Blood Pressure Monitor Journal

Name: _____ Time Asleep: _____

Date: _____ Time Awake: _____

Where are you?				What are you doing?						
What Time is it?	Home	Work/School	Other (specify)	ACE inhibitor medication	Light activity	Relaxing	Eating	Driving	Stressed	Other

Figure 1: Blood pressure journal

- Echocardiogram

This test is done to check the structure of the heart and how well it is functioning. This test uses sound to make a picture of your heart. The echocardiogram will take about 60 minutes. An electrocardiogram (ECG) will also be done at this time and your blood pressure will be measured.

- Neurocognitive Testing

These tests are done to assess brain function and thought process. We want to understand whether anticoagulation or fenestration has an impact on cognition. You will be asked to do the assessment on a computer and it will take about 30 minutes. We will explain how to do the test. We will also ask you whether you have a friend or sibling (similar age and gender) who you will be willing to nominate to participate in the neurocognitive testing (we will still need to get their permission too). The friend/sibling match helps us to control for other factors which might influence the results (e.g., age, gender etc.). This is not simply an intelligence test and you will not be provided with a score. Reports will be highly confidential. If we find that significant abnormalities exist we will provide a report to your treating specialist so that they can arrange appropriate follow-up that might help with your development and day-to-day functioning if you wish.

- Storage of Information in the Fontan Registry

Information that is collected during this study will be stored in the Fontan Registry database for future reference and for its use in future research with your consent. The information collected will be added to the current information that we have regarding your medical condition and will help give us more information about your health and your condition.

5. What are my alternatives to taking part?

Participation in a research project is voluntary. It is your choice to take part in this research. You do not have to take part if you do not want to.

If you give your consent and change your mind, you can withdraw from the project at any time. You do not need to tell us the reason why you want to stop participating in the project. If you leave the project we will use any information already collected unless you tell us not to.

Your decision will not affect any treatment or care you receive, or your relationship with The Royal Children's Hospital.

6. What are the possible benefits for me and other people in the future?

We are unable to say if there will be any direct benefit to you. The tests we are doing are for research purposes but these tests might pick up early signs of problems with your blood vessels that may not yet have been discovered. If this happens, we will offer appropriate medical follow-up and treatment.

We hope this project will show us what are the best medications and treatments for patients after having a Fontan procedure. This sort of information may change the way doctors perform Fontan operations as well as how doctors monitor people who have had a Fontan procedure.

7. What are the possible risks, side-effects, discomforts and/or inconveniences?

MRI risks

To date, there are no known long-term health risks related with MRI scans and it is considered a safe procedure when performed at a centre with appropriate guidelines, such as The Royal Children's Hospital.

The MRI scanner has a strong magnet. The magnetic attraction for some metal objects can pose a safety risk. It is important that metal objects are removed and not taken into the scanner room. You must tell us if you have metal implanted in your body, such as a pacemaker, coil or metal pins after being involved in an accident.

For the heart MRI a cannula will be placed in your arm for the administration of gadolinium (non-radioactive contrast dye), you will be offered a local anaesthetic (Angel) cream if required. Gadolinium poses a low risk for a minor contrast reaction (less than 0.05%) or for an anaphylactic reaction (0.03% - 0.1%). Most injections of intravenous contrast dye occur uneventfully.

While it is operating the scanner is noisy, but you will wear earphones to protect your ears. The scanner is shaped like a long tube and may cause some people to feel cramped or claustrophobic. Some people can

feel anxious during the scan. If you feel anxious we will stop the scan.

The scans we are taking are for research purposes. They are not meant to be used to help diagnose, treat or manage a particular medical condition. After the MRI scan a radiologist will look at your MRI scans for unusual features or unexpected findings. If the radiologist finds something that needs further examination, a referral to an appropriate medical doctor will be organised. The discovery of an unusual feature could have consequences for you and may affect your ability to work in certain professions or get life insurance cover. However, the discovery of an unusual feature may also help you to get treatment that may be of benefit. If anything unexpected is found on your MRI scan, we will contact you to talk about it.

Exercise test & blood pressure monitoring:

Exercise testing is a routine clinical investigation. Risks of exercise testing are very low in people with a Fontan circulation. Some of the more serious risks include abnormal heart beat, chest pain or discomfort, low blood pressure, fainting or heart attack. There is a small risk of muscle or joint injury. If you feel unwell during the testing you will be allowed to stop. The staff who will be performing the testing are trained to deal with any complications. Although there are no risks associated with the 24-hour blood pressure monitoring test, it is possible that you may find it uncomfortable and you may wake up at night when it takes readings.

Receiving health information:

Some people may find it distressing to receive information about their health.

If you wish to discuss any personal issues or require supportive counselling as a result of your participation in this study, we can refer you to an independent counsellor. We are also happy to discuss any concerns you may have.

Inconvenience

The main inconvenience is the time to come to The Royal Children's Hospital on two separate occasions to complete the tests for this research study. We will try to organise visits at a time that suits you best.

Reimbursement of costs:

1. All participants will be given a voucher to cover the cost to park at The Royal Children's Hospital on study visit days if they choose to park here.
2. We have some additional, limited funding to assist with travel and accommodation, which if required should be discussed with the research team. This assistance will be a maximum of \$1200 per participant (and includes travel and accommodation for that participant) and will be organised by the research team. Please note, once this funding runs out, we will not be able to provide financial assistance for this.

8. What will be done to make sure my information is confidential?

In this study we will collect and use personal and health information about you for research purposes. Any information we collect that can identify you will be treated as confidential and used only in this project unless otherwise specified. We can disclose the information only with your permission, except as required by law.

All information will be stored securely in the Heart Research department at the Murdoch Childrens Research Institute.

The following people may access information collected as part of this research project:

- the research team involved with this project
- The Royal Children's Hospital Human Research Ethics Committee

The stored information will be re-identifiable. This means that we will remove identifying information such as your name and give the information a special code number. Only the research team can match your name to the code number, if it is necessary to do so.

We are required to keep information collected as part of a research project for a certain length of time. Because participants in the project are under 18 years old, we must keep information until the youngest participant turns 25 years old. The research information may be destroyed or kept indefinitely in secure storage after this time.

access and correct the information we collect and store about you. Please contact us if you would like to access this information.

At the end of the study, results may be presented at conferences or published in medical journals. This will be done in such a way that you cannot be identified.

9. Will I be informed of the results when the research project is finished?

We will send you a summary of the overall project results. The summary will be of the whole group of research study participants, not your individual results.

If you would like more information about the project or if you need to speak to a member of the research team in an emergency please contact:

Name: A/Prof Yves d'Udekem

Contact telephone: (03) 9345 5203

If you have any concerns and/or complaints about the project, the way it is being conducted or your rights as a research participant, and would like to speak to someone independent of the project, please contact:

Director, Research Ethics & Governance, The Royal Children's Hospital Melbourne on telephone: (03) 9345 5044.

CONSENT FORM (Participant)

HREC Project Number: HREC 33274E

Research Project Title: Functional Outcomes after Fontan Surgery

Version Number: 11

Version Date: 01/08/2016

- I have read, or had read to me in my first language, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my involvement in this project.
- I voluntarily consent to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by The Royal Children's Hospital Melbourne Human Research Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007).
- I understand I will receive a copy of this Information Statement and Consent Form.

☐ I do	☐ I do not	consent to having a brain MRI scan
☐ I do	☐ I do not	consent to having a heart MRI scan
☐ I do	☐ I do not	consent to having contrast dye (i.e., gadolinium) injected for the heart MRI scan
☐ I do	☐ I do not	consent to having a cardiac exercise MRI scan
☐ I do	☐ I do not	consent to doing an exercise test
☐ I do	☐ I do not	consent to participating in 24-hour blood pressure monitoring
☐ I do	☐ I do not	consent to having an echocardiogram
☐ I do	☐ I do not	consent to taking part in computer-based neurocognitive testing
☐ I do	☐ I do not	consent to re-identifiable results/tests performed in this study to be stored in the Fontan Registry which may be used in future ethically approved research related to Fontan surgery .

Participant Name

Participant Signature

Date

Participant Mobile Phone Number

Other Phone Number

Participant email address

Name of Witness to Participant's
Signature

Witness Signature

Date

For neurocognitive testing - Sibling or Friend nomination (similar age & gender) **Name &**

contact number* (**will still need to be contacted and complete a separate consent form*)

☐ Tick if you have checked with your friend/sibling that it is OK to share their details with us

Declaration by researcher: I have explained the project to the participant who has signed above, and believe that they understand the purpose, extent and possible risks of their involvement in this project.

Research Team Member Name

Research Team Member Signature

Date

Note: All parties signing the Consent Form must date their own signature.

Information statement and consent form

HREC Project Number: HREC 33274

Research Project Title: Functional Outcomes after Fontan Surgery– A focus on Bone density

Principal Researcher: A/Prof Yves d'Udekem, Consultant Cardiac Surgeon

Version Number: 10 **Version Date:** 01/08/2016

Thank you for taking the time to read this **Parent/Guardian Information Statement and Consent Form**. We would like to invite your child to participate in a research project that is explained below. This document is 5 pages long. Please make sure you have all the pages.

If you speak a language other than English and would like this Information Statement and Consent Form in your language, please ask the person explaining this research project to you.

What is an Information Statement?

These pages tell you about the research project. It explains to you clearly and openly all the steps and procedures of the project. The information is to help you decide whether or not you would like your child to take part in the research. Please read this Information Statement carefully.

Before you decide if you want your child to take part or not, you can ask us any questions you have about the project. You may want to talk about the project with your family, friends or health care worker.

If you would like your child to take part in the research project, please sign the consent form at the end of this information statement. By signing the consent form you are telling us that you:

- understand what you have read
- had a chance to ask questions and received satisfactory answers
- consent to your child taking part in the project or its subcomponents.

We will give you a copy of this information and consent form to keep.

1. What is the research project about?

This phase of our research project is studying the effect of blood thinning medications, warfarin and aspirin, on bone health. There have been a few studies that suggest that warfarin in particular can cause bones to become weaker over the long-term.

Most patients who have had the Fontan procedure take life-long warfarin or aspirin. We would like to compare the bone health of patients who take warfarin to those who take aspirin. This has never been done before.

We hope that the information collected from this study will improve ongoing treatment of patients who have had the Fontan procedure by:

- Better understanding of the risks and benefits of blood thinning medications
- Allowing for early screening and intervention to prevent risks to bone health

We hope 157 adolescents and adults who have had the Fontan procedure will take part in this research project across Australia and New Zealand.

2. Who is funding this research project?

The project is being funded by a National Health and Medical Research Council research grant.

3. Why is my child being asked to be in this research project?

Your child is being asked to be in this project because they have had a Fontan procedure more than 5 years ago, are taking either aspirin or warfarin, and you have agreed to be contacted about research projects when you joined the Australia and New Zealand Fontan Registry.

4. What does participation in this research involve?

Please note that this study will be conducted at **Sunshine Hospital**. If you choose to participate, the recruitment coordinator will arrange an appointment for you over the phone.

This study involves medical tests that will measure your child's bone mineral density, which is a very good indicator of their bone health. Taken together, these tests will give us a very good understanding of your overall bone health. Although the time to complete each test is relatively short (15-30 minutes) please allow for a total of three hours to account for possible waiting time.

Upon your arrival to Sunshine hospital, the Bone Health Technician will greet you and explain each test to you in further detail to ensure you understand what is involved when participating in this research study.

To measure your bone mineral density we will use the following tests:

Dual-energy X-ray absorptiometry (DXA): For the DXA scan your child will be asked to lie on a table while their body is scanned using very low radiation x-ray beams.

Peripheral quantitative computed tomography (pQCT): For the pQCT scan, your child will be asked to sit in a chair and to place their arm or leg through a hole in the machine. Their limb will then be scanned using very low radiation x-ray beams.

Height and Weight: Height and weight measurements are required to interpret the DXA and pQCT results.

X-ray: An x-ray will be taken of your child's left wrist to calculate your 'bone age'.

Questionnaires: A number of short questionnaires are provided in this pack. The questionnaires cover important areas related to bone health including: calcium intake, stage of puberty, history of bone breaks and medications. The questionnaire about stage of puberty will ask your child to compare him/herself to pictures about pubertal stage (see attached questionnaire), as puberty plays an important role in bone development. The food frequency questionnaire (calcium intake) will help us determine how your child's diet is influencing their bone development.

Blood test: Vitamin D is an important contributor to bone health. Your child will also be offered a blood test to measure their vitamin D levels. *Please indicate on the consent form if you would like them to participate in this part of the study.*

Storage of Information in the Fontan Registry

Information that is collected during this study will be stored in the Fontan Registry database for future reference and for its use in future research, with your consent. The information collected will be added to the current information that we have regarding your child's medical condition and will help give us more information about your child's health and their condition.

5. What are my child's alternatives to taking part?

Participation in a research project is voluntary. It is your choice to let your child take part in this research. You do not have to agree if you do not want them to.

If you give your consent and change your mind, your child can withdraw from the project at any time. You do not need to tell us the reason why you or your child wants to stop participating in the project. If your child leaves the project we will use any information already collected unless you tell us not to.

Whatever your decision, it will not affect any treatment or care your child gets, or your family's relationship with The Royal Children's Hospital.

6. What are the possible benefits for my child and other people in the future?

We are unable to say if there will be any direct benefit to your child. The tests we are doing are for research purposes but these tests might pick up early signs of problems with your child's bone health. If this happens, we will offer appropriate medical follow-up and treatment.

We hope this project will show us which medications and treatments are best for patients after having a Fontan procedure.

7. What are the possible risks, side-effects, discomforts and/or inconveniences?

Radiation

This research study involves exposure to a very small amount of radiation. As part of everyday living, everyone is exposed to naturally occurring background radiation and receives a dose of about 2 millisievert (mSv) each year. The effective dose from this study is less than 0.06 mSv. At this dose level, no harmful effects of radiation have been demonstrated as any effect is too small to measure. The risk is believed to be minimal. **Please let us know if your child has participated in any other studies that involve radiation.**

The total dose of radiation your child will receive is very low and unlikely to have any negative effects. However, as x-ray beams can be harmful to unborn babies, please inform the radiographer if there is a possibility that your child may be pregnant before the scan. Pregnant participants will not be able to participate in this study.

Blood tests

There are no major risks associated with a blood test. It is possible to your child may feel some discomfort

during the blood test. They may feel a sting when the needle is put into their arm to take the blood. It is possible there may be some bruising, swelling or bleeding where the needle enters the skin. Some people can feel a little light-headed when blood is taken.

Inconvenience

The main inconvenience is the time to come to come in for the tests. Participants will be asked to come to Sunshine Hospital (Western Health) for all bone tests. We will try to organise visits at a time that suits you best.

Reimbursement of costs:

1. We can reimburse you for the cost to park at Sunshine Hospital. Please keep your receipts and send them to us if you would like this reimbursed.
2. We have some additional, limited funding to assist with travel and accommodation, which if required should be discussed with the research team. This assistance will be a maximum of \$1800 per child participant (which includes travel and accommodation for one child participant plus one parent/guardian) and will be organised by the research team. Please note, once this funding runs out, we will not be able to provide financial assistance for this.

8. What will be done to make sure my child's information is confidential?

In this study we will collect and use personal and health information about your child for research purposes. Any information we collect that can identify your child will be treated as confidential and used only in this project unless otherwise specified. We can disclose the information only with your permission, except as required by law.

All information will be stored securely in the Heart Research department at the Murdoch Children's Research Institute.

The following people may access information collected as part of this research project:

- the research team involved with this project
- The Royal Children's Hospital Human Research Ethics Committee

The stored information will be re-identifiable. This means that we will remove identifying information such as your child's name and give the information a special code number. Only the research team can match your child's name to their code number, if it is necessary to do so.

We are required to keep information collected as part of a research project for a certain length of time. Because the participants in this project are under 18 years old, we must keep information until the youngest participant turns 25 years old. The research information may be destroyed or kept indefinitely in secure storage after this time.

In accordance with relevant Australian and/or Victorian privacy and other relevant laws, you have the right to access and correct the information we collect and store about your child. Please contact us if you would like to access this information.

At the end of the study, results may be presented at conferences or published in medical journals. This will be done in such a way that your child cannot be identified.

9. Will we be informed of the results when the research project is finished?

We will send you a summary of the overall project results. The summary will be of the whole group of research study participants, not your child's individual results.

If you would like more information about the project or if you need to speak to a member of the research team in an emergency please contact:

Name: A/Prof Yves d'Udekem

Contact telephone: (03) 9345 5200

If you have any concerns and/or complaints about the project, the way it is being conducted or your child's rights as a research participant, and would like to speak to someone independent of the project, please contact:

Director, Research Ethics & Governance, The Royal Children's Hospital Melbourne on telephone: (03) 9345 5044

CONSENT FORM (Parent/Guardian)

HREC Project Number: HREC 33274

Research Project Title: Functional Outcomes after Fontan Surgery- A focus on Bone density

Version Number: 10

Version Date: 01/08/2016

- I consent to my child having bone densitometry tests.
- I have read, or had read to me in my first language, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my child's involvement in this project.
- I voluntarily consent for my child to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by The Royal Children's Hospital Melbourne Human Research Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007).
- I understand I will receive a copy of this Information Statement and Consent Form.

☐ I do	☐ I do not	consent to my child having a blood test
☐ I do	☐ I do not	consent to my child's re-identifiable results/tests performed in this study to be stored in the Fontan Registry which may be used in future ethically approved research related to Fontan surgery .

Child's Name

Parent/Guardian Name

Parent/Guardian Signature

Date

Parent/Guardian Mobile Phone Number

Other Phone Number

Parent/Guardian email address

Name of Witness to Parent/Guardian's Signature

Witness Signature

Date

Note: All parties signing the Consent Form must date their own signature.

Declaration by researcher: I have explained the project to the parent/guardian who has signed above, and believe that they understand the purpose, extent and possible risks of their child's involvement in this project.

Research Team Member Name

Research Team Member Signature

Date

Information statement and consent form

HREC Project Number: HREC 33274

Research Project Title: Functional Outcomes after Fontan Surgery – A focus on Bone density

Principal Researcher: A/Prof Yves d'Udekem, Consultant Cardiac Surgeon

Version Number: 10 **Version Date:** 01/08/2016

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Before you decide to take part or not, you can ask us any questions you have about the project. You may want to talk about the project with your family, friends or health care worker.

If you would like to take part in the research project, please sign the consent form at the end of this information statement. By signing the consent form you are telling us that you:

- understand what you have read
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**Murdoch Childrens
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1. What is the research project about?

This phase of our research project is studying the effect of blood thinning medications, warfarin and aspirin, on bone health. There have been a few studies that suggest that warfarin in particular can cause bones to become weaker over the long-term.

Most patients who have had the Fontan procedure take life-long warfarin or aspirin. We would like to compare the bone health of patients who take warfarin to those who take aspirin. This has never been done before.

We hope that the information collected from this study will improve ongoing treatment of patients who have had the Fontan procedure by:

- Better understanding of the risks and benefits of blood thinning medications
- Allowing for early screening and intervention to prevent risks to bone health

We hope 157 adolescents and adults who have had the Fontan procedure will take part in this research project across Australia and New Zealand.

2. Who is funding this research project?

The project is being funded by a National Health and Medical Research Council research grant.

3. Why am I being asked to be in this research project?

You are being asked to be in this project because you have had a Fontan procedure more than 5 years ago, are taking either aspirin or warfarin, and have agreed to be contacted about research projects when you joined the Australia and New Zealand Fontan Registry.

4. What does participation in this research involve?

Please note that this study will be conducted at **Sunshine Hospital**. If you choose to participate, the recruitment coordinator will arrange an appointment for you over the phone.

This study involves medical tests that will measure your bone mineral density, which is a very good indicator of your bone health.

Upon your arrival to Sunshine hospital, the Bone Health Technician will greet you and explain each test to you in further detail to ensure you understand what is involved when participating in this research study.

To measure your bone mineral density we will use the following tests:

Dual-energy X-ray absorptiometry (DXA): For the DXA scan you will be asked to lie on a table while your body is scanned using very low radiation x-ray beams.

Peripheral quantitative computed tomography (pQCT): For the pQCT scan, you will be asked to sit in a chair and to place your arm or leg through a hole in the machine. Your limb will then be scanned using very low radiation x-ray beams.

Height and Weight: Height and weight measurements are required to interpret the DXA and pQCT results.

Questionnaires: A number of short questionnaires are provided in this pack. The questionnaires cover important areas related to your bone health, including: calcium intake, history of bone breaks and medications. The food frequency questionnaire (calcium intake) will help us determine how your diet influences your bone development.

Blood test: Vitamin D is an important contributor to bone health. You will also be offered a blood test to measure your vitamin D levels. *Please indicate on the consent form if you would like to participate in this part of the study.*

Taken together, these tests will give us a very good understanding of your overall bone health. Although the time to complete each test is relatively short (15-30 minutes) please allow for a total of three hours to account for possible waiting time.

Storage of Information in the Fontan Registry

Information that is collected during this study will be stored in the Fontan Registry database for future reference and for its use in future research, with your consent. The information collected will be added to the current information that we have regarding your medical condition and will help give us more information about your health and your condition.

5. What are my alternatives to taking part?

Participation in a research project is voluntary. It is your choice to take part in this research. You do not have to take part if you do not want to.

If you give your consent and change your mind, you can withdraw from the project at any time. You do not need to tell us the reason why you want to stop participating in the project. If you leave the project we will use any information already collected unless you tell us not to.

Your decision will not affect any treatment or care you receive, or your relationship with The Royal Children's Hospital.

6. What are the possible benefits for me and other people in the future?

We are unable to say if there will be any direct benefit to you. The tests we are doing are for research purposes but these tests might pick up early signs of problems with your bone health. If this happens, we will offer appropriate medical follow-up and treatment.

We hope this project will show us which medications and treatments are best for patients after having a Fontan procedure.

7. What are the possible risks, side-effects, discomforts and/or inconveniences?

Radiation

This research study involves exposure to a very small amount of radiation. As part of everyday living, everyone is exposed to naturally occurring background radiation and receives a dose of about 2 millisievert (mSv) each year. The effective dose from this study is less than 0.06 mSv. At this dose level, no harmful effects of radiation have been demonstrated as any effect is too small to measure. The risk is believed to be minimal. **Please let us know if you have participated in any other studies that involve radiation.**

The total dose of radiation you will receive is very low and unlikely to have any negative effects. However, as x-ray beams can be harmful to unborn babies, please inform the radiographer if there is a possibility that you may be pregnant before the scan. Pregnant participants will not be able to participate in this study.

Blood tests

There are no major risks associated with a blood test. It is possible to you feel some discomfort during the blood test. You may feel a sting when the needle is put into your arm to take the blood. It is possible there may be some bruising, swelling or bleeding where the needle enters the skin. Some people can feel a little light-headed when blood is taken.

Inconvenience

The main inconvenience is the time to come to come in for the tests. Participants will be asked to come to Sunshine Hospital (Western Health) for all bone tests. We will try to organise visits at a time that suits you best.

Reimbursement of costs:

1. We can reimburse you for the cost to park at Sunshine Hospital. Please keep your receipts and send them to us if you would like this reimbursed.
2. We have some additional, limited funding to assist with travel and accommodation, which if required should be discussed with the research team. This assistance will be a maximum of \$1200 per participant (and includes travel and accommodation for that participant) and will be organised by the research team. Please note, once this funding runs out, we will not be able to provide financial assistance for this.

8. What will be done to make sure my information is confidential?

In this study we will collect and use personal and health information about you for research purposes. Any information we collect that can identify you will be treated as confidential and used only in this project unless otherwise specified. We can disclose the information only with your permission, except as required by law.

All information will be stored securely in the Heart Research department at the Murdoch Childrens Research Institute.

The following people may access information collected as part of this research project:

- the research team involved with this project
- The Royal Children's Hospital Human Research Ethics Committee

The stored information will be re-identifiable. This means that we will remove identifying information such as your name and give the information a special code number. Only the research team can match your name to the code number, if it is necessary to do so.

We are required to keep information collected as part of a research project for a certain length of time. Because participants in the project are under 18 years old, we must keep information until the youngest participant turns 25 years old. The research information may be destroyed or kept indefinitely in secure storage after this time.

In accordance with relevant Australian and/or Victorian privacy and other relevant laws, you have the right to access and correct the information we collect and store about you. Please contact us if you would like to access this information.

At the end of the study, results may be presented at conferences or published in medical journals. This will be done in such a way that you cannot be identified.

9. Will I be informed of the results when the research project is finished?

We will send you a summary of the overall project results. The summary will be of the whole group of research study participants, not your individual results.

If you would like more information about the project or if you need to speak to a member of the research team in an emergency please contact:

Name: A/Prof Yves d'Udekem

Contact telephone: (03) 9345 5203

If you have any concerns and/or complaints about the project, the way it is being conducted or your rights as a research participant, and would like to speak to someone independent of the project, please contact:

Director, Research Ethics & Governance, The Royal Children's Hospital Melbourne on telephone: (03) 9345 5044.

CONSENT FORM (Participant)

HREC Project Number: HREC 33274

Research Project Title: Functional Outcomes after Fontan Surgery - A focus on Bone density

Version Number: 10

Version Date: 01/08/2016

- I consent to having bone densitometry tests
- I have read, or had read to me in my first language, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my involvement in this project.
- I voluntarily consent to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by The Royal Children's Hospital Melbourne Human Research Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007).
- I understand I will receive a copy of this Information Statement and Consent Form.

☐ I do	☐ I do not	consent to having a blood test
☐ I do	☐ I do not	consent to re-identifiable results/tests performed in this study to be stored in the Fontan Registry which may be used in future ethically approved research related to Fontan surgery .

Participant Name

Participant Signature

Date

Participant Mobile Phone Number

Other Phone Number

Participant email address

Name of Witness to Participant's Signature

Witness Signature

Date

Declaration by researcher: I have explained the project to the participant who has signed above, and believe that they understand the purpose, extent and possible risks of their involvement in this project.

Research Team Member Name

Research Team Member Signature

Date

Note: All parties signing the Consent Form must date their own signature.

Thromboprophylaxis data collection form



Height: _____

Weight: _____

To be completed by bone technician

Date: _____

Name: _____

Date of Birth: _____

What kind of anticoagulation (blood-thinning) medication do you take?

- ☐ None
- ☐ Warfarin – Average daily dose: _____
- Date that you began warfarin treatment? _____
 - What is your target INR range? _____
- ☐ Aspirin – Daily dose: _____
- Date you began aspirin treatment? _____
- ☐ OTHER – _____ Daily dose: _____
- Date that you began OTHER treatment? _____

If you take warfarin:

On average, how many INR tests do you have per month? _____

Where do you have your INR monitoring done? *(please write down the percentage of tests done at each site)*

- Home INR monitoring _____%
- Pathology collection center INR monitoring _____%
- GP INR monitoring _____%

On average how many kilometers do you travel for your INR testing (from your home to INR service):

- Pathology collection center _____km
- GP _____km

What other medications do you take (name and dose)? _____

Have you had any other surgery after your Fontan procedure? Yes/No

Please give details of other surgery _____

Did you have any bleeding while in hospital? Yes/No

Please give details of the bleeding event: _____

What treatment did you receive for the bleeding? _____

Have you had any bleeding that required you to go to a doctor or to hospital? Yes/No

Please give details of the bleeding event: _____

What treatment did you receive for the bleeding _____

Have you ever had a blood clot or stroke? Blood clot/Stroke

➤ Where was the blood clot? _____

When did you have the blood clot or stroke? _____

What treatment did you receive for the blood clot or stroke? _____

Please detail any ongoing medical issues caused by the blood clot or stroke: _____

Did you have a fenestration as part of your Fontan procedure? Yes/No

Has the fenestration been closed? Yes/No

When was it closed? _____

Do you have any other medical conditions? _____

Have you had any palpitations (fast heart rate) in the last year? Yes/No

When did you last see your cardiologist? _____

When do you see you cardiologist again? _____

History of bone fractures

Have you broken any bones in the past	Yes	No
Forearm or wrist	Right	Left
Age broken forearm or wrist		
How was it broken?		
Finger	Right	Left
Age broken finger		
How was it broken?		
Hip	Right	Left
Age broken hip		
How was it broken?		
Ankle	Right	Left
Age broken ankle		
How was it broken?		
Toe	Right	Left
Age broken toe		
How was it broken?		
Leg	Right	Left
Age broken leg		
How was it broken?		
Back	Right	Left
Age broken back		
How was it broken?		

Food Frequency Questionnaire

IN THE PAST 3 MONTHS, HOW FREQUENTLY DID YOU HAVE THE FOLLOWING FOODS?

Please note: 1 glass of milk every day should be recorded as 7 serves per week; 2 per day should be recorded as 14 serves per week. If you never have a listed item, or have it less than once per week tick rarely or never. Make sure that you adjust for the serve size you eat. For example, if you have 2 tablespoons of milk in your tea and 3 cups of tea a day (ie: 6 tablespoons a day), you need to record 42 tablespoons in the 'how many serves per week' per column.

Food item	Serve size	How many serves per week?	Rarely or never
MILK			
Specify type = e.g., Rev, Plain, Flav (i.e., Milo)	1 glass = 200ml		
Type:			
In tea/coffee - type:	1 Tbsp = 20mls		
With cereal - type:	1/2 cup		
CHEESE			
1 slice hard cheese (i.e., Kraft sliced, Tasty, Coon)	Pre-cut Kraft Slice = 20g		
Soft, Cream, Cottage, Brie, Camembert	1 Tbsp = 20g		
YOGHURT type: Full/Reduced fat	Snack size tub = 200g		
ICE-CREAM	1 scoop = 50g		
SEAFOOD	1 serve = size of your hand		
Fish (e.g., flake, barramundi, fresh salmon)	1 fillet		
TINNED SALMON/TUNA	1/2 cup = 120g		
TINNED SARDINES	4-5 = 60g		
OTHER SEAFOOD			
(e.g., scallops, oysters)	6 scallops = 60g		
BREAD	2 slices or 1 small roll		
ALMONDS	20 nuts = 30g		
ORANGE JUICE (WITH ADDED CALCIUM)	1 glass = 200ml		
CALCIUM SUPPLEMENT	_____mg Cal/day		
FRUCHE	1 snack size tub = 200g		
CHOCOLATE	4 squares = 30g		

For participant's aged 18 years or below ONLY: Using the following diagrams, please select which image/s best reflect your developmental stage:

Male: Stage _____

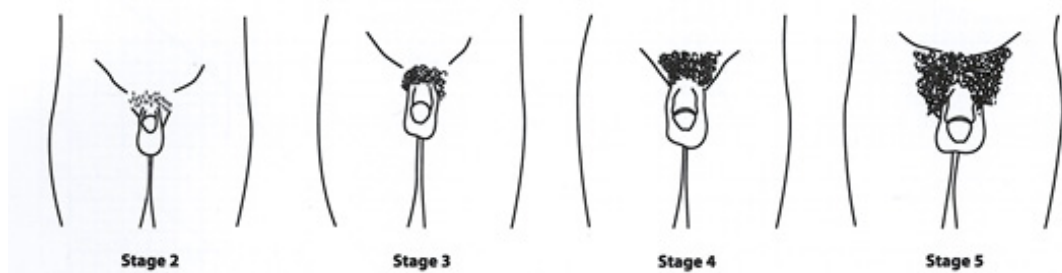
Female: Breast stage _____ Pubic hair stage _____

Self-assessment of Tanner stage

Have you started getting menstrual periods
Yes/No

Male

Genital and Pubic Hair Development Stages



Female

Breast Development Stages



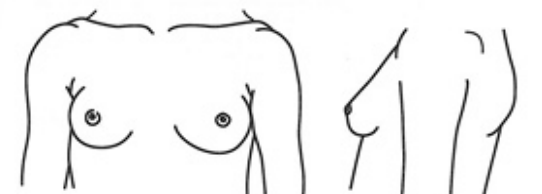
Stage 1. Prepubertal



Stage 2. Elevation of breasts and papilla



Stage 3. Further elevation and areola but no separation of contours

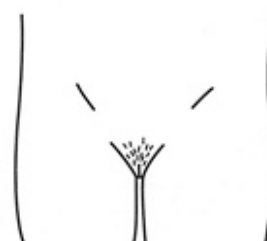


Stage 4. Areola and papilla form a secondary mound above level of the breast

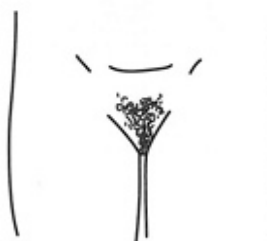


Stage 5. Areola recesses to the general contour of the breast

Pubic Hair Stages



Stage 2



Stage 3



Stage 4



Stage 5

Study recruitment invitation email for questionnaires

Dear Fontan Registry Participant,

The effects of blood thinning medications on bleeding and Quality of life.

You are invited to participate in a research study to assess bleeding and quality of life in people with a Fontan who take blood thinning medications (e.g. aspirin, warfarin).

Your participation will help us better understand if bleeding is a problem with particular blood thinners. Furthermore, your participation will allow us to compare the quality of life between people taking different types of blood thinners. It is hoped that this study will help us make more informed decisions when prescribing blood thinners in the future.

What do you need to do?

If you would like to participate in this research study, please click the link at the bottom of this email, or cut and paste it into your internet browser.

The survey will take approximately 20 minutes to complete.

Participation in this research study is voluntary, and this email should be disregarded if you are not interested in participating.

There is a potential that some of the questions asked may be confronting to some participants as it causes them to focus upon difficult aspects of their life. If you develop such feelings or feel distressed as a result of participation in this study, please contact your General Practitioner/healthcare provider or contact the study team directly, who can refer you to further support services.

We really appreciate your time and participation.

Kind regards,

Fontan Registry Research Team

Heart Research

SMS: 0431 659 911

T: (03) 9345 6161 (Karin), (03) 9345 4236 (Janina) or (03) 9936 6551 (Chantal)

E: info@fontanregistry.com

Anticoagulants and Bleeding Questionnaire

Warfarin and Aspirin Bleeding Assessment Tool

Thank you for agreeing to complete the 'Anticoagulants and Bleeding' survey. We really appreciate your time.

This survey will be used to compare the effect of anticoagulants (e.g. aspirin, warfarin) on bleeding in everyday life.

Please let us know if you have any issues with the survey questions, the link or the appearance of the survey in your browser.

www.mcri.edu.au

First Name

Surname

Email address

Phone number:

Study ID

Today's date

(Please enter DD-MM-YYYY)

What is your gender?

- ☐ Male
☐ Female

What type of anticoagulant medication do you currently take?

- ☐ Warfarin
☐ Aspirin
☐ Other (Please specify)
☐ None

Name of other anticoagulant medication

When did you begin taking [other_anticoag]?

(if unsure, please put approximate date)

What dose of [other_anticoag] do you take?

When did you begin taking warfarin?

(if unsure, please put approximate date)

What is your currently average daily dose of warfarin?

(mg)

What is your target INR range?

How many INR's have you had in the last 6 months?

(per 6 months)

Where do you have your INR monitoring done? (please answer 0 if INR testing is not performed at specified site)

Percentage of INR testing performed at Home:

 (%)Percentage of INR testing performed at Pathology
collection center:

 (%)

Percentage of INR testing performed at GP:

 (%)

**On average how many kilometres do you travel for your INR testing at the following sites?
(one way)**

Pathology collection center

(kms)

GP

(kms)

When did you begin taking Aspirin?

(if unsure, please put approximate date)

What dose of aspirin do you take?

Have you ever been diagnosed with a bleeding disorder?

- ☐ Yes
☐ No

What type of bleeding disorder were you diagnosed with?

Are you currently taking the contraceptive pill?

- ☐ Yes
☐ No

Which contraceptive pill are you taking?

In the last 5 years, have you had to stop your anticoagulant (warfarin/aspirin/other) for a period of time?

- ☐ Yes
☐ No

Which anticoagulant did you stop taking?

- ☐ Warfarin
☐ Aspirin
☐ Other

Name of other anticoagulant medication

What was the reason you needed to stop taking your anticoagulant?

- ☐ Bleeding
☐ Undergoing a procedure
☐ Other side-effect
☐ Other reason

Please explain the reason for stopping your anticoagulant.

Did you take a different anticoagulant to your current anticoagulant for a time?

- ☐ Yes
☐ No

When did you change anticoagulants?

(if unsure, please put approximate date)

Why did you change anticoagulants?

- ☐ Doctor's recommendation
☐ Bleeding
☐ Other side effect
☐ Blood clot
☐ My preference
☐ Other

Which different anticoagulant were you taking?

- ☐ Warfarin
☐ Aspirin
☐ Nothing
☐ Other

Name of other anticoagulant medication

Have you ever experienced any of the following bleeding events?**Nosebleeds**

Have you experienced nosebleeds?

- ☐ Yes
☐ No

Number of nosebleeds per year:

- ☐ < 1
☐ 1-5
☐ 6-12
☐ >12

Duration of average bleeding episode?

- ☐ < 1 minute
☐ 1-10 minutes
☐ >10 minutes

What anticoagulant were you taking at the time of the nosebleeds?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What was the other anticoagulant you were taking?

Did you require medical attention?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Cauterization/Packing/Surgery
☐ Blood product transfusion
☐ Vitamin K
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Bruising

Do you currently have bruising?

- ☐ Yes
☐ No

What is the average size of your bruises?

- ☐ < 1cm
☐ 1-5cm
☐ >5cm

Were the bruises caused by trauma (e.g. bumping into something, falling ect)?

- ☐ Yes
☐ No

Did the bruising require medical attention?

- ☐ Yes
☐ No

What type of medical treatment did you receive?

Have you had to stop taking your anticoagulant because of bruising?

- ☐ Yes
☐ No

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

Bleeding from minor wounds

Have you had bleeding from minor wounds (e.g. scratch, scrapes or small cuts)?

- ☐ Yes
☐ No

Number of bleeds from minor wounds per year?

- ☐ < 1
☐ 1-5
☐ >6

Duration of average bleeding episode?

- ☐ < 5 minutes
☐ >5 minutes

Did the bleeding require medical attention?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Surgery
☐ Vitamin K
☐ Blood product transfusion
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Bleeding from blood tests

Have you experienced bleeding from the needle site after blood tests?

- ☐ Yes
☐ No

If so, how often do you bleed?

- ☐ Every test
☐ Sometimes

How long do you bleed for?

- ☐ < 1 minutes
☐ 1-5 minutes
☐ 5-10 minutes
☐ >10 minutes

Does it ever require medical attention?

- ☐ Yes
☐ No

If so, what medical treatment do you receive?

- ☐ Pressure only
☐ Vitamin K
☐ Blood products
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment do you receive?

Bleeding in the mouth

Have you experienced bleeding in the mouth?

- ☐ Yes
☐ No

What type of mouth bleeding have you had?

- ☐ Tooth eruption
☐ Gums - spontaneous
☐ Gums - after brushing
☐ Bites to lip and tongue
☐ Other

Please specify other type of mouth bleeding

What anticoagulant were you taking at the time of the mouth bleeding?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What was the other anticoagulant you were taking?

Did the mouth bleeding require medical attention?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Surgery
☐ Vitamin K
☐ Blood product transfusion
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Have you had a tooth removed?

- ☐ Yes
☐ No

Did you experience bleeding after tooth removal?

- ☐ Yes
☐ No

How often do you have bleeding after tooth removal?

- ☐ No bleeding in at least 2 tooth removals
☐ Bleeding in 1 tooth removal
☐ Bleeding in 2 tooth removals
☐ Bleeding after 3 or more tooth removals
(If more than one tooth removed in one go, please count as one tooth removal)

What anticoagulant were you taking at the time of bleeding after tooth removal?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What was the other anticoagulant you were taking?

Did the bleeding after tooth removal require medical attention?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Resuturing or packing
☐ Vitamin K
☐ Blood product transfusion
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Gastrointestinal Bleeding

Have you experienced gastrointestinal (bleeding from stomach or bowel) bleeding?

- ☐ Yes
☐ No

What type of gastrointestinal bleeding did you have?

- ☐ Blood in vomit
☐ Blood in stool
☐ Ulcers
☐ Haemorrhoids
☐ Other

What other gastrointestinal bleeding did you have?

Was there a known cause of your gastrointestinal bleeding?

- ☐ Yes
☐ No

What was the cause of your gastrointestinal bleeding?

- ☐ Gastritis/Ulcer
☐ Colitis
☐ Mallory-Weiss tear
☐ Vascular malformations
☐ Other

Please specify other cause for gastrointestinal bleeding

What anticoagulant were you taking at the time of bleeding after tooth removal?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What was the other anticoagulant you were taking?

Did the gastrointestinal bleeding require medical attention?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Vitamin K
☐ Blood product transfusion
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Menstrual bleeding

Have you started getting menstrual periods?

- ☐ Yes
☐ No

How many days does your average period last?

How many days would you consider your period as heavy?

How often do you change your pad/tampon on your average period days?

(pads/tampons per day)

How often do you change your pad/tampon on your heaviest period days?

(pads/tampon per day)

Have you missed school/work due to heavy periods?

- ☐ Rarely
☐ Occasionally
☐ Often
☐ Always

Do you get flooding (blood running around pad or tampon)?

- ☐ Yes
☐ No

Has heavy menstrual bleeding required medical attention?

- ☐ Yes
☐ No

What anticoagulant were you taking at the time of seeking medical attention for menstrual bleeding?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What was the other anticoagulant you were taking?

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Hysterectomy
☐ tranexamic acid/Antifibrinolytic
☐ Dilatation and Curettage
☐ Iron therapy
☐ Vitamin K
☐ Stop anticoagulation
☐ Hormone therapy (e.g. "The Pill")
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What hormone therapy did you receive?

- ☐ Contraceptive pill
☐ Progesterone (mini pill)
☐ Progesterone IUD (Mirena)
☐ Progesterone injectable

What other medical treatment did you receive?

Have you been diagnosed with iron deficiency anaemia?

- ☐ Yes
☐ No

Do you have clots (visible on pad or tampon)?

- ☐ Yes
☐ No

Pregnancy

Have you been pregnant?

☐ Yes☐ No

How many live births have you had?

How many miscarriages have you had?

How many planned terminations have you had?

Live Births - Baby 1

What anticoagulant were you taking at conception of baby number 1?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What other anticoagulant were you taking at conception?

Did your anticoagulation treatment change during your pregnancy?

- ☐ Yes
☐ No

How did your anticoagulation treatment change during pregnancy?

- ☐ Stopped taking any anticoagulant
☐ Changed to another anticoagulant
☐ Other

How many weeks into your pregnancy did you stop taking your anticoagulant?

(weeks)

Which anticoagulant did you change to during your pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Enoxaparin
☐ Other

What was the other anticoagulant that you took during pregnancy?

What was the other way your anticoagulant treatment changed during pregnancy?

Did you have any and significant bleeding (haemorrhage) associated with the delivery of your baby?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Antifibrinolytic
☐ Vitamin K
☐ Dilatation and Curettage
☐ Iron therapy
☐ Blood product transfusion
☐ Hysterectomy
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Live Births - Baby 2

What anticoagulant were you taking at conception of baby number 2?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What other anticoagulant were you taking at conception?

Did your anticoagulation treatment change during your pregnancy?

- ☐ Yes
☐ No

How did your anticoagulation treatment change during pregnancy?

- ☐ Stopped taking any anticoagulant
☐ Changed to another anticoagulant
☐ Other

How many weeks into your pregnancy did you stop taking your anticoagulant?

(weeks)

Which anticoagulant did you change to during your pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Enoxaparin
☐ Other

What was the other anticoagulant that you took during pregnancy?

What was the other way your anticoagulant treatment changed during pregnancy?

Did you have any and significant bleeding (haemorrhage) associated with the delivery of your baby?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Antifibrinolytic
☐ Vitamin K
☐ Dilatation and Curettage
☐ Iron therapy
☐ Blood product transfusion
☐ Hysterectomy
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Live Births - Baby 3

What anticoagulant were you taking at conception of baby number 3?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What other anticoagulant were you taking at conception?

Did your anticoagulation treatment change during your pregnancy?

- ☐ Yes
☐ No

How did your anticoagulation treatment change during pregnancy?

- ☐ Stopped taking any anticoagulant
☐ Changed to another anticoagulant
☐ Other

How many weeks into your pregnancy did you stop taking your anticoagulant?

(weeks)

Which anticoagulant did you change to during your pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Enoxaparin
☐ Other

What was the other anticoagulant that you took during pregnancy?

What was the other way your anticoagulant treatment changed during pregnancy?

Did you have any and significant bleeding (haemorrhage) associated with the delivery of your baby?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Antifibrinolytic
☐ Vitamin K
☐ Dilatation and Curettage
☐ Iron therapy
☐ Blood product transfusion
☐ Hysterectomy
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Live Births - Baby 4

What anticoagulant were you taking at conception of baby number 4?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What other anticoagulant were you taking at conception?

Did your anticoagulation treatment change during your pregnancy?

- ☐ Yes
☐ No

How did your anticoagulation treatment change during pregnancy?

- ☐ Stopped taking any anticoagulant
☐ Changed to another anticoagulant
☐ Other

How many weeks into your pregnancy did you stop taking your anticoagulant?

(weeks)

Which anticoagulant did you change to during your pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Enoxaparin
☐ Other

What was the other anticoagulant that you took during pregnancy?

What was the other way your anticoagulant treatment changed during pregnancy?

Did you have any and significant bleeding (haemorrhage) associated with the delivery of your baby?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Antifibrinolytic
☐ Vitamin K
☐ Dilatation and Curettage
☐ Iron therapy
☐ Blood product transfusion
☐ Hysterectomy
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Live Births - Baby 5

What anticoagulant were you taking at conception of baby number 5?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What other anticoagulant were you taking at conception?

Did your anticoagulation treatment change during your pregnancy?

- ☐ Yes
☐ No

How did your anticoagulation treatment change during pregnancy?

- ☐ Stopped taking any anticoagulant
☐ Changed to another anticoagulant
☐ Other

How many weeks into your pregnancy did you stop taking your anticoagulant?

(weeks)

Which anticoagulant did you change to during your pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Enoxaparin
☐ Other

What was the other anticoagulant that you took during pregnancy?

What was the other way your anticoagulant treatment changed during pregnancy?

Did you have any and significant bleeding (haemorrhage) associated with the delivery of your baby?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Antifibrinolytic
☐ Vitamin K
☐ Dilatation and Curettage
☐ Iron therapy
☐ Blood product transfusion
☐ Hysterectomy
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Miscarriage 1

How many weeks into the pregnancy did you miscarry?

What anticoagulant were you taking at conception of pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What other anticoagulant were you taking at conception?

Did your anticoagulation treatment change during your pregnancy?

- ☐ Yes
☐ No

How did your anticoagulation treatment change during pregnancy?

- ☐ Stopped taking any anticoagulant
☐ Changed to another anticoagulant
☐ Other

How many weeks into your pregnancy did you stop taking your anticoagulant?

(weeks)

Which anticoagulant did you change to during your pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Enoxaparin
☐ Other

What was the other anticoagulant that you took during pregnancy?

What was the other way your anticoagulant treatment changed during pregnancy?

Did you have any and significant bleeding (haemorrhage) associated with the miscarriage?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Antifibrinolytic
☐ Vitamin K
☐ Dilatation and Curettage
☐ Iron therapy
☐ Blood product transfusion
☐ Hysterectomy
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Miscarriage 2

How many weeks into the pregnancy did you miscarry?

What anticoagulant were you taking at conception of pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What other anticoagulant were you taking at conception?

Did your anticoagulation treatment change during your pregnancy?

- ☐ Yes
☐ No

How did your anticoagulation treatment change during pregnancy?

- ☐ Stopped taking any anticoagulant
☐ Changed to another anticoagulant
☐ Other

How many weeks into your pregnancy did you stop taking your anticoagulant?

(weeks)

Which anticoagulant did you change to during your pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Enoxaparin
☐ Other

What was the other anticoagulant that you took during pregnancy?

What was the other way your anticoagulant treatment changed during pregnancy?

Did you have any and significant bleeding (haemorrhage) associated with the miscarriage?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Antifibrinolytic
☐ Vitamin K
☐ Dilatation and Curettage
☐ Iron therapy
☐ Blood product transfusion
☐ Hysterectomy
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Miscarriage 3

How many weeks into the pregnancy did you miscarry?

What anticoagulant were you taking at conception of pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What other anticoagulant were you taking at conception?

Did your anticoagulation treatment change during your pregnancy?

- ☐ Yes
☐ No

How did your anticoagulation treatment change during pregnancy?

- ☐ Stopped taking any anticoagulant
☐ Changed to another anticoagulant
☐ Other

How many weeks into your pregnancy did you stop taking your anticoagulant?

(weeks)

Which anticoagulant did you change to during your pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Enoxaparin
☐ Other

What was the other anticoagulant that you took during pregnancy?

What was the other way your anticoagulant treatment changed during pregnancy?

Did you have any and significant bleeding (haemorrhage) associated with the miscarriage?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Antifibrinolytic
☐ Vitamin K
☐ Dilatation and Curettage
☐ Iron therapy
☐ Blood product transfusion
☐ Hysterectomy
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Miscarriage 4

How many weeks into the pregnancy did you miscarry?

What anticoagulant were you taking at conception of pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What other anticoagulant were you taking at conception?

Did your anticoagulation treatment change during your pregnancy?

- ☐ Yes
☐ No

How did your anticoagulation treatment change during pregnancy?

- ☐ Stopped taking any anticoagulant
☐ Changed to another anticoagulant
☐ Other

How many weeks into your pregnancy did you stop taking your anticoagulant?

(weeks)

Which anticoagulant did you change to during your pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Enoxaparin
☐ Other

What was the other anticoagulant that you took during pregnancy?

What was the other way your anticoagulant treatment changed during pregnancy?

Did you have any and significant bleeding (haemorrhage) associated with the miscarriage?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Antifibrinolytic
☐ Vitamin K
☐ Dilatation and Curettage
☐ Iron therapy
☐ Blood product transfusion
☐ Hysterectomy
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Miscarriage 5

How many weeks into the pregnancy did you miscarry?

What anticoagulant were you taking at conception of pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What other anticoagulant were you taking at conception?

Did your anticoagulation treatment change during your pregnancy?

- ☐ Yes
☐ No

How did your anticoagulation treatment change during pregnancy?

- ☐ Stopped taking any anticoagulant
☐ Changed to another anticoagulant
☐ Other

How many weeks into your pregnancy did you stop taking your anticoagulant?

(weeks)

Which anticoagulant did you change to during your pregnancy?

- ☐ Warfarin
☐ Aspirin
☐ Enoxaparin
☐ Other

What was the other anticoagulant that you took during pregnancy?

What was the other way your anticoagulant treatment changed during pregnancy?

Did you have any and significant bleeding (haemorrhage) associated with the miscarriage?

- ☐ Yes
☐ No

What kind of medical treatment did you receive?

- ☐ Consultation only
☐ Antifibrinolytic
☐ Vitamin K
☐ Dilatation and Curettage
☐ Iron therapy
☐ Blood product transfusion
☐ Hysterectomy
☐ Stop anticoagulation
☐ Other

For how long did you stop your anticoagulation? (e.g. x days, x weeks or x months)

(Please specify days, weeks or months)

What other medical treatment did you receive?

Have you ever had a blood clot or stroke?

- ☐ Blood clot
☐ Stroke
☐ None

What anticoagulant were you taking at the time the blood clot occurred?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What was the other anticoagulant you were taking?

Where was the blood clot?

When did the blood clot occur?

(Please enter DD-MM-YYYY)

What treatment did you receive for the blood clot?

Please detail any ongoing medical issues caused by the blood clot

What anticoagulant were you taking at the time the stroke occurred?

- ☐ Warfarin
☐ Aspirin
☐ Other
☐ None

What was the other anticoagulant you were taking?

When did the stroke occur?

(Please enter DD-MM-YYYY)

What treatment did you receive for the stroke?

Please detail any ongoing medical issues caused by the stroke

Do you have any other medical conditions?

- ☐ Yes
☐ No

Please give details of other medical conditions

Have you had heart palpitations or arrhythmias in the last year?

- ☐ Yes
☐ No

How many times have you had palpitations or
arrhythmia in the past year?

When did you last see your cardiologist?

(Please enter DD-MM-YYYY)

When do you see your cardiologist again?

(Please enter DD-MM-YYYY)

Do you have any additional comments regarding
bleeding you have experienced or any glitches in
this survey?

LETTER TO THE EDITOR

Comment on: Generation and optimization of the self-administered pediatric bleeding questionnaire and its validation as a screening tool for von Willebrand disease

To the Editor:

We would like to congratulate Casey et al. on the development of their self-administrable pediatric bleeding questionnaire (Self-PBQ) as a screening tool for von Willebrand disease.¹ To date, similar screening tools have required expert administration, generally in a clinical setting. The development of a self-administrable tool has many benefits including reducing the time burden on clinicians and the possibility of administration outside of the clinic.

Although we acknowledge the usefulness of such a tool, we raise a number of concerns with the validity of the Self-PBQ. Namely, we are concerned with the wording of a number of items. One such example is item 1.9 where a possible answer is, “given a medication intravenously (IV) or with a needle into the skin at least once (desmopressin—DDAVP, Octostim or Stimat)”. The reference to *intravenously* and *desmopressin—DDAVP, Octostim or Stimat* is unlikely to be comprehended by a layperson. The agreement demonstrated in the validation of the Self-PBQ with the expert administered version may be influenced either direction due to the wording, that is, if a question is not understood in one version of the tool the participant is unlikely to understand the same question when asked in another version of the same tool. Therefore, the participant's responses will most likely be in agreement between the tools, despite the limited understanding of the question.²

The authors noted that the tool was converted to a grade 4 level of reading, however the specifics of how this was achieved was not mentioned. It is critical that self-administered tools aimed at children undergo lay translation in consultation with an appropriate expert specializing in educational theory.^{2,3}

It is fundamental to the utility of any self-administered screening tool that comprehension by the target population is achievable. Without patients adequate understanding of the questions being

posed to them, the development of a meaningful and useful tool is unachievable.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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No difference between aspirin and warfarin after extracardiac Fontan in a propensity score analysis of 475 patients

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Abstract

OBJECTIVES: The most effective method of long-term thromboprophylaxis after the Fontan procedure is not clear. We compared the rates of thromboembolic events between patients receiving aspirin and warfarin after an extracardiac conduit Fontan procedure in a bi-national registry.

METHODS: All patients who underwent an extracardiac conduit Fontan procedure from 1997 to 2010 in Australia and New Zealand were identified. Medication status and routine follow-up and echocardiographic data were obtained from all patients. Survival analysis with propensity score matching and adjustment was performed to determine the treatment effect of warfarin compared with that of aspirin beyond the first year of follow-up, after which time patients had settled on their long-term thromboprophylaxis strategy.

RESULTS: Of 570 eligible patients, the data of 475 patients who were regularly followed up without mechanical valve replacement were available for analysis. Long-term thromboprophylaxis consisted of warfarin in 301 patients (63%), aspirin in 157 (33%) and none in 17 (4%). The 10-year rate of freedom from all thromboembolic events was 91% [95% confidence interval (CI) 88–94%]. Thromboembolic events beyond the first year of follow-up occurred in 18 patients (6 on aspirin and 12 on warfarin). After (i) propensity score adjustment and (ii) matching yielding 164 pairs, the hazard rates of thromboembolic events beyond the first year were not statistically different between the warfarin and aspirin groups [(i) hazard ratio (HR) 2.3, 95% CI 0.7–7.4, $P = 0.2$ and (ii) HR 1.5, 95% CI 0.5–4.7, $P = 0.5$, respectively].

CONCLUSIONS: No difference in the hazard rates of late thromboembolic events was observed between aspirin and warfarin beyond the first year after the extracardiac conduit Fontan procedure.

Keywords: Fontan procedure • Thromboprophylaxis • Warfarin • Aspirin • Congenital heart disease

INTRODUCTION

The Fontan circulation is an ideal environment for thrombosis. While some form of thromboprophylaxis is deemed necessary to prevent the occurrence of thromboembolic events [1], no consensus

yet exists on the optimal medication. The evidence to support either aspirin or warfarin is lacking [2].

The Australia and New Zealand Fontan Registry was created with the aim of using retrospective follow-up data from multiple centres to create a dataset that could answer research questions

based on varying treatment practices in the region [3]. In Australia and New Zealand, since 1997, all centres have adopted the extracardiac conduit as the exclusive mode of Fontan completion. While creating the registry, we registered the variation in thromboprophylaxis practice between centres. We hypothesized that, after propensity score matching in this contemporary cohort of patients operated with a standard technique, no difference would be observed between patients treated with warfarin and aspirin.

METHODS

The design and administration of the Australia and New Zealand Fontan Registry have been previously described [3]. Since 1997, the extracardiac conduit became the predominant technique of Fontan procedure performed in both countries, and from 2006 has been the sole Fontan technique used. The analysis of early and late outcomes of the whole cohort of 570 extracardiac Fontan patients has recently been published [4]. From this dataset, a total of 30 patients were excluded who died prior to discharge (7 patients), were referred from outside Australia or New Zealand (17) or had mechanical prosthetic valves (6). We attempted to obtain the discharge summary, yearly clinical summary and echocardiogram or magnetic resonance imaging report for the remaining eligible local patients. All transthoracic echocardiograms were acquired by cardiologists with post-fellowship training in paediatric or grown-up congenital cardiology, or by sonographers with dedicated training in congenital imaging. These images were then reviewed and reported by the treating cardiologist. The frequency of imaging was generally yearly, but was determined by the treating cardiologists according to institutional preference and clinical need. Anticoagulation status and occurrence of thromboembolism were determined from the clinical summary and imaging reports. Patients who were transplanted or died during the first year after their Fontan (3 patients), those who were lost to follow-up for any period of more than 5 years (51) and those with <1 year of follow-up available (12) were excluded from the analysis, leaving a cohort of 475 patients (88%) with sufficient complete follow-up.

Pre- and perioperative variables were obtained from the registry database. The study conformed to the 'STROBE STATEMENT: Guidelines for reporting observational studies' [1, 5] and to the standards set by the Declaration of Helsinki. Ethical review board approvals were granted from each participating institution.

Thromboprophylaxis status

Thromboprophylaxis regime varied according to institutional preference. Institutions with a preference for long-term anticoagulation with aspirin routinely administered warfarin for 3–6 months before transitioning to low-dose aspirin (5 mg/kg/day, equating in practice to doses between 50 and 150 mg). For this reason, we limited the analysis to the risk of thromboembolic events beyond the first year, once all patients had settled on their long-term thromboprophylaxis strategy.

Of 475 patients included in the study, 410 (86%) were initially anticoagulated with warfarin. Those who were destined to receive lifelong low-dose aspirin (157 patients, 33%) were transitioned during the first year of follow-up. Patients were determined to be in a treatment group (aspirin or warfarin) based on the medication they had settled on at their 1-year post-Fontan follow-up visit.

Participants who crossed from one anticoagulant treatment to another beyond the first year had their follow-up terminated for the analysis at the time of crossover (20 patients). Those who were on aspirin plus warfarin (1 patient) were assigned to the warfarin group.

End-points

The primary end-point was time to first thromboembolic event, which was defined as any thrombus evident on imaging (including thrombus within the Fontan circuit, deep venous thrombosis and pulmonary embolism) or any clinical event associated with thrombotic embolism [stroke, transient ischaemic attack (TIA), pulmonary embolism and end-organ infarction]. Stroke and TIA were defined as neurological deficit lasting ≥ 72 and < 72 h, respectively. All pulmonary embolic events were confirmed by echocardiography, computed tomography or ventilation–perfusion scan.

Bleeding was defined as Bleeding Academic Research Consortium (BARC) Types 2 (overt haemorrhage that is actionable) to 5 (fatal bleeding) [2, 6]. Minor bleeding events (BARC Types 0 and 1) were excluded.

The timing of events was divided into: (i) pre-Fontan, (ii) early postoperative occurring prior to hospital discharge, (iii) during the first year after Fontan and (iv) events beyond the first year.

A composite end-point of time to first bleeding or thromboembolic event is also reported.

Statistical analysis

All analyses were performed in Stata 13 (StataCorp LP, College Station, TX, USA). Analysis of the association between anticoagulant regimen and time to first thromboembolic event was performed using Cox proportional hazards regression. Kaplan–Meier survival curves were generated for each end-point. Smoothed hazard functions were generated using a Gaussian kernel density estimate and visual adjustment of the Silverman kernel width to mitigate over-smoothing. No patient suffered recurrent thromboembolic events, so repeated events analysis was not performed.

To counteract potential bias caused by marked baseline heterogeneity in measured variables between the aspirin and warfarin groups (Table 1), a propensity score was calculated. Clinically relevant factors were selected *a priori*, and additional factors were included if univariable logistic regression demonstrated an association with treatment assignment [odds ratio (OR) > 2.5 or < 0.4 or a *P*-value < 0.10]. The model was considered saturated if the area under the receiver operating characteristic (ROC) curve was greater than 0.8. The final model included the factors summarized in [Supplementary Material](#). The final model did not include the centre as a random effect, as the residual bias was too large. Pre-Fontan saturation was also excluded from the propensity model due to missing data in 20 patients on aspirin. Propensity scores were (i) included in a multivariable model to counteract the effect of heterogeneity between groups and (ii) used to match patients who had close propensity scores [3, 7, 8]. We thus constructed two survival models for each end-point: (i) including the propensity score as a covariate for all individuals and (ii) restricting the analysis to propensity score-matched pairs. Matched pairs were created by performing 1:1 nearest-neighbour matching, and a clustered sandwich estimator of variance was used in the survival model to calculate confidence intervals. Residual bias for

Table 1: Patient characteristics according to the thromboprophylaxis group

	None (17 patients)	Aspirin (157 patients)	Warfarin (301 patients)
Male, n (%)	9 (53%)	85 (54%)	184 (61%)
Dextrocardia, n (%)	0 (0%)	17 (11%)	19 (6%)
Isomerism, n (%)	0 (0%)	13 (8%)	21 (7%)
Hypoplastic left heart syndrome, n (%)	3 (18%)	6 (4%)	62 (21%)
Ventricular morphology, n (%)			
Left	10 (59%)	90 (57%)	156 (52%)
Right	6 (35%)	53 (34%)	114 (38%)
Biventricular/indeterminate	1 (6%)	14 (9%)	31 (10%)
Pre-Fontan procedures			
Number of prior palliations, mean (SD)	2.2 (0.7)	2.2 (0.8)	2.5 (1.1)
Prior pulmonary artery banding, n (%)	5 (29%)	48 (31%)	67 (22%)
Pre-Fontan haemodynamics			
Oxygen saturation (%), mean (SD) ^a	75 (20)	84 (6)	81 (6)
Pulmonary artery pressure (mmHg), mean (SD) ^b	11.5 (2.4)	10.8 (2.7)	11.1 (2.6)
Aortopulmonary or veno-venous collaterals, n (%)	2 (12%)	19 (12%)	103 (34%)
Arterio-venous malformations, n (%)	1 (6%)	4 (3%)	24 (8%)
Fontan operative characteristics			
Concomitant procedure, n (%)	3 (18%)	15 (10%)	41 (14%)
Fenestration, n (%)	5 (29%)	17 (11%)	168 (56%)
Age at Fontan, median (IQR)	5.7 (5.1–7.0)	4.8 (3.9–5.8)	4.7 (3.8–5.8)
Associated interventions before or at time of Fontan			
Atrioventricular valve repair, n (%)	1 (6%)	5 (3%)	22 (7%)
Aortic arch intervention, n (%)	6 (35%)	40 (25%)	119 (40%)

SD: standard deviation; IQR: interquartile range.

^aMissing in 28 patients.^bMissing in 56 patients.

a covariate in the propensity model was considered a greater than 5% standardized percentage bias in the matched cohort, which is defined as the percentage difference of the sample means in the treated and non-treated subsamples as a percentage of the square root of the average of their sample variances [3, 9]. The overall bias of the model was assessed by determining the median standardized bias. This was calculated by deriving the standardized biases for each covariate in the propensity model, and finding the median value. A model was considered to have an acceptable level of overall bias if the median bias after matching was <5%.

The end-points analysed were (i) time to first thromboembolic event and (ii) a composite of time to first bleeding or thromboembolic event. Time was measured starting from Fontan completion with entry to the survival model at the first-year follow-up visit.

Post hoc sensitivity analysis was performed using the 'stpower' command in Stata 13, to calculate the minimum effect size or hazard ratio that could be detected based on the number of matched pairs obtained and the number of events actually observed.

RESULTS

The whole cohort consisted of 570 patients with an extracardiac conduit. After excluding the 7 early deaths, 17 patients referred from overseas and 6 patients with mechanical valve prostheses, there were 540 patients remaining. Follow-up that was complete was available in 475 patients (88%), comprising follow-up of 3479 patient-years and a median duration of 7 years (interquartile range 4.7–9.7).

The characteristics of the whole cohort of 475 patients with complete follow-up are presented in Tables 1 and 2 and characteristics included in the propensity score model of the matched cohort are presented in Table 3.

Thromboprophylaxis regimen

At the time of discharge from hospital, 410 patients (86%) were receiving warfarin, 52 (1%) aspirin, 3 (1%) both aspirin and warfarin, and 10 (2%) no thromboprophylaxis. By the time of the clinic visit beyond 1 year after the Fontan procedure, patients had settled on their long-term thromboprophylaxis strategy, which consisted of warfarin in 300 patients (63%), aspirin in 157 (33%) and aspirin with warfarin in 1 patient. The remaining 17 patients (4%) received no thromboprophylaxis during the period of follow-up analysed.

There were 20 patients (4%) who crossed between groups after the first year of follow-up. Seven patients who were on aspirin switched to warfarin: 5 patients after a developing a thromboembolic event, 1 an arrhythmia and 1 protein-losing enteropathy. Eight patients ceased warfarin therapy, due to patient preference in 5 patients, medical comorbidity in 1, bleeding in 1 and poor compliance in 1. One patient who suffered a thromboembolic event switched from warfarin alone to warfarin plus aspirin. One who was on warfarin plus aspirin transitioned to aspirin alone after an episode of haemoptysis. One who was not on any therapy commenced on aspirin after occurrence of atrial arrhythmia. The final patient was non-compliant with aspirin, and it was thus ceased.

In the overall cohort, treatment assignment analysis by univariable logistic regression revealed that the following factors were associated with long-term warfarin anticoagulation: fenestration

Table 2: Patient characteristics according to thromboembolic events

	None (444 patients)	During first year (16 patients)	Beyond first year (18 patients)
Male, <i>n</i> (%)	262 (59%)	13 (81%)	7 (39%)
Dextrocardia, <i>n</i> (%)	38 (9%)	0 (0%)	0 (0%)
Isomerism, <i>n</i> (%)	32 (7%)	1 (6%)	1 (6%)
Hypoplastic left heart syndrome, <i>n</i> (%)	63 (14%)	4 (25%)	3 (17%)
Ventricular morphology, <i>n</i> (%)			
Left	245 (55%)	6 (38%)	10 (56%)
Right	159 (35%)	6 (58%)	6 (33%)
Biventricular/indeterminate	40 (9%)	4 (25%)	2 (11%)
Pre-Fontan procedures			
Number of prior palliations, mean (SD)	2.4 (1.0)	2.3 (0.7)	2.3 (1.1)
Prior pulmonary artery banding, <i>n</i> (%)	112 (25%)	3 (19%)	7 (39%)
Pre-Fontan haemodynamics			
Oxygen saturation (%), mean (SD) ^a	82 (7)	81 (6)	80 (4)
Pulmonary artery pressure (mmHg), mean (SD) ^b	11.0 (2.6)	11.2 (2.1)	11.5 (2.6)
Aortopulmonary or veno-venous collaterals, <i>n</i> (%)	116 (26%)	4 (25%)	2 (11%)
Arterio-venous malformations, <i>n</i> (%)	27 (6%)	1 (6%)	0 (0%)
Fontan operative characteristics			
Concomitant procedure, <i>n</i> (%)	58 (13%)	2 (13%)	1 (6%)
Fenestration, <i>n</i> (%)	172 (39%)	8 (50%)	5 (28%)
Age at Fontan, median (IQR)	4.8 (3.9–5.8)	4.4 (3.5–5.6)	5.2 (4.4–6.2)
Associated interventions before or at time of Fontan			
Atrioventricular valve repair, <i>n</i> (%)	28 (6%)	0 (0%)	1 (6%)
Aortic arch intervention, <i>n</i> (%)	153 (35%)	4 (25%)	8 (44%)
Pre-Fontan thromboembolic event, <i>n</i> (%)	6 (1%)	2 (13%)	0 (0%)
Warfarin at time of event, <i>n</i> (%)	272 (61%)	14 (88%)	12 (67%)

SD: standard deviation; IQR: interquartile range.

^aMissing in 28 patients.^bMissing in 56 patients.**Table 3:** Characteristics of patients in the propensity score-matched cohort according to the thromboprophylaxis group

	Aspirin (164 patients)	Warfarin (164 patients)	Residual bias (%)	t-Test P-value
Male, <i>n</i> (%)	91 (55%)	93 (57%)	3	
Dextrocardia, <i>n</i> (%)	19 (12%)	15 (9%)	9	
Systemic right ventricle, <i>n</i> (%)	55 (34%)	40 (24%)	19	
Hypoplastic left heart syndrome, <i>n</i> (%)	6 (4%)	4 (2%)	4	
Common atrioventricular valve, <i>n</i> (%)	22 (13%)	20 (12%)	4	
Isomerism, <i>n</i> (%)	14 (9%)	15 (9%)	2	
Pre-Fontan procedures, mean (SD)	2.2 (0.8)	2.3 (0.9)	4	
Prior pulmonary artery banding, <i>n</i> (%)	52 (32%)	42 (26%)	14	
Pre-Fontan haemodynamics				
Aortopulmonary or veno-venous collaterals, <i>n</i> (%)	20 (12%)	47 (29%)	23	<0.001
Arterio-venous malformations, <i>n</i> (%)	4 (2%)	11 (7%)	24	
Fontan operative characteristics				
Fenestration, <i>n</i> (%)	17 (11%)	41 (25%)	35	<0.001
Age at Fontan, median (IQR)	4.9 (3.9–5.9)	4.7 (4.0–5.7)	3	
Associated interventions before or at time of Fontan				
Atrioventricular valve repair, <i>n</i> (%)	8 (5%)	8 (5%)	0	
Aortic arch intervention, <i>n</i> (%)	41 (25%)	38 (23%)	4	
Thromboembolic risk factors				
Pre-Fontan thromboembolism	4 (3%)	2 (1%)	9	
Early postoperative thromboembolism	1 (1%)	1 (1%)	0	
Events during the first year of follow-up	2 (1%)	3 (2%)	4	

SD: standard deviation; IQR: interquartile range.

Only *P*-values for factors with residual bias have been presented.

[OR 10.4, 95% confidence interval (CI) 6.0–18], lower pre-Fontan saturation (OR 0.90 for every 1% increase, 95% CI 0.86–0.93), hypoplastic left heart syndrome (HLHS) (OR 6.5, 95% CI 2.6–16), pre-Fontan collaterals (OR 2.9, 95% CI 1.7–5.1), prior or concomitant aortic arch intervention (OR 1.9, 95% CI 1.3–3.0), number of prior procedures (OR 1.3 for each additional procedure, 95% CI 1.1–1.6) and absence of a prior pulmonary artery band (OR 0.7, 95% CI 0.4–1.0). Additionally, there were trends towards associations for pre-Fontan arterio-venous malformations (OR 2.6, 95% CI 0.9–7.5), absence of dextrocardia (OR 0.6, 95% CI 0.3–1.1), prior or concomitant valve repair (OR 2.0, 95% CI 0.9–4.5), early post-operative thromboembolic event (OR 4.3, 95% CI 0.5–34) and thromboembolic event during the first year of follow-up (OR 3.8, 95% CI 0.8–17).

Thromboembolic events

Thromboembolic events were noted prior to Fontan completion in 8 patients (1.5%), in the early postoperative period in 9 (1.7%), during the first year in 7 (1.3%) and during late follow-up after the first postoperative year in 18 (3.5%). Pre-Fontan events consisted of embolic stroke in 5 patients, pulmonary embolism in 2 and central vein thrombosis in 1. Early postoperative events consisted of embolic stroke in 3 patients, watershed infarcts in 3, conduit thrombosis in 2 and renal embolism in 1. The Fontan circuit was fenestrated in 7 of the 9 patients who suffered early postoperative events, and all 4 early systemic embolic infarcts occurred in patients with a fenestrated Fontan circuit. Events occurring after hospital discharge but during the first year of follow-up consisted of conduit thrombus in 5 patients and symptomatic pulmonary embolism in 2. All conduit thrombi were asymptomatic and were detected on routine screening echocardiography. Six of the 7 patients who experienced events during the first year were on warfarin at the time, and as a result, 2 of these patients received combined anticoagulation and antiplatelet therapy for a period of 3–6 months with eventual thrombus resolution.

Thromboembolic events beyond the first year of follow-up consisted of stroke in 3, TIA in 2, conduit thrombus in 10 and pulmonary embolism in 3. Nine out of 10 conduit thrombi were detected on routine screening echocardiography and caused no symptoms, whereas the remaining patient was being investigated for exertional breathlessness by transoesophageal echocardiography. At

the time of their event, 12 patients (67%) were taking warfarin and 6 (33%) were taking aspirin. Out of the 5 patients who suffered a stroke or TIA, the Fontan circuit was fenestrated in 1, whereas in the other 4 it was not fenestrated; 3 were on warfarin and 2 on aspirin at the time of their event. No patient suffered recurrent events.

Of the 18 patients who suffered a thromboembolic event while on warfarin, 8 (44%) had an international normalized ratio (INR) of <2 at the time of or immediately prior to the event. Among patients who had reintervention, there were 19 reoperations and 38 catheter-based interventions. Of the 19 patients who were reoperated, 2 suffered thromboembolic events: one a TIA while on aspirin and the other a conduit thrombosis while on warfarin. No patients who had a catheter-based intervention suffered an event.

Bleeding events

Bleeding that satisfied the study definition of BARC Types 2–5 occurred in 12 patients (3%). These consisted of post-traumatic intracranial haemorrhage in 3, spontaneous intracerebral haemorrhage in 1, major epistaxis in 1, haemoptysis in 1, gastrointestinal bleeding in 3, severe menorrhagia with iron deficiency anaemia in 1, vaginal haemorrhage in 1 and superficial post-traumatic haemorrhage in 1. Eleven of the bleeding events were associated with warfarin (91%), and 4 (36%) occurred while the INR was greater than 3.

Survival analysis

The rate of freedom from all thromboembolic events was 99% (95% CI 98–100%) at 30 days, 97% (96–99%) at 1 year, 94% (91–96%) at 5 years and 91% (88–94%) at 10 and 12 years (Fig. 1). The 12-year rate of freedom from bleeding events was 91% (95% CI 83–95%). The 12-year rate of freedom from thromboembolism or bleeding was 84% (77–90%). The unadjusted rates of thromboembolic events occurring beyond the first year after the Fontan were 0.54 (95% CI 0.24–1.2) per 100 patient-years for aspirin and 0.57 (95% CI 0.32–1.0) for warfarin. The 12-year rates of freedom from the composite end-point for the aspirin and warfarin groups were 93% (95% CI 86–97%) and 79% (95% CI 68–87%),

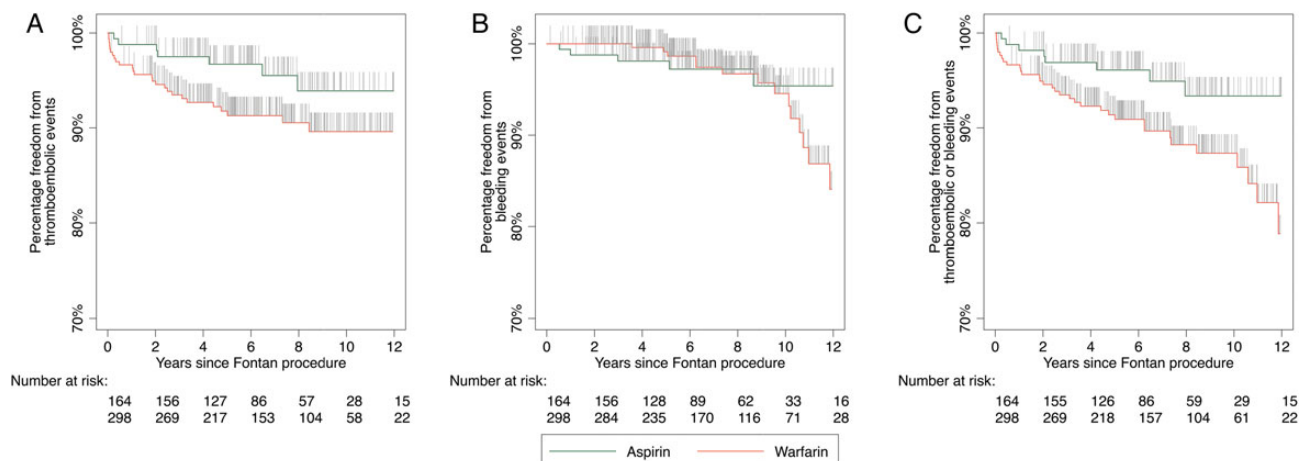


Figure 1: Freedom from (A) thromboembolic events, (B) bleeding events and (C) a composite of both events; aspirin (green) versus warfarin (red).

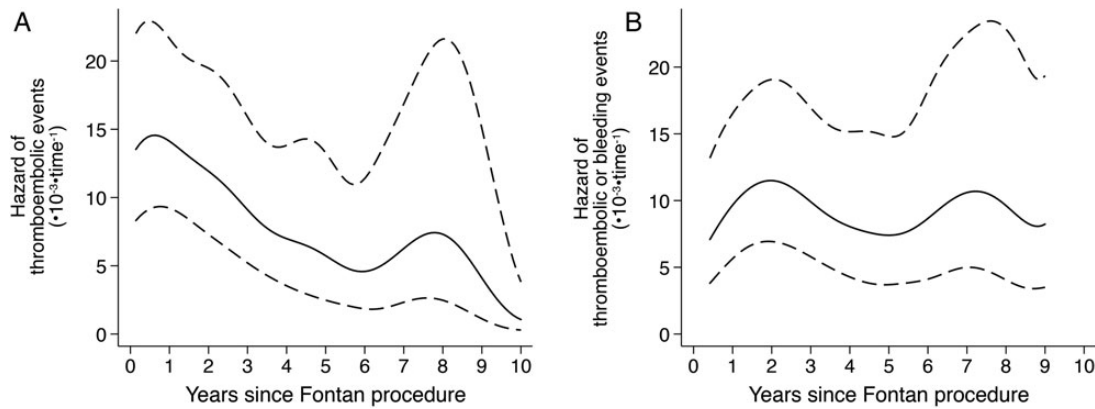


Figure 2: Smoothed hazard function for (A) thromboembolic events, demonstrating an early high-risk phase followed by a progressive decline in risk as previously shown by others [1], and (B) thromboembolic or bleeding events. Dotted lines represent 95% confidence intervals.

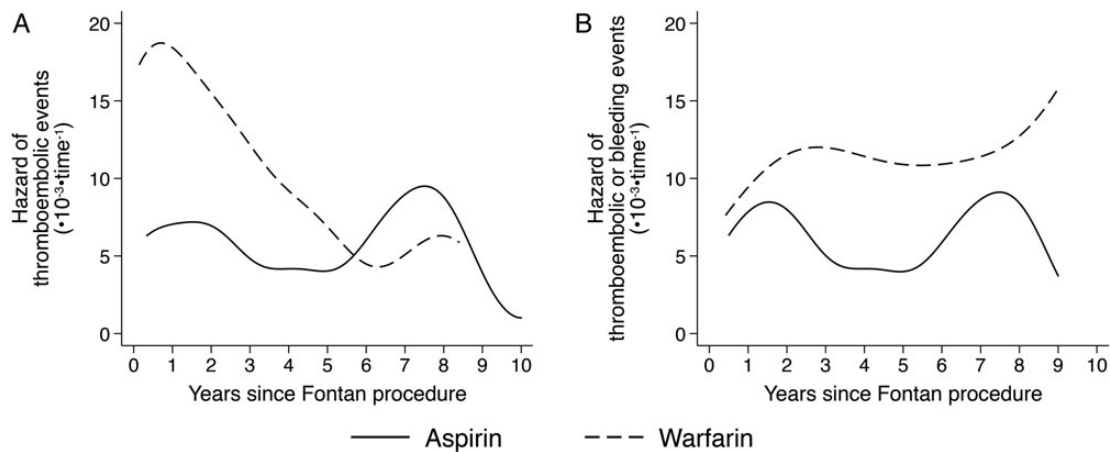


Figure 3: Smoothed hazard functions by the thromboprophylaxis group for (A) thromboembolic events and (B) thromboembolic or bleeding events.

Table 4: Results of Cox regression analysis with propensity score adjustment and matching

	HR	95% CI	P-value
Thromboembolic events occurring beyond Year 1			
Unadjusted (n = 451)	1.3	0.5–3.7	0.6
Propensity score adjusted (n = 451)	2.3	0.7–7.4	0.2
Propensity-matched cohort (n = 328)	1.5	0.5–4.7	0.5
Thromboembolic or bleeding events occurring beyond Year 1			
Unadjusted (n = 450)	1.8	0.6–4.8	0.3
Propensity score adjusted (n = 450)	2.2	0.7–6.9	0.2
Propensity-matched cohort (n = 328)	1.7	0.6–5.2	0.4

Hazard ratios are of the warfarin group compared with the aspirin group.
HR: hazard ratio; CI: confidence interval.

respectively. There was a time-related decline in the overall hazard function for thromboembolic events (Figs 2 and 3).

The results of the propensity score-derived models are presented in Table 4. The model was saturated with an area under the ROC

curve of 0.84. The mean propensity scores for patients in the warfarin and aspirin groups were 0.77 (95% CI 0.74–0.79) and 0.44 (95% CI 0.41–0.48, $P < 0.001$), respectively. The median standardized biases before and after matching were 18 and 4%, respectively.

After matching on the propensity score, 164 pairs of patients (328 patients in total) in the warfarin and aspirin groups were identified. The variables included in the propensity score model are presented in [Supplementary Material](#) and, after the matching process, there was residual bias between the aspirin and warfarin groups, respectively, in the following characteristics: the proportion of patients with arterio-venous or veno-venous collaterals (12 vs 29%, $P < 0.001$) and the proportion with a fenestrated Fontan (11 vs 25%, $P < 0.001$).

The Cox regression results for the propensity score-based analyses (Table 4) illustrate higher point estimates for the risk of late thromboembolic events in patients treated with warfarin compared with those treated with aspirin [hazard ratio (HR) 2.3, 95% CI 0.7–7.4, $P = 0.2$ after propensity score adjustment, and HR 1.5, 95% CI 0.5–4.7, $P = 0.5$ in the matched cohort]; however, the confidence limits are wide. The results of the analysis of the composite outcome (thromboembolism or bleeding) were similar.

Sensitivity analysis

Post hoc sensitivity analysis was performed to determine the minimum effect size that could be detected with the sample obtained in the propensity-matched cohort. This showed that, with 164 patients in each group and 18 events in total, a minimum hazard ratio of 3.8 could be detected with an 80% power and a significance level of 0.05.

DISCUSSION

The absence of thromboprophylaxis is associated with thromboembolic events after the Fontan procedure [4, 10], and anticoagulation or antiplatelet therapy has been widely recommended [11]. There is no consensus on the optimal method of thromboprophylactic therapy after Fontan surgery. Whether a patient receives aspirin or warfarin remains institutionally based in Australia and New Zealand. The extracardiac technique has been uniformly adopted in this region since 1997 [4]. Because of the standardization of our Fontan technique and the concomitant variation in thromboprophylaxis practice in our region, we had a unique opportunity to review the rates of thromboembolic events in patients with a single contemporary technique with two different thromboprophylaxis approaches. The recruitment of patients to prior randomized controlled trials has proved difficult and could not establish differences in long-term events [12].

The primary outcome in this study was any thromboembolic event occurring beyond the first year of follow-up, and our results suggest that there was no difference between warfarin and aspirin upon the rates of thromboembolic or bleeding events. In fact, point estimates demonstrated a slightly higher risk of thromboembolic events in the warfarin group; however, the study was underpowered to detect a clinically meaningful difference, as only differences above HR 3.8 could be detected. A lack of superiority of warfarin has been previously suggested, but the impact of warfarin on long-term outcomes had remained unclear [2, 13]. The routine administration of warfarin after Fontan surgery should thus be put into question [14]. The burden of warfarin anticoagulation compared with aspirin administration is undeniable. There is a growing concern that the long-term use of warfarin is associated with low bone mineral density and increased fracture risk [15, 16]. It has been previously demonstrated that close to half of the INR

measurements of patients on warfarin after Fontan surgery are outside target values [1]. While it is uncertain whether our findings will change clinical practice, they should at the minimum reassure those willing to consider the use of long-term aspirin beyond the first year for the sake of patient convenience, and those interested in initiating randomized trials of these two treatments.

Limitations

This study is limited by its retrospective nature because only events that became clinically relevant were reported and asymptomatic thrombi may not always have been detected. Nevertheless, the rate of thromboembolism in this series is similar to other contemporary epidemiological studies [17]. Individual patients' longitudinal INR measurements were not collected, so the adequacy of anticoagulation in those receiving warfarin could not be assured.

Because the majority of our patients were transitioned during the first year to aspirin from an initial treatment with warfarin, our study does not provide the appropriate design to analyse the difference between anticoagulation regimens in this high-risk period.

Propensity score matching attempts to account for covariates that predict receiving treatment, by selecting matched pairs from each group with similar probabilities of treatment assignment. Treatment with warfarin was associated with a number of unfavourable characteristics (HLHS, pre-Fontan collaterals and fenestration). Thus, the proportion of patients in the matched cohort with these characteristics is much smaller (Tables 1 versus 3) than in the overall cohort. Additionally, residual bias in the proportion of patients with fenestration and pre-Fontan collaterals remained even after propensity score matching. These factors limit the generalizability of the findings in the analysis for patients with certain adverse features such as HLHS, fenestration and pre-Fontan collaterals.

Owing to the small number of thromboembolic events, a propensity score-matched cohort that included the centre could not be analysed and a centre effect cannot be ruled out. Finally, sensitivity analysis showed that only large effect sizes could be detected with the sample size and the number of events observed in this cohort. Smaller differences as have been observed may or may not be due to random variation and remaining selection bias. Further data are required to conclusively rule out smaller differences.

Conclusion

In this bi-national, multicentric, propensity score-matched study of thromboprophylaxis beyond 1 year after the extracardiac Fontan procedure, no differences were found in the rates of thromboembolism between patients taking aspirin and warfarin.

SUPPLEMENTARY MATERIAL

[Supplementary material is available at EJCTS online.](#)

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Full Length Article

Differences in the mechanism of blood clot formation and nanostructure in infants and children compared with adults



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ABSTRACT

Introduction: Infants and children have a lower incidence of thrombosis compared with adults. Yet, the mechanism of blood clot formation and structure in infants and children, as the end product of coagulation, has not been studied. This study aimed to establish differences in the mechanism of thrombin generation, fibrin clot formation and response to thrombolysis in infants and children compared with adults.

Materials and methods: We studied thrombin generation, fibrin clot formation, structure and fibrinolysis in healthy infants, children and adults.

Results: Younger populations had a decreased potential to generate thrombin, at a slower velocity compared with adults, correlating positively with age. Clot formation at venous shear rate was decreased in infants and children compared with adults, with increased time for fibrin formation, decreased fibrin formation velocity, resulting in decreased tendency for fibrin formation in younger populations. These differences were less pronounced at arterial shear rate. Studies of the fibrin clot structure in paediatric age groups showed a significantly larger pore size compared with adults, suggestive of a clot that is less resistant to fibrinolysis. The presence of tissue plasminogen activator (tPA) resulted in a significant decrease in the pore size of infants and children, but not in adults.

Conclusions: This is the first study to suggest that the mechanism of blood clot formation and nanostructure, as well as response to thrombolytic therapy is different in infants and children compared with adults.

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1. Introduction

The extremely low incidence of thrombosis in infants and children suggests that they are protected from clinical events. Apart from the enormous differences in rates of spontaneous thrombosis, thrombosis secondary to vascular access occurs at lower rates in children compared to adults, although such secondary thrombosis are the major proportion of thrombosis observed in children. The lower prevalence in thrombosis, for the same iatrogenic insult, relates to physiological differences in the hemostatic system of children and adults. There is compelling evidence from multiple studies, that the hemostatic system is profoundly affected by age [1–6]. Vitamin K dependent coagulation factors are reduced by 50% in newborns compared with adults and remain about 10–20%

lower during childhood. In neonates antithrombin levels are reduced by 20%, but throughout the rest of childhood are increased by 10% compared with adults. Protein C and protein S levels are also reduced in neonates, with protein C remaining reduced throughout childhood [1].

The age-related differences in the quantitative and functional measurements of the majority of hemostatic proteins [1–5], are described conceptually as Developmental Haemostasis. Recently, the platelet phenotype has also been shown to change with age [6]. However, the majority of studies of Developmental Haemostasis to date have focused on individual components of the hemostatic system. Very few studies have focused on the functionality of the hemostatic system as a whole.

Thrombin generation tests are known to correlate with the overall function of the hemostatic system and are related to a bleeding and/or thrombotic risk [7]. Such tests are increasingly being recognized as a versatile diagnostic tool in thrombosis and haemostasis. Thrombin generation has previously been found to be decreased in infants and children compared with adults [5,8,9] and has been suggested to be

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the result of an increased inhibition of thrombin by the naturally occurring coagulation inhibitor alpha-2-macroglobulin ($\alpha 2M$) [10]. However, the exact mechanism behind the age-specific differences in thrombin generation is not well understood.

Formation of a clot via the thrombin-mediated conversion of fibrinogen into fibrin is the final step in the process of coagulation. The fibrinolytic and viscoelastic properties of the clot are directly dependent on the structure of the fibrin clot [11]. The thrombin concentration present during the conversion influences fibrin clot nanostructure. In the presence of low amounts of thrombin, fibrin fibres are thick and highly susceptible to lysis. On the other hand, in the presence of high thrombin levels, fibres are thin but tightly packed and impermeable [12]. The fibrin network architecture regulates the distribution of lytic enzymes [13], thus fibrin clots composed of compact (small pores), highly branched networks and with thin fibres are more resistant to fibrinolysis. Plasma from adults with a personal or family history of thrombosis produces such thin, highly-branched, impermeable fibrin networks that are resistant to fibrinolysis [14]. Adults with myocardial infarction and stroke have clots that are less permeable and hence more resistant to fibrinolysis [15,16], whilst adults with venous thromboembolism (VTE) have decreased clot permeability, lower compaction and prolonged clot lysis time compared with healthy controls [17].

The exact mechanism of the protection of children for thrombosis is unknown and improved understanding of this mechanism may provide new insights in the prevention and treatment of hemostatic disturbances in children and adults. This study investigates the clot nanostructure of children compared with adults and the effect of tissue plasminogen activator (tPA) on the properties of the fibrin clot.

2. Materials and methods

2.1. Samples

Blood samples used in this study were collected and processed according to established protocols [1,5,6,18]. Specifically, samples were obtained from healthy children attending the hospital for minor day-surgery without a family history of coagulation disorders (e.g. thrombosis and haemorrhage). Children were assigned to 4 different groups according to age: 1 month to 1 year, 1 to 5 years, 6 to 10 years and 11 to 16 years, based on previous studies of developmental haemostasis [1,5,6]. The paediatric cohort excluded adolescents taking oral contraceptive and smokers. Adult samples were obtained from healthy volunteers aged between 20 and 45 years of age who were not taking any medication (including oral contraceptive), were not smokers and did not have a family history of coagulation disorders. The incidence of thrombosis is not significantly different in adults within this age range and hence these were analysed as one age group. Family history was assessed via a brief interview with the parents of the children and the adult volunteers themselves.

This study was approved by the Royal Children's Hospital Ethics in Human Research Committee, reference number 20,031. Written informed consent was obtained from parents of children and from adult participants themselves.

Blood samples were collected in S-Monovette® tubes (Sarstedt, Australia), containing 1 volume of citrate per 9 volumes of blood. Samples were then centrifuged at 3000 g for 10 min at 10 °C (Megafuge 1.0R, Heraeus), and platelet poor plasma was stored at –80 °C until testing. Informed consent was obtained from the parents of children and from the adult participants themselves. All participants were healthy individuals, without previous thromboembolic events, family history of bleeding or thrombosis, and not subjected to any form of anticoagulant therapy. All laboratory analyses of plasma samples were performed in a blinded fashion.

2.2. Thrombin generation

Thrombin generation (TG) was measured using the Calibrated Automated Thrombography (CAT) as described previously [19–22]. In detail, coagulation was triggered with 1 and 5 pM tissue factor (TF) in the presence of 4 μM phospholipids (60% dioleoyl phosphatidyl choline (PC), 20% dioleoyl phosphatidyl serine (PS) and 20% dioleoyl Phosphatidyl ethanolamine (PE); prepared as described elsewhere [23]. Parameters measured from the TG curve included: lag time, maximal thrombin concentration (peak), time to peak, as well as the area under the curve (endogenous thrombin potential, ETP) and velocity index ($VI = \text{Peak height} / (\text{time to Peak} - \text{lag time})$). The raw data from TG was also used to calculate the overall velocity constant of the total thrombin decay, the thrombin decay constant ($k_{\text{dec,Thrombin}}$). The thrombin decay constant was split mathematically into specific decay by $\alpha 2M$ ($k_{\text{dec},\alpha 2M}$) and by AT ($k_{\text{dec,Serpin}}$), as previously described [24]. The latter includes the inhibitory action of a group of miscellaneous serpins that play a minor role in the inhibition of thrombin during thrombin generation.

2.3. Assessment of fibrin formation in the setting of continuous flow using rheometry

Fibrin clot formation was measured in the presence of laminar flow using rheometer MCR301 (Anton Paar, Oosterhout, the Netherlands). All measurements were performed with a CP40 measuring system (Anton Paar, angle 1°), at the zero gap position suggested by the manufacturer. A quartz glass plate (Anton Paar) was used as sample holder. 220 μl plasma was pre-incubated with 55 μl TF/phospholipids (PL) (yielding the indicated final concentration) for 5 min at 37 °C. The measurement was started upon addition of $CaCl_2$ (final concentration 16.67 mM). All experiments were performed at 37 °C. Resulting viscosity plots are characterized by the parameters base level (viscosity level before coagulation), lag time (time it takes before an increase of the viscosity is observed), plateau level (maximum viscosity level), plateau-base level (increase in viscosity due to the clotting of the sample), time-to-plateau (TTP, time to reach the plateau level), and velocity (slope of the ascending curve between the lag time and the TTP).

2.4. Fibrin clot formation and fixation for SEM

Fibrin clots were the final result of a thin layer thrombin generation method on paper discs, as described previously [25]. The decision to use thin layer thrombin generation versus the standard CAT approach for preparation of fibrin clots was the final reaction volume of 5 μl versus 120 μl , respectively. The volume of 5 μl is easily fixed and results in significantly improved SEM images compared to larger sample volumes. In short, a 96 well microplate was used in which paper discs (Whatman 589/1, Whatman GmbH) were placed at the bottom of the wells. The final reaction mixture included 50% plasma with the following final concentrations: 4 μM phospholipids (60% dioleoyl PC, 20% dioleoyl PS and 20% dioleoyl PE, prepared as described elsewhere [23]), 5×10^{-9} mmol/L tissue factor and 16.7 mmol/L $CaCl_2$; with and without additional 50 ng/ml tPA. The concentration of tPA was guided by previous studies [26,27].

The resulting fibrin clots were prepared using a modification of a previously published method [27]. This protocol was initiated at exactly 10 min post thin layer thrombin generation for all samples across all age groups, standardizing the time from initiation of clotting to fibrin clot preparation, which is especially important for samples prepared in the presence of tPA. In detail, the fibrin clots were fixed with 2.5% glutaraldehyde; washed with PBS and fixed with osmium tetroxide OsO_4 1% in sodium cacodylate 200 mM, pH 7.4. The clots were dehydrated in ethanol and hexamethyldisilazane and were placed on carbon adhesive

tabs. The surface of each clot was coated with gold using the Q150 R coating system (Quorum Technologies, Lewes, East Sussex, UK).

2.5. Scanning electron microscopy

Fibrin clots were studied using Scanning Electron Microscopy (SEM) on a Phenom Pro desktop SEM (Phenom-World, Eindhoven, Netherlands). The age-specific differences in fibrin fibre thickness and pore size were quantified using the Fibermetric software (Phenom-World, Eindhoven, Netherlands). A total of 36 representative images for each sample were measured at magnification 10,000. For each image 500 fibrin fibres were counted with the maximum fibre thickness defined as 1 μm , to exclude the impact of the filter paper fibres on which the clot was located.

2.6. Statistical analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS Version 19.0). Results are expressed as mean and standard deviation. Data were checked for normality. Statistical differences between the age groups were determined using the independent-samples T test or the Mann Whitney U test depending on the distribution of the data. Comparison of results

and correlations were done using Pearson correlation for normally distributed data and Spearman's correlation for non-normally distributed data. Comparisons were made between each of the paediatric groups and the adult group. A p-value of less than 0.05 is considered as significant.

3. Results

3.1. Thrombin generation

Thrombin generation was measured at 1 and 5 pM tissue factor (TF) in plasma samples from 86 infants, children and adults. Samples were tested individually and the results were grouped according to age: 1 month to 1 year ($N = 13$, 10M/3F), median 0.86 years, range 0.63 to 0.99 years; 1–5 years ($N = 20$, 10M/10F), median 1.42 years, range 1.02 to 4.63 years; 6–10 years ($N = 19$, 9M/10F), median 7.53 years, range 5.35 to 9.84; 11–16 years ($N = 16$, 8M/8F), median 10.95, range 10.12 to 15.41 years and adults ($N = 18$, 9M/9F), median 23.30, range 20.47 to 42 years. In children the endogenous thrombin potential (ETP) ($p < 0.0001$), peak thrombin ($p < 0.005$) and velocity index for thrombin generation ($p < 0.05$) were consistently significantly lower compared with adults (Fig. 1A, C and E), at both concentrations of TF. The difference from children to adults in ETP ranged from 27% to 35%, whilst the difference in peak thrombin generated ranged from 28% to

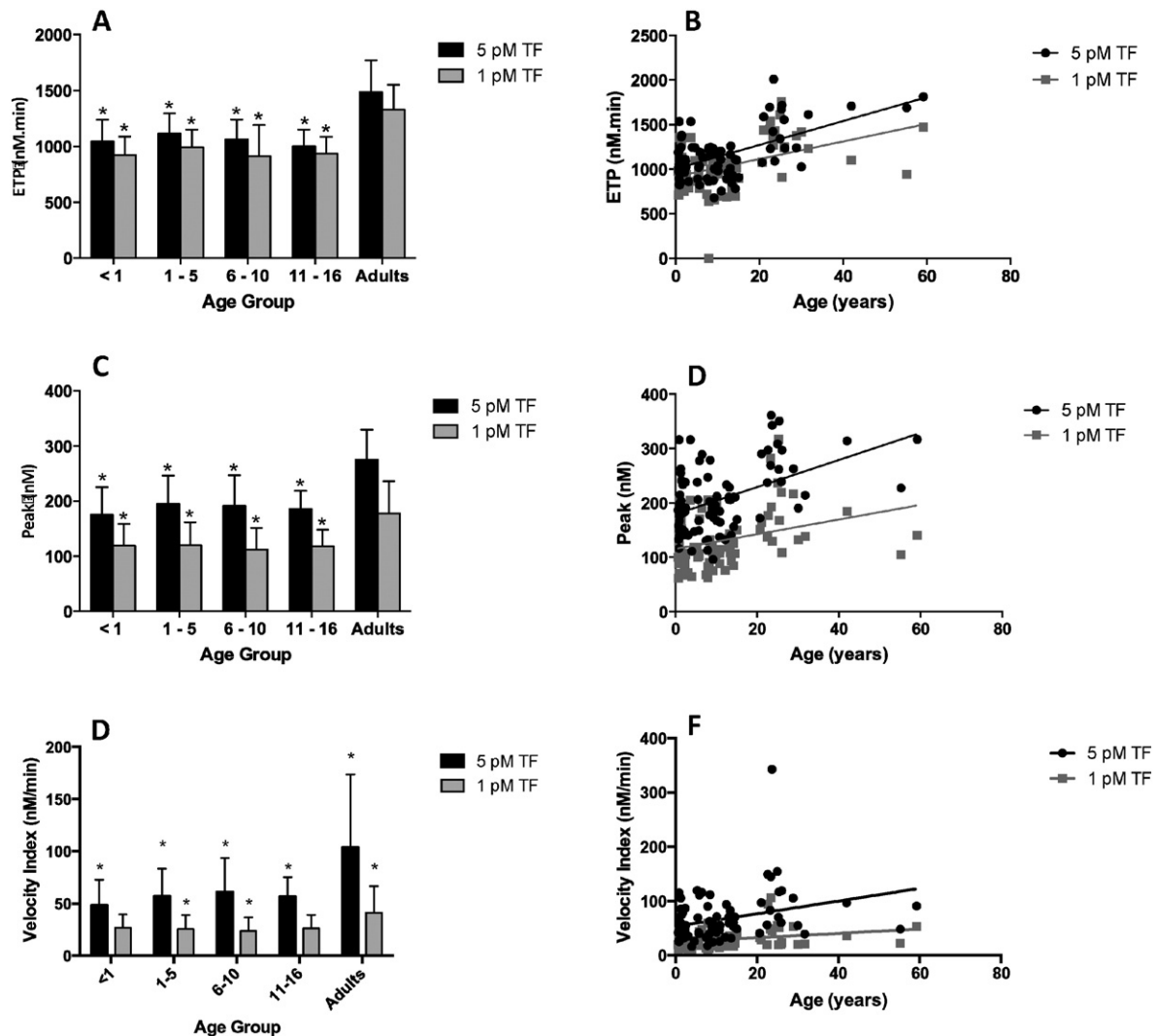


Fig. 1. Age-specific differences in thrombin generation in the presence of 5 pM and 1 pM tissue factor. (A) Endogenous Thrombin Potential (ETP); (B) Correlation of ETP with age ($r = 0.569$ at 1 pM; $r = 0.608$ at 5 pM); (C) Peak thrombin; (D) Correlation of Peak thrombin with age ($r = 0.414$ at 1 pM; $r = 0.503$ at 5 pM); (E) Velocity Index; (F) Correlation of Velocity index with age ($r = 0.272$ at 1 pM; $r = 0.314$ at 5 pM). Results are presented as mean and standard deviation. * $p < 0.05$.

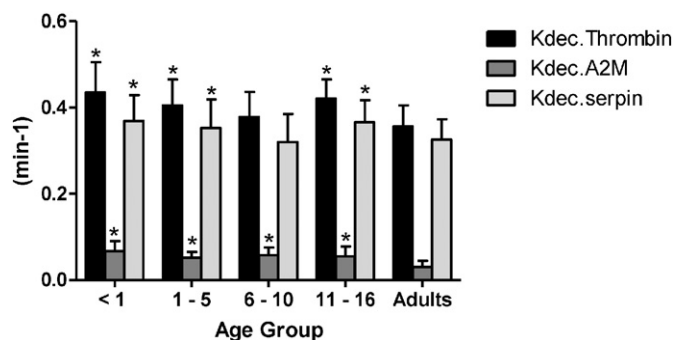


Fig. 2. Age-specific differences in thrombin generation decay constants. Kdec, total decay constant; Kdec.a2M, alpha-2-macroglobulin specific decay constant; Results are presented as mean and standard deviation. * $p < 0.05$, ** $p < 0.005$.

37% respectively. At 1 pM TF there was also a prolongation in lag-time for the 1–5 and 6–10 age groups ($p < 0.05$); whilst at 5 pM TF there was a prolongation in lag time for 1–5 year olds and time to peak for 1 month to 1 year olds, as well as 1–5 year olds compared with adults ($p < 0.05$), (data not shown). In addition, there was a positive correlation of both ETP, peak thrombin and velocity index with age (Fig. 1B, D and F) ($p < 0.05$).

The thrombin generation curve is determined by two main underlying processes prothrombin conversion and thrombin inactivation. The rate of thrombin inactivation was estimated from the thrombin generation curve and it was divided into a α_2 Macroglobulin (α_2 M)-dependent and a serpin-dependent component (Fig. 2; results presented for 5 pM TF). The rate constant of the total thrombin decay was significantly higher in infants and children (<1, 1–5, 11–16 year olds) compared with adults ($p < 0.005$). The α_2 M-specific thrombin decay constant was significantly higher in younger populations, across all age groups ($p < 0.001$) and represented 14% of the total thrombin inhibition, compared with 9% in adults. The serpin-specific thrombin decay constant was higher in infants and children (<1, 1–5, 11–16 year olds) compared with adults ($p < 0.05$).

3.2. Assessment of fibrin formation during continuous physiological flow

We used a plate and cone principle-based rheometer to study age-related differences in fibrin formation (changes in viscosity), whilst applying continuous laminar flow.

To understand the impact of continuous flow, we studied fibrin formation at both venous (300 s^{-1}) and arterial (800 s^{-1}) shear conditions. Taking into account the small sample volume available from the paediatric groups, we prepared plasma pools from the different groups, each consisting of 8 to 10 individual samples. The viscosity level in the adult pooled sample reached at 7 min was set at 100% for both shear rates. We observed that fibrin formation at venous shear rate was decreased in infants and children compared with adults (Fig. 3A, Table 1), whilst this difference was much less pronounced at an arterial shear rate of 800 s^{-1} (Fig. 3B, Table 1).

Accounting for the fact that major differences were observed at the venous as compared with the arterial shear rate, we then studied fibrin formation at physiological venous shear rate (300 s^{-1}) in individual plasma samples. Plasma samples of children aged 1 month to 1 year ($N = 8$), 1–5 years ($N = 8$), 6–10 years ($N = 6$), 11–16 years ($N = 8$) and adults ($N = 8$), were tested in triplicate. We observed a tendency for reduced fibrin formation in all paediatric groups compared with the adult group, and this reached statistical significance for the 1 year olds (Table 1 and Fig. 3C). No difference in fibrin formation was found between the paediatric age groups. The lag time for the fibrin formation was significantly increased, whilst there was a tendency for a reduced velocity index in children compared with adults.

3.3. Nanostructure of fibrin clots

Four randomly selected plasma samples (analysed in triplicate, with three clots from each replicate and hence a total of 36 images per age group) were chosen from each of the five age groups and were used for the SEM study of fibrin clots. We observed significant differences in fibrin clot nanostructure between all paediatric age groups and adults at baseline. Fig. 4 shows representative images for adults and infants respectively at baseline (Fig. 4A and B). Table 2 summarizes the

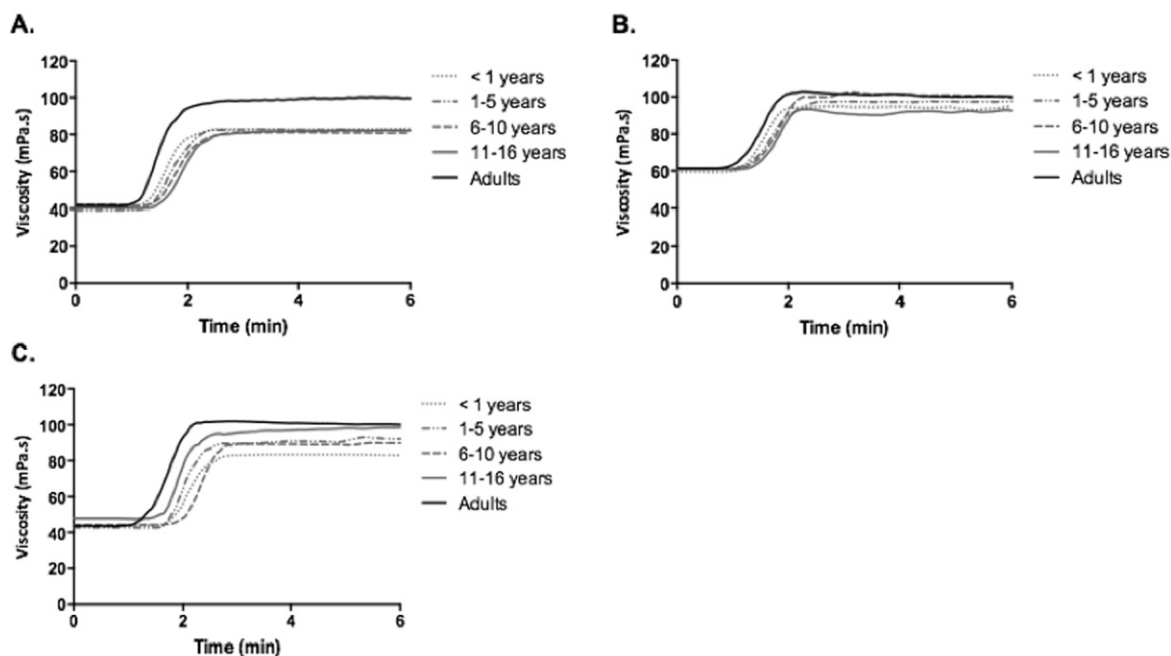


Fig. 3. Rheometry–fibrin formation at 300/s (A) and 800/s (B) for pooled samples and 300/s for individual samples (C). The three panels represent viscosity relative to the adult pool. The viscosity level in the adult pooled sample reached at 7 min for each shear rate was set at 100%. Panel C is a representative of individual results.

quantitative changes in fibrin fibre thickness and pore size. There was no difference in fibrin fibre thickness between infants and children compared with adults at baseline. However, the pore size was significantly larger in paediatric age groups compared with adults (<1 year olds, $p < 0.05$; 1–5 year olds, $p < 0.0001$; 6–10 year olds, $p < 0.005$; 11–16 year olds, $p < 0.0001$). These results demonstrate that the fibrin clot, as the final step in haemostasis, is weaker in infants and children compared to the clot formed in adults.

3.4. Effect of tissue plasminogen activator (tPA) on fibrin clot nanostructure

The presence of tPA did not have an effect on fibrin fibre thickness in infants, children and adults; however, it did result in a significant decrease in the pore size of infants and children (<1 year, $p < 0.05$; 1–5 years, $p < 0.005$; 11–16 years, $p < 0.005$), but not in adults (Fig. 4C and D and Table 2).

4. Discussion

Developmental haemostasis has been studied for many years; however, this is the first study to demonstrate, including under flow conditions, that the end product of coagulation is a different clot structure in infants and children compared with adults. In addition the clot formed in infants and children responds differently to fibrinolysis. These breakthrough findings significantly enhance our understanding of the physiology of thrombosis formation in children, and may have major implications for prophylaxis treatment strategies for this serious condition across all age groups.

In this study we demonstrated that thrombin generation, as one of the main steps towards clot formation is decreased in infants and children (including adolescents) compared with adults. More specifically, it takes longer for thrombin to start to be generated, it is generated at a lower speed of reaction and the maximum amount of thrombin is decreased in younger populations. Our findings are consistent with previous studies of thrombin generation in children, using either similar [5] or the same [8] methodology. The decreased potential to generate thrombin in infants and children with the significant correlation of the speed of conversion of prothrombin to thrombin, the maximum concentration, as well as the overall potential for thrombin generation with age is consistent with the decreased incidence of spontaneous thrombosis in these populations [28]. Importantly, we demonstrate a shift in the balance of thrombin inhibition by $\alpha 2M$ versus Antithrombin, such that the main mechanism of thrombin inhibition in infants and children is $\alpha 2M$ mediated. This confirms our previous suggestion that $\alpha 2M$ is an important inhibitor of thrombin in early life and during childhood [10], as opposed to Antithrombin, which is the main thrombin inhibitor in adults.

This was the first study to investigate the specific mechanism of inhibition of thrombin that takes place during the thrombin generation reaction. The concentrations of fibrinogen to be converted to fibrin and prothrombin to be converted to thrombin are not different between infants and children compared with adults [1]. Hence, the decrease in thrombin generation in these young populations is not dependent on the availability of these two proteins. The fact that all three decay constants, Total Thrombin, $\alpha 2M$ -Thrombin and Serpin (mainly Anti-thrombin)-Thrombin are significantly higher in infants and children compared with adults provides an explanation of the mechanism for decreased thrombin generation in the younger populations. Higher decay constants indicate that infants and children are able to clear thrombin from the pro-coagulant plasma more rapidly compared with adults, a mechanism which may play an important role in the thromboprotective mechanism functioning in the young populations. The extent of the age-specific differences in decay constants, specifically $\alpha 2M$ -Thrombin and Serpin-Thrombin cannot be completely attributed to the 50% increase in concentration of $\alpha 2M$ or the 37% reduction in concentration of Antithrombin in infants and children [1]. In fact, this difference in

Table 1
Fibrin formation in pooled and individual samples under conditions of arterial (800 s^{-1}) and venous (300 s^{-1}) flow. Results are presented as mean and standard deviation.

Age (yrs)	Pooled samples 300 s^{-1} (mimicking venous flow)						Pooled samples 800 s^{-1} (mimicking arterial flow)					
	Base level (mPa·s)	Lag time (min)	Plateau level (mPa·s)	Time to plateau (min)	Plateau-base level (mPa·s)	Velocity index (mPa·s/min)	Base level (mPa·s)	Lag time (min)	Plateau level (mPa·s)	Time to plateau (min)	Plateau-base level (mPa·s)	Velocity index (mPa·s/min)
<1	1.31 ± 0.06	1.14 ± 0.05	2.65 ± 0.02	2.45 ± 0.07	1.34 ± 0.08	1.52 ± 0.26	1.20 ± 0.03	1.07 ± 0.07	1.93 ± 0.07	2.08 ± 0.20	0.73 ± 0.10	1.04 ± 0.10
1–5	1.25 ± 0.01	1.19 ± 0.12	2.60 ± 0.01	2.39 ± 0.12	1.35 ± 0.01	1.60 ± 0.23	1.24 ± 0.02	1.13 ± 0	1.98 ± 0.07	2.28 ± 0.12	0.75 ± 0.07	0.86 ± 0.08
6–10	1.33 ± 0.09	1.31 ± 0.02	2.55 ± 0.10	2.59 ± 0.07	1.22 ± 0.15	1.47 ± 0.15	1.23 ± 0.03	1.21 ± 0.05	2.01 ± 0.08	2.24 ± 0.02	0.77 ± 0.10	0.98 ± 0.06
11–16	1.28 ± 0.02	1.34 ± 0.02	2.52 ± 0.03	2.41 ± 0.03	1.24 ± 0.04	1.62 ± 0.27	1.23 ± 0.01	1.30 ± 0.07	1.89 ± 0.01	2.11 ± 0.19	0.66 ± 0.02	0.96 ± 0.25
Adults	1.32 ± 0.01	0.90 ± 0.04	3.04 ± 0.04	1.99 ± 0.12	1.72 ± 0.03	2.75 ± 0.33	1.24 ± 0.03	0.89 ± 0.05	2.04 ± 0.01	1.86 ± 0.06	0.80 ± 0.03	0.88 ± 0.15
Age (yrs)	Individual 300 s^{-1} (mimicking venous flow)						Individual 800 s^{-1} (mimicking arterial flow)					
	Base level (mPa·s)	Lag time (min)	Plateau level (mPa·s)	Time to plateau (min)	Plateau-base level (mPa·s)	Velocity index (mPa·s/min)	Base level (mPa·s)	Lag time (min)	Plateau level (mPa·s)	Time to plateau (min)	Plateau-base level (mPa·s)	Velocity index (mPa·s/min)
<1	1.25 ± 0.03***	1.38 ± 0.24**	2.41 ± 0.26*	2.59 ± 0.35*	2.41 ± 0.26*	1.52 ± 0.26*	1.16 ± 0.25*	1.07 ± 0.07	1.93 ± 0.07	2.08 ± 0.20	0.73 ± 0.10	1.61 ± 0.37*
1–5	1.31 ± 0.03	1.41 ± 0.24***	2.70 ± 0.36	2.67 ± 0.20**	2.70 ± 0.36	1.60 ± 0.23	1.39 ± 0.34	1.13 ± 0	1.98 ± 0.07	2.28 ± 0.12	0.75 ± 0.07	1.62 ± 0.45*
6–10	1.29 ± 0.04*	1.48 ± 0.21**	2.62 ± 0.15	2.74 ± 0.22*	2.62 ± 0.15	1.47 ± 0.15	1.33 ± 0.12	1.21 ± 0.05	2.01 ± 0.08	2.24 ± 0.02	0.77 ± 0.10	1.71 ± 0.31*
11–16	1.32 ± 0.06	1.40 ± 0.18**	2.65 ± 0.31	2.50 ± 0.28*	2.65 ± 0.31	1.62 ± 0.27	1.33 ± 0.26	1.30 ± 0.07	1.89 ± 0.01	2.11 ± 0.19	0.66 ± 0.02	2.24 ± 1.37
Adults	1.34 ± 0.03	1.00 ± 0.19	2.91 ± 0.34	2.21 ± 0.25	2.91 ± 0.34	2.75 ± 0.33	1.57 ± 0.33	0.89 ± 0.05	2.04 ± 0.01	1.86 ± 0.06	0.80 ± 0.03	2.17 ± 0.76

Note: statistical analysis could not be performed for the results of the pooled samples.

s^{-1} , reciprocal seconds; mPa·s, milli-pascal-second; min, minutes.

* $p < 0.05$ compared with adults.

** $p < 0.005$ compared with adults.

*** $p < 0.001$ compared with adults.

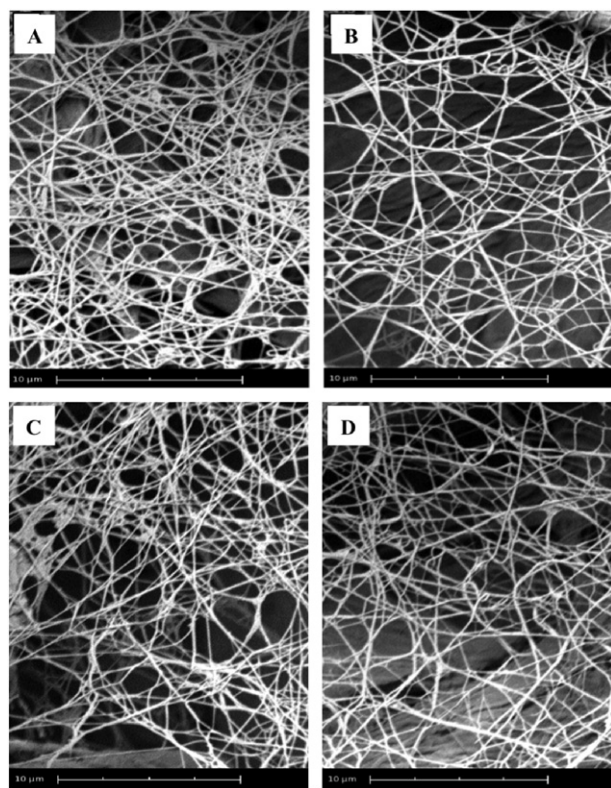


Fig. 4. Representative images of fibrin clots. (A) Adult at baseline; (B) <1 year old at baseline; (C) Adult in the presence of tPA; (D) <1 year old in the presence of tPA.

decay constants that is independent of protein concentration suggests the presence of age-specific structural differences (e.g. post translational modifications) and hence differences in the function of coagulation proteins in children.

The rheometry experiments demonstrate that the extent of age-specific differences in fibrin formation is increased in venous versus arterial flow. Whilst previous studies have applied oscillatory forces and characterized clot formation by the clotting time and viscoelastic properties of the clot, major drawbacks of the oscillation mode (e.g. such as in the ROTEM) compared to rotational mode are the extreme low shear rates reached, which are more comparable to static measurements. Apart from oscillatory forces resulting in nearly static conditions, the rheometer approach allows the application of rotational forces on the clotting sample. In rotational mode the test occurs mostly under laminar flow reproducing that occurring in the human body. By selecting the

rotational mode and applying different shear rates, our approach is closer to physiology compared to conventional coagulation tests performed under static conditions. Under conditions of venous flow fibrin clot formation in the younger populations is retarded compared to adults, and takes place at a slower pace, as demonstrated by the increased lag time and decreased velocity index, respectively. Additionally, the final clot itself is weaker (lower viscosity/lower plateau-base level), as compared with adults. To our knowledge this is the first study investigating fibrin clot formation under flow conditions in healthy infants and children, as well as in adults. Age-specific differences in blood clot formation under flow were consistent with the differences observed in thrombin generation under static conditions. This was specifically true of parameters such as lag time, velocity index, as well as peak thrombin or plateau-base level. Importantly, our observed age-specific differences in fibrin formation under flow are consistent with the decreased incidence of thrombosis in infants and children and at least in part explain the mechanism behind these differences.

The fibrin clot images observed in our study are comparable to the fibrin clot images obtained using similar methods in human plasma [29, 17]. We demonstrate significant differences in fibrin clot nanostructure between infants and children compared with adults, results that reflect differences observed during the rheometry experiments. Fibrin clots in infants and children were characterized by a larger pore size (more permeable), and are therefore likely to be less resistant to fibrinolysis compared with fibrin clots in adults. This is based on the fact that fibrin clots composed of compact (small pores) are more resistant to fibrinolysis [13].

This is the first study to evaluate the effect of tPA on fibrin clots nanostructure in human plasma of healthy individuals. The fact that tPA did not have an effect on the fibrin clots of infants is not surprising considering that the concentration of plasminogen is 39%, tPA is 40% and thrombin activatable fibrinolysis inhibitor (TAFI) function is 60% of the adult value; whilst plasminogen activator inhibitor (PAI) function is 50% higher compared with adults [1]. These findings may validate the current clinical practice (derived empirically) of giving fresh frozen plasma to infants and children prior to thrombolysis to provide adequate substrate for the tPA.

Overall, these findings suggests that the fibrin clots in infants and children are structurally weaker compared with those of adults, and could be one of the potential explanations for the fact that thrombosis is less common in the young populations. In addition, the results are also consistent with the clinical anecdotal evidence that spontaneous resolution of thrombosis in infants and children is more common compared with adults.

Future studies should focus on studying blood clot formation and nanostructure in studies that include larger sample size, as well as in different clinical scenarios but also in whole blood, in order to assess the effect of platelets and other cellular elements.

Table 2

Quantitative changes in fibrin fibre thickness and pore size at baseline and in the presence of tPA.

Age (years)	Baseline				+ tPA			
	Fibrin fibres (nm)	Fibrin fibres (% adult)	Pore size (µM ²)	Pore size (% adult)	Fibrin fibres (nm)	Fibrin fibres (% baseline)	Pore size (µM ²)	Pore size (% baseline)
<1	354 (272–423)	98.33	0.062* (0.039–0.119)	114.81	345 (300–397)	97.46	0.050**** (0.035–0.071)	80.65
1–5	355 (314–408)	98.61	0.068*** (0.042–0.099)	125.93	360 (290–440)	101.41	0.059***** (0.042–0.081)	86.76
6–10	360 (317–420)	100	0.065** (0.038–0.095)	120.37	363 (317–418)	100.83	0.060 (0.041–0.101)	92.31
11–16	360 (321–390)	97.78	0.067*** (0.043–0.141)	124.07	362 (296–399)	102.84	0.056***** (0.033–0.094)	83.58
Adults	360 (321–390)	100	0.054 (0.038–0.066)	100	349 (319–514)	96.94	0.051 (0.034–0.084)	94.44

Results are presented as medians and range.

* $p < 0.05$ compared with adults.

** $p < 0.005$ compared with adults.

*** $p < 0.0001$ compared with adults.

**** $p < 0.05$ compared with baseline.

***** $p < 0.005$ compared with baseline.

Authorship contributions

V.I., R.A.D. and B.d.L. conceived the study; V.K. and C.A. recruited participants; V.I., L.P., H.K., R.K. and S.B. performed laboratory analysis, collected and interpreted data; V.I., L.P., R.K., V.K. and C.A. performed the statistical analysis; V.I. wrote the manuscript; R.A.D., C.H., B.d.L. and P.M. interpreted the data and critically reviewed the manuscript. All authors edited the manuscript for content.

Conflict of interest disclosures

The authors declare no competing financial interests. H.K., L.P., R.K., S.B., C.H. and B.d.L. are employees of the research company Synapse BV, a member of the Diagnostica Stago group. C.H. is consultant to Diagnostica Stago. B.d.L., C.H., L.P. and H.K. are inventors on a European patent application encompassing the rheometry method described in this manuscript.

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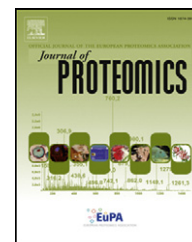
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Differences in the resting platelet proteome and platelet releasate between healthy children and adults



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ABSTRACT

Major age-related diseases such as cardiovascular disease and cancer are the primary causes of morbidity and mortality in Australia and worldwide. In our recent study characterising differences in the plasma proteome between healthy children and adults, a large number of proteins differentially expressed with age were found to be of platelet origin. This study aimed to characterise differences in the resting platelet proteome and the platelet releasate of healthy children compared to healthy adults.

This study represents the setup of a procedure for the proteomic analysis of platelets from children. Differentially expressed platelet proteins were identified using Two-dimensional Differential In-Gel Electrophoresis and mass spectrometry.

Significant differences in the expression of nine proteins (1.1%) in the resting platelet proteome were observed in children compared to adults. Serotransferrin, fibrinogen alpha chain, glyceraldehyde-3 phosphate dehydrogenase, serum albumin, transgelin-2, calponin-2/LIM and SH3 domain protein 1 and human chorionic gonadotropin 2039797 were *up-regulated*, whereas thrombospondin-1 was *down-regulated* in children. Eleven proteins (1.5%) were differentially expressed in the platelet releasate of children compared to adults, where transferrin was also *upregulated* and TSP-1 was *down regulated*. Identified proteins are involved in processes including tissue and organ development, cell proliferation regulation and angiogenesis. Our results provide novel insights into platelet physiology as well as growth, development and ageing in healthy individuals.

Biological significance

The incidence of major diseases such as cardiovascular disease (CVD) and cancer increase with increasing age and are the major causes of morbidity and mortality both in Australia and worldwide. As the aged population continues to increase dramatically, so too will the financial strains associated with the long term care of the elderly population. Compared to adults, children have a significantly lower incidence of major diseases such as thromboembolic disease. This

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suggests that children have a protective mechanism against the development of disease. Therefore, studies focussing on the molecular changes of proteins, the machinery of the cell, between children and adults are the key to determining the underlying mechanisms responsible for the onset of major diseases. A well-defined example of how protein expression can change with age is that of the plasma proteome. Significant differences in the expression of numerous plasma proteins between healthy children and adults have been recently demonstrated. Interestingly, a large number of differentially expressed proteins were found to be of platelet origin. This finding forms the basis for the current study, presenting as strong evidence for the age-specific differences of the platelet proteome.

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1. Introduction

The incidence of major diseases such as cardiovascular disease (CVD) and cancer increase with increasing age and are the major causes of morbidity and mortality both in Australia and worldwide [1]. As the aged population continues to increase dramatically, so too will the financial strains associated with the long term care of the elderly population. Compared to adults, children have a significantly lower incidence of major diseases such as thromboembolic disease [2]. This suggests that children have a protective mechanism against the development of disease. Therefore, studies focussing on the molecular changes of proteins, the machinery of the cell, between children and adults are the key to determining the underlying mechanisms responsible for the onset of major diseases. A well-defined example of how protein expression can change with age is that of the plasma proteome. Recently, Ignjatovic et al. [3] demonstrated significant differences in the expression of numerous plasma proteins between healthy children and adults. Interestingly, a large number of differentially expressed proteins were found to be of platelet origin. This finding forms the basis for the current study, presenting as strong evidence for the age-specific differences of the platelet proteome.

The platelet is an anucleate cell fragment and an essential blood constituent [4]. In addition to many pre-formed proteins associated with alpha, dense and T granules, the platelet also inherits template messenger RNAs, ribosomes and translational machinery from parent megakaryocytes, giving it the ability to autonomously and continuously synthesise proteins [5]. Platelets also acquire proteins (e.g. coagulation factors such as Factor V) from the plasma environment [6]. Platelets play a major role in haemostasis where they are involved in the formation of vascular plugs and in wound repair [7]. Platelets also play a role in biological processes such as inflammation, immunity and angiogenesis, and contribute to disease pathologies including arterial thrombosis and haemorrhage [8].

Platelets have a complex cytoskeletal and canalicular network which allows them to quickly release granular proteins, up-regulate specific membrane receptors and interact with other cell types when activated during circulation. The process of secretion, termed as 'exocytosis', is an essential part of the platelet activation event. The platelet releasate, i.e. proteins released by platelets, are delivered to the specific milieu of the vascular injury site. Platelet releasate proteins can be isolated from the supernatant of the platelet suspension after stimulation by the specific agonist [9–11].

The platelet proteome is extremely complex and dynamic. In the past decade, studies of the platelet proteome have focussed on mapping the entire platelet proteome [12], characterising the platelet proteome in the healthy elderly [13,14], as well as in different disease states such as smoking [15], coronary atherosclerosis [16] and myocardial infarction [17]. Despite impressive advances in platelet research, the majority of such studies have involved adult patients, with no investigation of the resting platelet proteome and platelet releasate in the paediatric population.

We have recently demonstrated that the platelets of children have a different phenotype [18] and respond differently to activation [19] compared to the platelets from adults. Despite these recent as well as previous publications, there are still many gaps in our knowledge regarding developmental changes of the platelet proteome, the physiological and pathophysiological impact of such changes and their influence on disease progression.

The present study represents the first step to narrowing the knowledge gap and improving prevention and treatment for diseases of ageing. We hypothesised that there are major differences in the platelet proteome and platelet releasate of healthy children compared to adults. We tested this hypothesis by characterising the age specific differences of the resting platelet proteome, as well as the platelet releasate in healthy children compared to healthy adults.

2. Material and methods

2.1. Sample information

Blood samples were obtained from healthy children and adults using established protocols [3,18,20]. Specifically, written informed consent was obtained and the study was approved by the Royal Children's Hospital Ethics in Human Research Committee (#20031E). Written consent was provided by a parent or guardian for paediatric samples, and by participants themselves for adult samples. Blood samples were obtained from healthy children about to undergo minor elective surgery (non-haematological related illness such as tonsillectomy, circumcision) at the Royal Children's Hospital. The children were otherwise healthy, with no family history of bleeding disorders or thromboembolic events and were not receiving any anti-platelet medication or anticoagulant therapy at least within 10 days prior to sample collection.

Adult samples were obtained from healthy volunteers who did not have any family history of bleeding and thromboembolic disorders, and who were not receiving any anti-platelet medication or anticoagulant therapy at least within 10 days prior to sample collection. Blood (~5 mL) was drawn from all participants by research assistants from the Department of Haematology via a venipuncture using a 23-gauge needle. Blood samples were collected in S-Monovette® tubes (Sarstedt, Australia), containing 1 volume of citrate per 9 volumes of blood.

2.2. Isolation of the resting platelet proteome

Immediately following blood collection, platelets were separated from plasma using centrifugation (Biofuge Fresco, Heraeus, Australia) and the platelet proteome was isolated according to the protocol established by Garcia [21].

2.3. Isolation of the platelet releasate

Platelet releasate was obtained following activation of the platelets with thrombin receptor activating peptide-6 (TRAP-6) with an adaptation of the protocol by Garcia [21] and Coppinger et al. [9]. Washed platelets were resuspended in HTS buffer (pH 7.4, supplemented with 1.8 mM CaCl_2 freshly added) to obtain platelet counts between $200\text{--}300 \times 10^6/\text{L}$. Suspensions were divided into two tubes. TRAP was added to a final concentration of 5 M to one tube to activate platelets and release its granular contents. Equal volume of HTS buffer instead of TRAP was added to the other, control tube. Prostaglandin E1 (PGE1) was added to both tubes and platelet suspensions were centrifuged at low speed 2000 rpm (Biofuge Fresco, Heraeus, Australia) at 4 °C for 10 min. The supernatant was centrifuged again at high speed 13,000 rpm at 4 °C. It should be noted that in our protocol we did not remove microparticles from the TRAP-induced platelet releasate by ultracentrifugation and therefore some proteins identified in the platelet releasate would originate from microparticles. Our decision is based on the fact that similarly to proteins released from platelets, microparticles are physiologically active in mediating platelet functions. 2 μM or 1 μL of phosphatase inhibitor and phenylmethylsulfonylfluoride (PMSF) was added to both tubes. The releasate protein was concentrated using Amicon Ultra 3 kDa filter (Millipore, Ireland) following procedures as described by the manufacturer. The concentrated releasate protein was stored in aliquots at -80°C .

2.4. Two-dimensional-Differential In-Gel Electrophoresis (2D-DIGE)

The following 2D-DIGE protocol was performed for both the resting platelet proteome and platelet releasate samples. The internal standard consisted of an equal amount of each one of the platelet samples (22 resting platelet proteome or 16 platelet releasate samples), was labelled with cyanine 2 (Cy2) dye (GE Healthcare Life Sciences, Australia) and run on each gel to control for gel-to-gel variation. Each sample (20 μg and 13 μg of protein for the resting platelet proteome and releasate samples respectively) was randomly labelled with either a Cy3 or Cy5 dye and randomized to a gel. Two samples were run on each gel.

The Cy2, Cy3 and Cy5 samples (a total of 60 μg of platelet sample/480 pmol and 39 mg of releasate sample/312 pmol of Cy dye for the resting and releasate samples respectively) for each gel were pooled and loaded onto the 1st Dimension Immobilized pH Gradient (IPG) Strip. The 7 cm, pH 4–7 Immobiline Drystrips (GE Healthcare Life Sciences, Australia) were rehydrated overnight (≥ 16 h) with sample rehydration buffer (urea, 2% CHAPS, 0.5% 4–7 IPG buffer/ampholytes and 0.002% bromophenol blue) to a final volume of 155 μL . Isoelectric focusing was carried out using the Ettan IPGphor3 Isoelectric focusing system (GE Healthcare Life Sciences, Australia) until 5000 V hr was reached.

The IPG Strips were conditioned for 15 min in equilibration buffer containing NuPAGE® LDS sample buffer (1 M Tris, urea, glycerol, 1% bromophenol blue, 10% SDS) and dithiothreitol (DTT). The strips were then alkylated for 15 min in equilibration buffer containing Iodoacetamide (IAA) instead of the DTT and loaded onto 4–12% IPG Novex Bis-Tris gels (Invitrogen, USA). Second dimension was run using the XCell SureLock® Mini-Cell Electrophoresis System (Life Technologies, China). All gels were run at 200 V constant electromagnetic force (EMF) for approximately 1 h in $\times 1$ MOPS SDS Running Buffer ($\times 20$ NuPage MOPS SDS Running Buffer (Life Technologies, USA) and Milli-Q H_2O).

2.5. Gel imaging

The gels were scanned three times using a Typhoon Trio Variable mode imager (GE Healthcare Life Sciences, Australia), with a resolution of 100 μm and PMT of 500 V. Cy2, Cy3 and Cy5 images were scanned using a 488, 532 and 633 nm laser respectively.

2.6. Image analysis

Image analysis was performed using the DeCyder version 7.0 software following the manufacturer's instructions (GE Healthcare Life Sciences, Australia). The Differential In-Gel Analysis (DIA) module was used to detect and quantify protein spots. The Biological Variation Analysis (BVA) module was used to match each gel relative to an internal standard (Cy2 labelled) and to statistically analyse differences in normalised spot abundance between gels of the two age groups. The filtering parameters were set to determine spots of interest that: had a $p\text{-value} \leq 0.05$ for the t-test (unpaired Student's t-test) employed to test the variation between the two age groups, a >1.5 fold change in abundance between the groups and were present in $\geq 73\%$ of the spot maps.

2.7. Spot excision and identification

Proteins of interest were excised manually from the gel using a pipette tip. Gel pieces were destained, dehydrated and proteins digested using an in-gel protocol established by Shevchenko et al. [22].

2.8. Mass spectrometry

Peptides liberated from the gel plugs following in-gel trypsin digestion were analysed using the Orbitrap hybrid tandem mass

spectrometer (Thermo Fisher Scientific). LC-MS/MS spectra were searched against the UniProt protein database and the NCBI nr Homo sapiens database for possible protein identifications. Using peptide mass fingerprinting and the deduced de novo amino acid sequence, protein hits were returned and identified by MASCOT (v2.4, Matrix Science, U.K.). Only protein identities for samples with significant molecular weight search scores ($p < 0.05$) were reported. The identities of proteins of interest were verified through comparison to platelet proteome reference maps available online on Swiss-2D PAGE (<http://world-2dpage.expasy.org/swiss-2dpage/>).

2.9. Extended Data Analysis (EDA)

The EDA component of the DeCyder software (GE Healthcare Life Sciences, Australia) was used for more specific analysis of the expression patterns of the platelet proteome proteins between the two age groups. The principal component analysis (PCA) and hierarchical clustering were used to group protein expression changes of differentially expressed proteins between the two age groups.

2.10. Enzyme Linked Immunosorbent Assay (ELISA) validation

Human transferrin present in the resting platelet proteome samples was quantified using a double-antibody sandwich assay kit (Genway Biotech Inc.TM, California). For the quantification of transferrin, 40 μ g of each platelet proteome sample was diluted 1:25 and 1:100 with sample diluent. Platelet proteome samples and protein standards were added in duplicate to pre designated wells on the 96-well microtiter plate (100 μ L/well) and incubated at room temperature for 30 min. All solutions were aspirated, washed repeatedly and treated with both streptavidin HRP conjugate solution and 3,3',5,5'-tetramethylbenzidine (TMB). The reaction was stopped using sulphuric acid and the absorbance was read by a microplate reader at 450 nm.

3. Results

The resting platelet proteome was isolated from blood samples of 11 children (mean age: 4.32 years $\pm$ 1.60) and 11 adults (mean age: 35.14 years $\pm$ 9.55). The platelet releasate was isolated from 8 children (median age: 5.2 years) and 8 adults (median age: 34.4 years). Age was the sole factor differing between the two populations, ensuring that any differences in protein expression were due to inherent biological variation.

3.1. Resting platelet proteome

3.1.1. Protein spot maps and protein identities

Representative 2D-DIGE Images for an adult and paediatric sample are shown in Fig. 1A and B respectively. Some of the obvious differences between the two samples are highlighted in both figures (circles), while the overlay of the two images in Fig. 1C also highlights the differences in the platelet proteome between the two groups. Yellow spots highlight areas of identical

expression between children and adult platelet proteome samples. Proteins differentially expressed between the two samples are represented by either red or green spots.

An average of 837 $\pm$ 54 protein spots for the resting platelet proteome samples were detected from the eleven analytical gels. Nine protein spots (1.1%), were found to be differentially expressed between children and adults. Of these nine protein spots, eight proteins were identified (Table 1A).

The majority of proteins were up-regulated in the platelet proteome of children compared to adults. Thrombospondin-1 (TSP-1) was the only protein that was down-regulated in the platelet proteome of children compared to adults.

3.1.2. ELISA validation

There was a tendency towards higher concentrations of transferrin in the resting platelet proteome of paediatric samples (64.09 $\pm$ 60.28 ng transferrin/ μ g platelet protein) compared to adults (29.65 $\pm$ 8.32 ng transferrin/ μ g platelet protein), p -value = 0.24.

3.1.3. Location for proteins of interest

The locations of differentially expressed proteins in the resting platelet proteome are shown in Fig. 2. The majority of the identified proteins found to be differentially expressed with age were up-regulated in the platelet proteome of children compared to adults, with TSP-1 being the only down-regulated protein.

3.1.4. Proteins with common expression patterns in the resting platelet proteome

The hierarchical clustering of the identified proteins of the resting platelet proteome is represented in Fig. 3. In children (red spots), differentially expressed proteins were mostly increased as emphasised by the red bands whereas in adults (blue spots), differentially expressed proteins were mostly decreased as highlighted by the green bands. A clear separation between the two age populations is evident where the top dendrogram branches into main two groups in Fig. 3. The majority of samples from children are localised to the group on the left and the majority of adult samples are localised to the group on the right.

The PCA, used to reduce the complexity of the proteomic data, examines the global trends of expression in differentially expressed proteins in the resting platelet proteome from children and adults (Fig. 4). Fig. 4 shows that samples were grouped based on the variance of their protein expression, highlighting a distinct separation between the two age groups. The majority of the variance (60.3%) in protein expression between children and adults was captured by the first principal component, with an additional 12.1% of the variance by the second component.

3.1.5. Gene ontology

The gene ontology of platelet proteins that are differentially expressed in the resting platelet proteome of children compared to adults are presented in Fig. 5, highlighting the molecular functions (Fig. 5A) and biological processes (Fig. 5B) of these proteins.

Fig. 5A highlights the molecular functions of proteins that are differentially expressed in children compared to adults. The majority of these proteins (82%) play a role in binding

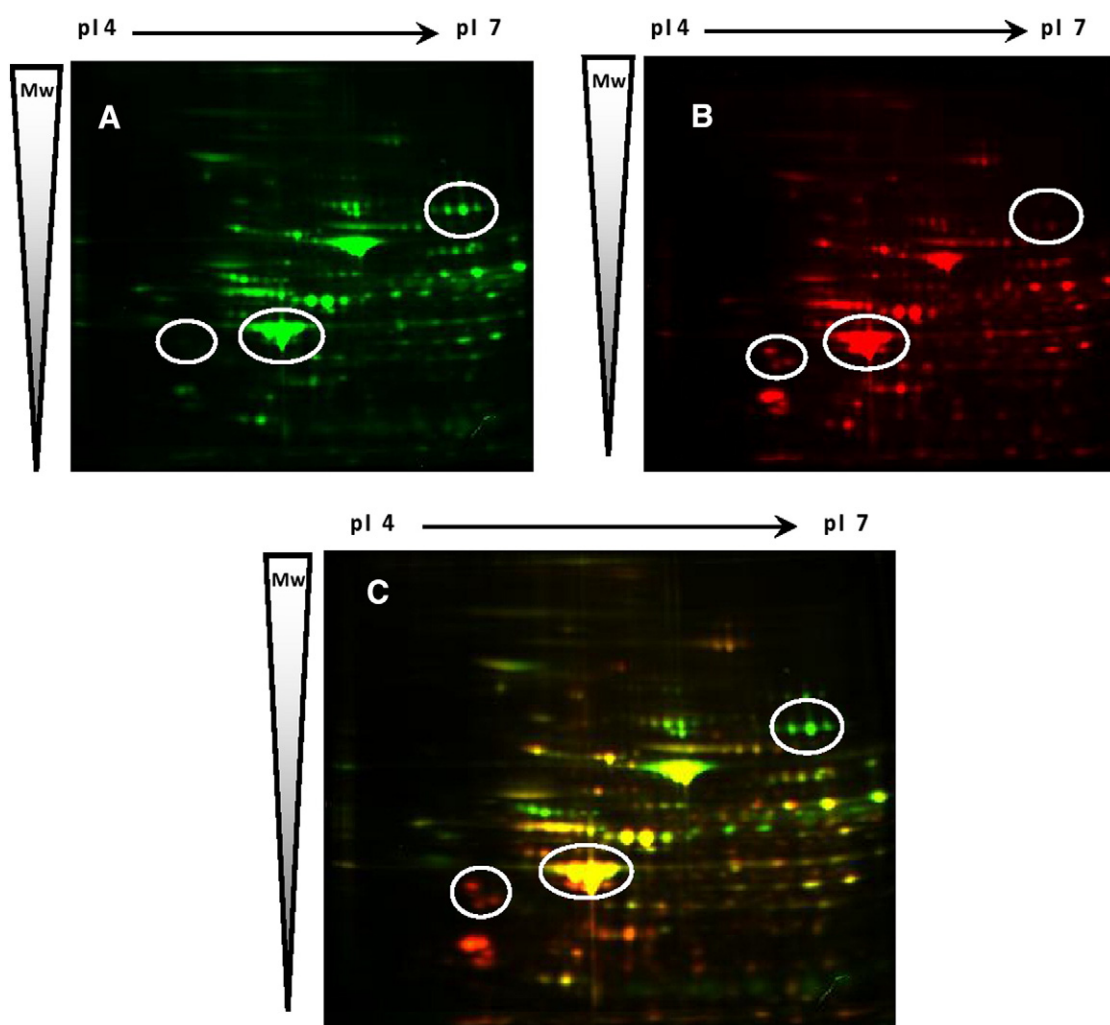


Fig. 1 – Representative 2D-DIGE gel of human platelet proteins in the resting platelet proteome. A total of 60 μg of platelet protein was run. (A) Cy3 labelled adult sample (20 μg labelled platelet protein). (B) Cy5 labelled paediatric sample (20 μg labelled platelet protein). (C) Superimposition of (A) and (B). Yellow spots highlight areas of identical expression between children and adult platelet proteome samples. Proteins differentially expressed between the two samples are represented by either red or green spots (highlighted by white circles).

including that of heparin, actin, DNA, microtubules, toxic substances, drugs and proteoglycans. The remaining proteins (18%) are involved in antioxidant activity among other molecular functions.

Some of the biological processes associated with differences in platelet protein expression with age include; regulatory processes (46%) such as that of cell proliferation and angiogenesis; responses (21.8%) to hypoxia and drugs; developmental processes (3.5%) including muscle and organ development; platelet function (5.2%); and also immunity (2.6%) (Fig. 5B).

Furthermore, differentially expressed proteins in the resting platelet proteome were found to originate from four different areas. The majority (46.5%) of the proteins were found to originate from intracellular regions including the mitochondrion, the secretory granule lumen, cytosol and the perinuclear region of the cytoplasm. The proteins were also found to originate from membrane (20.9%) and endosomal

origins (20.9%), as well as from extracellular regions (4.7%) such as the extracellular space.

3.2. Platelet releasate

3.2.1. Protein spot maps and protein identities

The average number of protein spots detected in the platelet releasate samples was 718 ± 144 from the eight analytical gels. Eleven of the proteins (1.5%) were differentially expressed in paediatric compared to adult samples (Table 1B). A representative image of platelet releasate from children and adults, as well as the overlay is shown in the Supplementary figure.

Proteins identified to have higher expression in the platelet releasate in children are Plasminogen Activator Inhibitor-1 (PAI-1), transferrin, beta actin, tropomyosin 3 and tropomyosin 4. TSP-1, fibrinogen gamma chain, gelsolin, factor XIII peptide A

Table 1A – List of differentially expressed proteins in the resting platelet proteome identified from the 2D-DIGE profiling of human platelets children compared to adults.

Protein	Master #	Protein ID ^a	Protein AC ^b	t-Test ^c	Ave ratio ^d	Protein score ^e
1	115	Thrombospondin-1	P07996	0.012	–1.51	166
2	258	Serotransferrin	P02787	0.037	1.78	205
3	361	Fibrinogen alpha chain	P02671	0.028	1.57	129
4	724	Glyceraldehyde-3-phosphate dehydrogenase	P04406	0.013	1.68	150
5	415	Serum albumin	P02768	0.014	1.99	2543
	392	Serum albumin	P02768	0.0062	2.90	996
6	765	Transgelin-2	P37802	0.050	2.64	364
7	715	Calponin-2	Q99439	0.030	1.69	249
		LIM and SH3 domain protein 1	Q14847	0.030	1.69	176
8	792	Human chorionic gonadotropin HCG2039797 (possible J 56 gene segment)	A0N4V7	0.019	1.68	45

AC — accession; Ave ratio — average ratio.

^a Protein identities confirmed by the OrbiTrap.

^b UniProt database was searched.

^c Unpaired t-test calculated using DeCyder v7.0 (GE Healthcare Life Sciences).

^d Average volume ratios children/adults of protein expression. Positive values represent an up-regulation of that protein in children compared to adults and a negative value signifies a down-regulation.

^e The protein score shows the combined scores of all observed mass spectra that can be matched to amino acid sequences within a specific protein; a more confident match is represented by a higher score.

and B, and osteonectin were found to have lower expression in children compared to adults.

4. Discussion

This is the first study of the resting platelet proteome and platelet releasate in children. The results of this study provide important insights into normal growth and development, showing significant differences in the expression of proteins in both the resting platelet proteome and platelet releasate of healthy children compared to healthy adults. In addition, this study represents the setup of a procedure for the proteomic analysis of platelets from children.

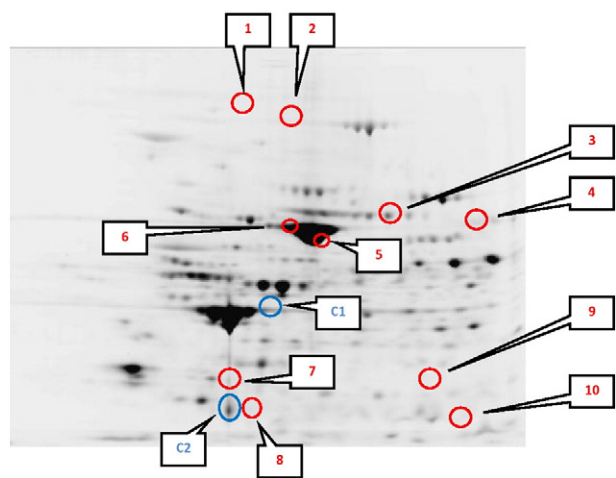


Fig. 2 – Representative Cy2 spot map image (20 mg of labelled platelet protein) showing the locations of the proteins of interest of the resting platelet proteome highlighted in red and the control proteins represented in blue.

The present study utilised sample numbers based on and consistent with previous studies investigating the platelet proteome [14–16,23]. The carefully controlled selection of subjects in this study, as well as standardized blood collection, processing and storage protocol across the two age groups minimized the variation to age and not other external factors.

For the first time, small format gels were utilised as opposed to larger format gels in order to accommodate for the smaller volume of blood that can be drawn from children and therefore the smaller amounts of protein available for analysis. Both, the platelet proteome and releasate were successfully resolved using the small format gels, showing a vast array of platelet protein spots. This approach resulted in comparable resolution of both proteomes to previous studies using larger format gels [13,14].

Platelet proteins that were differentially expressed with age play a role in processes such as binding of molecules and proteins such as heparin, actin, DNA, microtubules, toxic substances, drugs and proteoglycans. Protein binding is essential in facilitating numerous biological processes and hence changes in protein binding alter disease susceptibility and progression. Understanding the roles that differentially expressed binding proteins play will aid in the understanding of the normal process of growth and development and will provide further insight into determining the onset and progression of disease. Furthermore, these proteins are also known to be involved in a number of biological processes primarily involving the regulation of cell proliferation, angiogenesis and gene expression; tissue and organ development; and cellular responses to stimuli such as drugs, nutrients and hypoxia. Considering that the majority of proteins were up-regulated in the platelet proteome of children compared to adults, these proteins most likely play an essential role in promoting the ongoing process of normal growth and development. This is supported by the fact that the paediatric subjects utilised in this study are representative of the early childhood period, a critical stage in human growth and development.

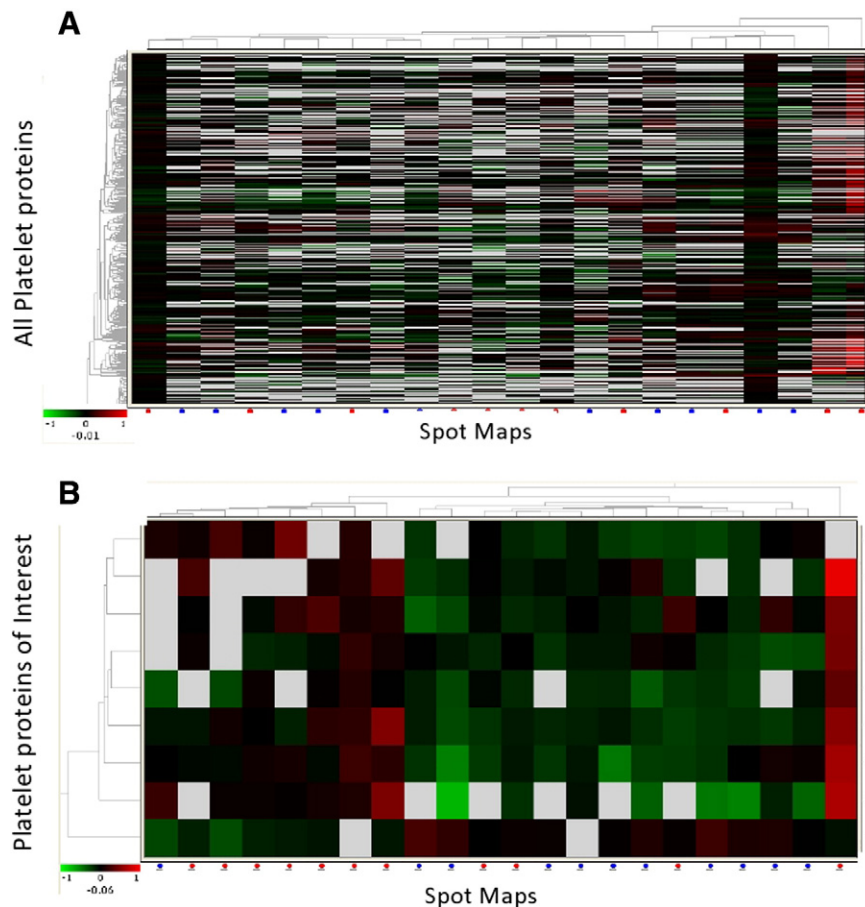


Fig. 3 – Hierarchical clustering of the resting platelet proteome. (A) All proteins; (B) proteins that are differentially expressed in children compared to adults. Each differentially expressed protein is represented by a single horizontal row. Hierarchical clustering of individual samples is displayed on top whereas individual protein clustering is shown on the left. Relative expression values are represented as an expression matrix (heat map) through use of a relative scale (–1 green to +2 red) with green bars signifying a decrease in protein expression and red bars an increase in protein expression.

Throughout childhood, the physical growth and maturation of organ systems (e.g. the brain) takes place as well as the extensive development of the immune system. The function and increased expression of these proteins could hence be crucial in enhancing cell signalling, stimulation as well as proliferation particularly in early life. Furthermore, the specific platelet protein expression in children could play an important role in securing protection against the development of diseases, whereby children have a much lower incidence of major disease (e.g. thromboembolic disease) compared to adults [2].

Two proteins differentially expressed in both the resting platelet proteome and platelet releasate; TSP-1 and serotransferrin will be discussed below (Table 2). TSP-1 is a major alpha-granule protein of human platelets, which plays a crucial role in promoting platelet aggregation. It is synthesised by the parent megakaryocyte through mRNA [24].

As established by previous studies, platelet aggregation is known to contribute to CVD [25], atherosclerosis [26] and thromboembolic disease [27]. TSP-1 promotes platelet aggregation by inhibiting the effect of nitric oxide of which prevents platelet aggregation. In this regard, differences in TSP-1 may contribute to known differences in platelet phenotype and

activation in children compared to adults. In a recent study of a shear gradient-dependent thrombus formation, TSP-1 plays a role in the propagation of thrombus growth in the arterial vasculature, hence promoting obstructive and pathogenic thrombosis events [28,29]. In children, TSP-1 is found significantly down regulated and this concurs with the clinical observation that children are less prone to spontaneous thrombosis. Therefore, decreased levels of TSP-1 in children may contribute to the state of thromboprotection observed in the younger population, whereby the incidence of thrombotic and haemorrhagic events in children is much lower than that of the adults. TSP-1 is also known to play an important role in cancer aetiology and progression in adults, being implicated in tumour cell adhesion, migration, invasion and angiogenesis [30]. Considering that age is a significant risk factor associated with cancer aetiology, decreased TSP-1 expression in childhood may contribute to the decreased incidence of particular cancers in children. Previous research has established that the inhibition of TSP-1 abolishes colon cancer invasion, highlighting the integral role TSP-1 in promoting the progression of colonic cancer [30].

There was a trend for increased expression of serotransferrin in the resting platelet proteome and platelet releasate of children

Spot Maps (Score Plot)

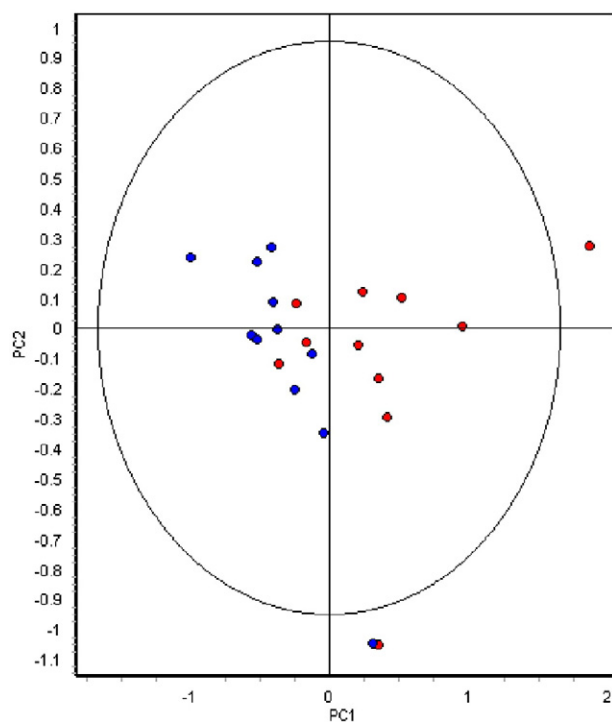


Fig. 4 – Principal component analysis of differentially expressed proteins within the resting platelet proteome. PC1 — principal component 1; PC2 — principal component 2; (A) scatter plot of spot maps of differentially expressed proteins within the resting platelet proteome from children (red) and adults (blue).

compared to adults, as confirmed the ELISA validation. The fact that the p-value was higher than 0.05 is most likely due to the inter-individual variability observed in the paediatric population. Platelet activation is known to promote the progression of acute coronary syndromes (ACS) and subsequent thrombus formation in adults [31]. Serotransferrin suppresses platelet activation through its antioxidant properties. Therefore, increased expression levels of serotransferrin in children may contribute to differences in platelet function and phenotype, as well as conferring protection from the development and progression of thromboembolic disease. In a study conducted by Lopez-Farre et al. [31], serotransferrin was reduced in the platelets of ACS patients in comparison to patients with stable coronary ischemic disease. This finding suggests that during the acute phase of a coronary ischemic event, decreased expression of antioxidant proteins such as serotransferrin facilitates platelet activation. Oxidative stress is known to play an active role in promoting atherosclerosis [32] and the development of CVD [33]. As an antioxidant, serotransferrin is protective against platelet-induced thrombotic events. Furthermore, decreased serotransferrin levels have been linked to the development of age-related disease such as Alzheimer's disease of which is also promoted by oxidative stress [34]. The decreased expression of serotransferrin could potentially act as a biomarker for the susceptibility to disease such as Alzheimer's disease and thromboembolic disease. Future research could focus on the development of pharmacological therapies targeting differences in the expression of platelet serotransferrin. This may be a viable strategy to target diseases induced by platelet activation and oxidative stress such as atherosclerosis and other CVD.

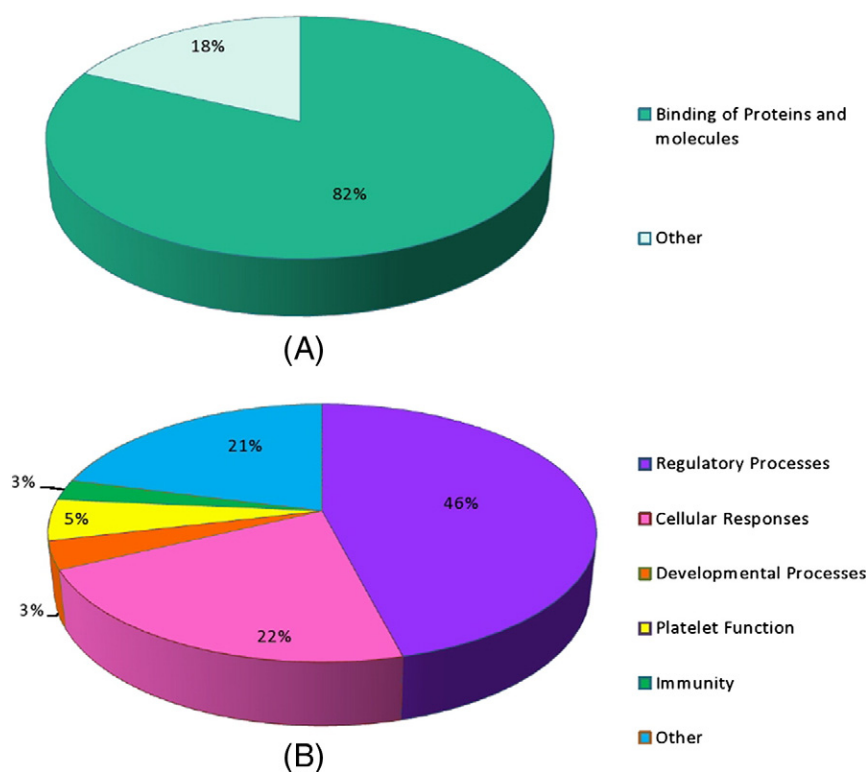


Fig. 5 – (A) Molecular functions for identified platelet proteins in the resting platelet proteome that are differentially expressed with age; (B) biological processes associated with identified platelet proteins in the resting platelet proteome that are differentially expressed with age.

Table 1B – List of differentially expressed platelet releasate proteins.

Master # ^a	UniProt name ^b	Description ^c	Fold change ^d	P-value ^e
206	TSP1_HUMAN	Thrombospondin1 (TSP-1)	–1.73	0.041
206	GELS_HUMAN	Gelsolin	–1.73	0.041
267	F13A_HUMAN	Coagulation factor XIII A chain	–2.17	0.015
267	F13B_HUMAN	Coagulation factor XIII B chain	–2.17	0.015
260	GELS_HUMAN	Gelsolin	–3.56	<0.01
245	B4E1B2_HUMAN	Serotransferrin	+2.01	0.015
271	B4E1B2_HUMAN	Serotransferrin	+14.12	<0.01
480	FIBG_HUMAN	Fibrinogen gamma chain	–4.07	<0.01
484	FIBG_HUMAN	Fibrinogen gamma chain	+2.51	0.048
542	ACTB_HUMAN	Beta-actin	+2.71	0.031
597	PAI1_HUMAN	Plasminogen activator inhibitor 1	+1.85	0.047
661	B2RDE1_HUMAN	Tropomyosin 3	+2.00	0.042
661	TPM4_HUMAN	Tropomyosin alpha-4 chain	+2.00	0.042
759	SPRC_HUMAN	Osteonectin	–1.82	<0.01

List of differentially expressed platelet releasate proteins.

^a Master spot numbers are reference numbers assigned by DeCyder software, corresponding to proteins that showed differential expression.

^b UniProt name is the assigned name from Universal Protein Resource database.

^c Protein identification was determined by mascot search based on a minimum of two significant and bold peptides with sequence score above its identity threshold.

^d Fold change is expressed as log ratio of protein abundance (adults/children). A positive value means that protein expression is up-regulated in children.

^e P-value indicates the Student's test and a difference observed between two groups; p-value < 0.05 means statistically significance.

5. Study limitations/challenges

The maximum blood volume able to be collected from paediatric patients was 5 mL. This significantly limited the quantity of the total platelet protein available for the downstream proteomic analysis. Typically at least 25 mL of adult blood is obtained for similar platelet releasate experimental approaches. For this reason, small format gels were utilised, as opposed to large format gels. Although the use of small format gels partly circumvented the requirement of greater protein quantities, this approach limited the ability to separate proteins with two-dimensional gel electrophoresis (2-DE). The use of larger gels may have allowed for greater horizontal and vertical separation of the platelet proteins, enabling more effective protein separation and identification of additional differentially expressed proteins. While this study has identified

differences in the expression of platelet proteins with age, the gap in knowledge surrounding the specific age of onset of these changes remains; and can only be filled with longitudinal studies. Inclusion of additional age ranges such as infants; post-pubertal children and adolescents may provide further insight into the specific age of onset of changes in platelet protein expression.

6. Conclusion

The age-specific changes in the platelet proteome and platelet releasate established in this study provide vital insights into normal growth and development, creating a platform for comparisons with disease states affecting platelets in both children and adults. The proteins differentially expressed

Table 2 – The functions of TSP-1 and serotransferrin and their association with different disease settings, their own expression levels and their clinical significance in children.

Protein	Role	Disease setting	Expression levels (previous studies in adults)	Outcome/clinical significance in children
TSP-1	- Platelet aggregation - Tumour cell adhesion, migration, invasion and angiogenesis	- Cardiovascular Disease (CVD), atherosclerosis, thromboembolic events - Cancer	↑ ↑	↓ TSP-1 levels may contribute to thromboprotection in children ↓ TSP-1 levels may contribute to the decreased incidence of particular cancers in children
Serotransferrin	- Suppresses platelet activation - Antioxidative effects	- Acute coronary syndromes (ACS), thrombus formation - Alzheimer's disease	↓ ↓	↑ Serotransferrin levels may contribute to thromboprotection in children ↑ Serotransferrin levels is protective against Alzheimer's disease

with age in this study may contribute to known differences in platelet phenotype and activation and thromboprotection in children. These proteins should be further investigated across a wider age spectrum to include age groups such as infants and adolescents, as well as older adults. This could reveal more about the involvement of the proteins of interest identified in this study in the aetiology of disease, their ability to act as biomarkers for early disease detection and their potential implication as therapeutic targets for the treatment of disease.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2015.04.003>.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

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Cardiopulmonary bypass changes the plasma proteome in children undergoing tetralogy of Fallot repair

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Abstract

Introduction: Cardiopulmonary bypass (CPB) can be associated with deleterious clinical effects. However, the impact of CPB on inflammatory, immunological and other homeostatic pathways remains poorly understood. We investigated the impact of CPB on the plasma proteome in children undergoing tetralogy of Fallot repair.

Methods: Blood samples were taken from 20 children prior to and at the end of CPB and 6h, 12h and 24h after CPB. Plasma was analysed by liquid chromatography-mass spectrometry (LC-MS) in a label-free, untargeted approach. Data were analysed using Genedata software to identify peptides that were differentially expressed ($p < 0.01$ above a false discovery rate). Proteins were identified from peptides that demonstrated differential expression.

Results: The proteins that were found to be differentially expressed were haptoglobin isoform I preproprotein, isoform 2 of semaphorin-6C, vitamin D-binding protein, inter-alpha-trypsin inhibitor, ceruloplasmin, apolipoprotein B100 and fibrinogen alpha.

Conclusion: CPB alters the plasma proteome with differences most apparent at 6h and 12h post CPB. There was a return to baseline with no proteins differentially regulated by 24h.

Keywords

cardiopulmonary bypass; proteins; paediatric; cardiac surgery; mass spectrometry; proteomics

Introduction

Cardiopulmonary bypass (CPB) is essential during the repair of congenital cardiac defects. Morbidity associated with CPB remains a significant problem, with cardioplegic arrest and CPB being associated with central nervous system damage, coagulopathies and end-organ dysfunction, which may be a result of systemic inflammation, the introduction of gaseous microemboli and the pro-thrombotic state that is associated with CPB.^{1,2}

The complete gene and protein expression changes in response to CPB remain to be determined. Plasma is an

ideal body fluid to examine as it is easily obtained and the protein changes may reflect biological processes occurring in a variety of different organs. Previous proteomic studies on plasma from children undergoing CPB have involved patients with various cardiac conditions³⁻⁶ and small numbers of patients, making it difficult to interpret findings.

The deleterious effects of CPB may be of particular concern in children undergoing cardiac surgery with CPB. This may be further complicated by the chronic

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hypoxia experienced in patients with tetralogy of Fallot (TOF). We tested the hypothesis that CPB induces significant changes to the proteomic response in the plasma of children undergoing cardiac surgery for TOF repair.

Methods

The study was approved by the Royal Children's Hospital Ethics in Human Research Committee (29133) and conducted in accordance with the National Health and Medical Research Council of Australia Statement on ethical conduct in human research. Informed written consent was obtained from the parents of the participants.

Patients were included if they were undergoing complete TOF repair. Patients with chromosomal abnormalities, congenital lung malformations and haematological disorders were excluded.

Cardiac surgery via a median sternotomy was performed with standard anaesthesia, cardioplegic arrest and CPB. CPB was performed using a <1000 ml/min circuit (Cellplex Pty Ltd, Dandenong, VIC, Australia). The prime was composed of 500 mL Plasma-Lyte, 100 mL water for injection, 25 mL 8.4% sodium bicarbonate, 30 mg heparin, 2.5 mmol calcium, 100 mL 20% albumin and 1 unit of packed red blood cells (280 mL). The prime was then run through the haemofilter circuit to wash the unit.

Blood samples were collected from each patient prior to CPB, after the induction of anaesthesia, at the end of CPB prior to modified ultrafiltration and at 6h, 12h and 24h post CPB. Blood was collected in S-Monovette® tubes (Sarstedt Australia Pty Ltd, Mawson Lakes, South Australia, Australia), containing one volume per nine volumes of blood of 0.106 mol/L (3.2%) trisodium citrate anticoagulant. Plasma was obtained by centrifugation of the blood for 10 min at 10°C and 3000 g and frozen at -80°C for batch testing.

Prior to the proteomic analysis, samples for all participants included in this study were pooled for practical reasons into pools of four for each time point, such that there were 5 pooled samples per time point. A protease inhibitor cocktail (Sigma-Aldrich Co. LLC, Castle Hill, New South Wales, Australia) was added to the plasma sample in a ratio of 1 µL to each 50 µL of plasma.

Plasma fractionation

Ion exchange chromatography, using strong cation and anion exchange columns (Thermo Scientific, West Palm Beach, FL, USA), was employed to exclude albumin and IgG, based on the isoelectric point. Samples were processed using the manufacturer's directions with 50 mM Tris HCl buffer at pH 6.8 (strong cation exchange

column) and buffer 50 mM sodium acetate at pH 5.2 (strong anion exchange column).

Protein quantification

The Bradford assay (Bio-Rad, Hercules, CA, USA) was used to determine the protein content of each sample.⁷

Trichloroacetic acid protein precipitation

Protein precipitation involved adding one volume of 100% (w/v) trichloroacetic acid to four volumes of the protein sample. Samples were incubated at 4°C for 10 minutes and pelleted by centrifugation at 14,000 g for five minutes. The supernatant was removed and the pellet washed twice with 200 µL of cold acetone and centrifuged at 14,000 g for five minutes. Pellets were dried and redissolved in 60 µL of 6 M urea.

In-solution trypsin digestion

Fifty micrograms of protein from each sample was taken for in-solution trypsin digestion as previously described.⁸

Mass spectrometry

Samples were prepared and liquid chromatography with tandem mass spectrometric (LC-MS/MS) detection methods was carried out in a label-free, untargeted approach on a LTQ Orbitrap Velos (Thermo Scientific, West Palm Beach, FL, USA) as previously described.⁸ Raw data were collected using Thermo Xcalibur Software (Thermo Scientific).

Data analysis and statistical analysis

Data were analysed using Genedata software (Genedata, Basel, Switzerland). Initial data processing was carried out using Genedata Expressionist Refiner MS for the alignment of MS data, noise reduction and peak extraction (clustering). Once clustered, peak area intensity measurements of precursor ions were normalised using the arithmetic mean.

In order to determine the effect of CPB on the plasma proteome, samples from each time point were compared to the baseline samples. An 'absent/present' search was performed, which aimed to identify the number of peptides that were absent or present in the samples from the various timepoints compared to the baseline.

An effect size analysis was conducted to identify those peptides that demonstrated a fold change of ≥ 2 . A t-test was conducted to identify those peptides that were significantly changed. Bootstrapping was conducted to determine the accuracy of sample estimates. In

Table 1. Demographics and clinical data.

	Mean $\pm$ SD
Age (months)	7.4 $\pm$ 3.3
Weight (kg)	7.7 $\pm$ 1.8
Gender (n)	9M/11F
Pre-operative oxygen saturation (%)	92.6 $\pm$ 5.6
Cardiopulmonary bypass time (min)	104 $\pm$ 7
Aortic cross-clamp time (min)	83 $\pm$ 22
Minimum temperature during bypass (°C)	31.6 $\pm$ 2.1
CPB circuit prime volume (mL)	472 $\pm$ 44

CPB: cardiopulmonary bypass.

addition, a permutation Q test was conducted to determine a false discovery rate (FDR); only p-values less than the permutation Q value were accepted as significant. The results from the effect size analysis and t-test analysis were combined in the form of a Venn diagram and, from this diagram, those peptides demonstrating fulfilment of both criteria, that is, a fold change and a statistically significant change, were selected for inclusion into a target list for further testing by tandem MS.

An ANOVA was performed in order to identify peptides that were changing over time. A bootstrap was applied and the FDR calculated over which the significant peptides could be selected for analysis. Those peptides that were showing significant differential expression patterns above the FDR were selected for the formation of a target list.

Targeted mass spectrometry analysis

For the targeted LC-MS runs, the mass spectrometer was operated in data-dependent mode, as described above, using collision-induced dissociation in the linear-trap quadrupole (CID-LTQ), but also in the data-dependent mode, using accurate mass collision-induced dissociation Fourier Transform (CID-FT) and higher energy C trap dissociation Fourier Transform (HCD-FT) for MS/MS fragmentation at a resolution of 7500 in the Orbitrap. Regardless of the MS/MS mode, the full MS scan was set at 60,000 resolutions and the ten most intense peptide ions with charge states greater or equal to two were isolated at a minimum threshold value of 5,000 from the assigned target list. A dynamic exclusion of two repeats over 30 seconds with exclusion duration of 45 seconds was set.

Data mining

Data from the Thermo Xcalibur (Thermo Scientific) were imported into Proteome Discoverer version 1.3 (Thermo Fisher Scientific, West Palm Beach, FL, USA)

and MASCOT v.2.3.01 (Matrix Science, London, UK), using settings previously detailed.⁸ A MASCOT search was carried out on the NCBI mammalian and human databases (National Centre for Biotechnology Information, Bethesda, NY, US). A precursor mass tolerance of 20 ppm and a fragment mass tolerance of 0.8 Da were selected. Dynamic modifications included oxidation and carbamidomethylation. The search was performed against a decoy database to allow determination of a FDR. The strict criterion was set at 0.01 and the relaxed as an FDR of 0.05. The characteristics of the peptides in the target lists, namely mass to charge ratio (m/z), retention time, charge and mass and fragmentation patterns were used to identify matches within the databases.

Results

Twenty patients were recruited into the study. Their demographics and clinical data are presented in Table 1.

The data in the following section are presented in accordance with the guidelines for reporting of proteomic MS data.⁹ The MS performed on the samples from all the time points resulted in 335,964 clusters from the MS analysis of which 96,725 were determined to be peptides, based on a charge state of ≥ 2 .

The initial absent/present search demonstrated 106 peptides that were unique to the control baseline sample and 28 peptides that were uniquely present in the end-CPB samples. At 6 hours, there were 204 peptides unique to the baseline sample and 5 unique to the 6 hour sample. At 12 hours, 87 peptides were unique to the baseline sample and 32 uniquely present in the 12 hour sample. At 24 hours, there were fewer peptides unique to the baseline sample, with 19 peptides and 40 unique to the 24 hour samples. These data are displayed in Figure 1.

The results of the effect size and t-test analyses are presented in the form of Venn diagrams (Figure 2) for comparison of the baseline samples with those from the end-CPB, 6 h, 12 h and 24 h time points. This figure details the number of peptides that are down-regulated or up-regulated by a factor of ≥ 2 . The number of peptides that are significantly different in their expression intensity (p<0.01 above an FDR) are indicated at the intersection of the circles. Identified proteins are described in Table 2 and Table 3.

In order to assess the effect of CPB over the time course, an ANOVA was performed on the control group samples over the five time points. The peptides selected were those that were significantly changing at a p-value of less than 10^{-7} . Twenty peptides were significantly changing over the course of the study. The proteins identified are detailed in Table 4.

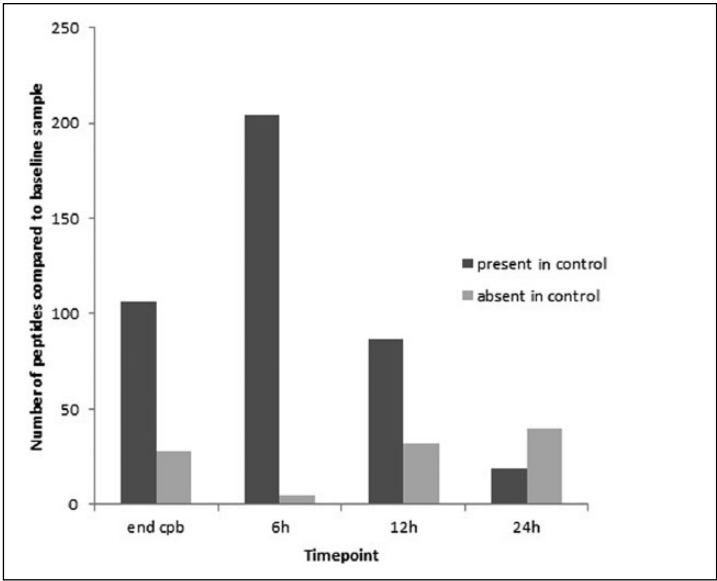


Figure 1. Pairwise comparison of the ‘absent/present’ analysis. Comparison of the baseline samples with the samples from the end of cardiopulmonary bypass (end cpb), 6 h post cardiopulmonary bypass (6h), 12 h post cardiopulmonary bypass (12h) and 24 h post cardiopulmonary bypass (24h). The two bars at each time point show the number of peptides unique to the baseline sample (dark grey bar) and the number of peptides unique to the samples from the time point specified on the x axis (light grey bars). To be considered, the peptide was required to be detected in all five pooled samples at each time point.

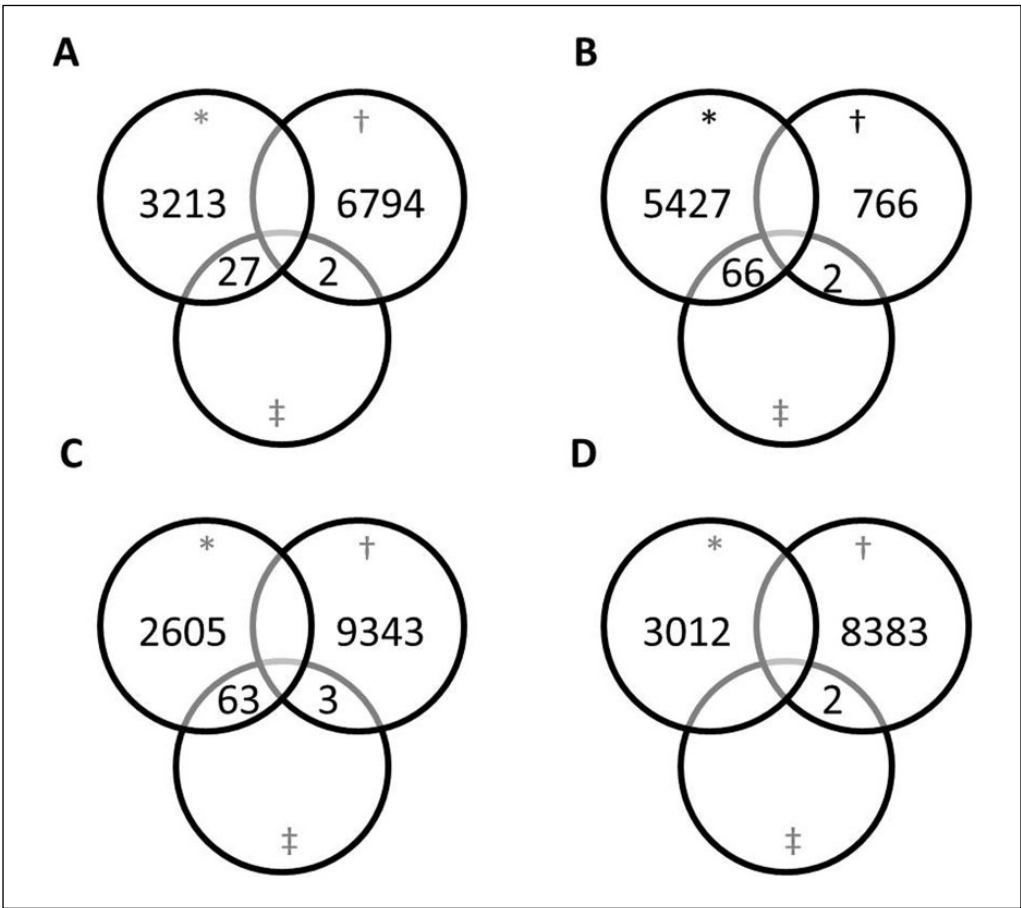


Figure 2. Venn diagram detailing the number of differentially expressed peptides in the samples compared to the baseline samples at each time point. A. At the end of cardiopulmonary bypass. B. 6 h post cardiopulmonary bypass. C. 12 h post cardiopulmonary bypass. D. 24 h post cardiopulmonary bypass. * - down-regulated, † - up-regulated, ‡ - significantly differentially expressed.

Table 2. Proteomic data for the peptides that demonstrated differential expression and were matched to proteins. Data includes properties of the peptides and the identifying information for the proteins when the baseline samples were compared to the samples from each of the other time points.

Cluster number	Charge	Mass	m/z	RT (min)	Accession Number	Protein	Sequence	Score
BASELINE V END CPB								
UP-REGULATED								
075871	2	798.48	400.25	21.45	194391072	Vitamin D-binding protein	VLEPTLK	14250
DOWN -REGULATED								
000775	6	3431.57	572.93	27.11	Q9H3T2-2 SEQUEST	Isoform 2 of Semaphorin-6C	ALLTQVAVDGMAGPH-sNITVmFLGsNDGTVLK	650
016645	4	1707.87	427.97	18.69	4826762	haptoglobin isoform 1 preproprotein	LRTEGDGVYTLNNEK	12772
035719	4	3431.58	858.90	27.14	P00738	Haptoglobin	AVGDKLPEcEADDGcPKP-PEIAHGYVEHSVR	10324
054758	3	1707.83	570.28	21.87	4826762	haptoglobin isoform 1 preproprotein	LRTEGDGVYTLNNEK	12772
076505	2	857.48	429.75	21.16	4826762	haptoglobin isoform 1 preproprotein	QLVEIEK	12772
077334	2	919.45	460.73	29.49	4826762	haptoglobin isoform 1 preproprotein	GSPWQAK	12772
078159	2	979.48	490.75	26.96	4826762	haptoglobin isoform 1 preproprotein	VGYVSGWGR	12772
081832	2	1202.62	602.32	31.34	4826762	haptoglobin isoform 1 preproprotein	VTSIQDWVQK	12772
086085	2	1438.65	720.33	22.31	4826762	haptoglobin isoform 1 preproprotein	TEGDGVYTLNNEK	12772
BASELINE V 6H								
UP-REGULATED – NONE IDENTIFIED								
DOWN-REGULATED								
000775	6	3431.57	572.93	27.11	Q9H3T2-2	Isoform 2 of Semaphorin-6C	ALLTQVAVDGMAGPH-sNITVmFLGsNDGTVLK	650
016645	4	1707.87	427.97	18.69	4826762	haptoglobin isoform 1 preproprotein	LRTEGDGVYTLNNEK	12772
051183	3	1438.68	480.57	18.30	4826762	haptoglobin isoform 1 preproprotein	TEGDGVYTLNDKK	12772
054758	3	1707.83	570.28	21.87	4826762	haptoglobin isoform 1 preproprotein	VTSIQDWVQK	12772
056004	3	1793.99	599.00	35.63	P00738	Haptoglobin	VVLHPNYSQVDIGLIK	985.28
056910	3	1856.89	619.97	19.77	4826762	haptoglobin isoform 1 preproprotein	AVGDKLPEcEAVcGKPK	12772
062091	3	2171.04	724.69	34.63	P00450	Ceruloplasmin	LISVDTEHSNITLQnGPDR	144.97
076505	2	857.48	429.75	21.16	4826762	haptoglobin isoform 1	QLVEIEK	12772
077334	2	919.45	460.73	29.49	186590	inter-alpha-trypsin inhibitor heavy chain	RLSNENHIGIAQR	
077375	2	922.52	462.27	16.61	180249	ceruloplasmin	VDKDNEDFQESNR	144.97

(Continued)

Table 2. (Continued)

Cluster number	Charge	Mass	m/z	RT(min)	Accession Number	Protein	Sequence	Score
078159	2	979.48	490.75	26.96	11761629	fibrinogen alpha chain isoform alpha preproprotein	mADEAGSEADHEGTHSTK	22554.86
081832	2	1202.62	602.32	31.34	306569733	mutant Apo B 100	LGNNPVSK	
083902	2	1310.59	656.30	22.80	4826762	haptoglobin isoform I preproprotein	TEGDGVYTLNDK	12772
085789	2	1417.81	709.91	34.10	4826762	haptoglobin isoform I preproprotein	DIAPTLTYVGKK	12772
086085	2	1438.65	720.33	22.31	11761629	fibrinogen alpha chain isoform alpha preproprotein	TVIGPDGHHK	22554.86
BASELINE V 12H								
UP-REGULATED – NONE IDENTIFIED								
DOWN-REGULATED								
016645	4	1707.87	427.97	18.69	296653	hp2-alpha	LRTEGDGVYTLNNEK	691.06
035719	4	3431.58	858.90	27.14	296653	hp2-alpha	AVGDKLPecEADDGcPKP-PEAHGYVEHSVR	691.06
050969	3	1417.81	473.61	34.09	4826762	haptoglobin isoform I preproprotein	DIAPTLTYVGKK	12772
054762	3	1707.87	570.30	18.67	4826762	haptoglobin isoform I preproprotein	LRTEGDGVYTLNNEK	12772
076505	2	857.48	429.75	21.16	4826762	haptoglobin isoform I preproprotein	QLVEIK	12772
077334	2	919.45	460.73	29.49	4826762	haptoglobin isoform I preproprotein	GSPWQAK	12772
077375	2	922.52	462.27	16.61	4826762	haptoglobin isoform I preproprotein	ILGGHLDK	12772
078159	2	979.48	490.75	26.96	4826762	haptoglobin isoform I preproprotein	VGYVSGWGR	12772
078255	2	985.57	493.79	16.51	4826762	haptoglobin isoform I preproprotein	KQLVEIK	12772
081832	2	1202.62	602.32	31.34	4826762	haptoglobin isoform I preproprotein	VTSIQDWVQK	12772
086085	2	1438.65	720.33	22.31	4826762	haptoglobin isoform I preproprotein	TEGDGVYTLNNEK	12772
089388	2	1707.83	854.92	21.86	4826762	haptoglobin isoform I preproprotein	LRTEGDGVYTLNNEK	12772
BASELINE V 24H								
UP-REGULATED – NONE IDENTIFIED								
DOWN-REGULATED – NONE								

Cluster number refers to the number allocated to each peptide fragment in the Genedata software; Charge refers to the charge of the peptide; mass indicates the mass of the peptide (Da); m/z indicates the mass to charge ratio; RT: Retention time (min), the time at which the peptide elutes off the LC column; Accession number refers to the corresponding protein from the NCBI human protein database; Protein indicates the protein that the peptide was matched to; Sequence is the sequence of amino acids; Protein score is a score assigned by MASCOT software to indicate the confidence with which the proteins identified match the NCBI human protein database.

Table 3. The integrated peaks are for the proteins found to be differentially expressed between the baseline sample and samples taken at the end of cardiopulmonary bypass (CPB) and 6 h, 12 h and 24 h post CPB. The numbers represent the average of the intensity for all peptides identified for the identified protein at the specified time point.

Time Point	Protein	Baseline	Time point	P value
End CPB				
Up-regulated	Vitamin D Binding Protein	160.7 ± 18.5	3345.1 ± 432.2	0.0001
Down-regulated	Isoform 2 Of Semophornin 6c	66703.4 ± 12974.9	654.8 ± 58.4	0.001
	Haptoglobin Isoform I Preproprotein	26595.1 ± 3276.2	540.1 ± 74.4	0.0001
6 h post CPB				
Up-regulated	None identified			
Down-regulated	Isoform 2 Of Semophorin 6c	66703.4 ± 12974.9	534.3 ± 131.5	0.003
	Haptoglobin Isoform I Preproprotein	12410.3 ± 2442.4	58.4 ± 25.2	0.003
	Ceruloplasmin	23035.2 ± 5195.1	69.9 ± 47.2	0.005
	Inter-alpha Trypsin Inhibitor	42998.5 ± 5679.1	522.2 ± 85.4	0.0009
	Fibrinogen Alpha	25761.7 ± 7125.1	59.8 ± 21.5	0.0006
	Apolipoprotein B100	34736.0 ± 4591.8	331.9 ± 66.9	0.0008
12 h post CPB				
Up-regulated	None identified			
Down-regulated	Haptoglobin Isoform I Preproprotein	21702.6 ± 4860.3	67.2 ± 21.4	0.0001
24 h post CPB				
Up-regulated	None identified			
Down-regulated	None identified			

Table 4. Proteins demonstrating differential expression that were successfully identified from the ANOVA analysis examining the effect of CPB over the first 24 h post CPB.

Cluster number	Charge	Mass	m/z	RT(min)	Accession Number	Protein	Sequence	Score
076505	2	857.48	429.75	21.16	4826762	Haptoglobin isoform I preproprotein	QLVEIEK	12772
077334	2	919.45	460.73	29.49	4826762	Haptoglobin isoform I preproprotein	GSFPWQAK	12772
077375	2	922.52	462.27	16.61	4826762	Haptoglobin isoform I preproprotein	ILGGHLDK	12772
078159	2	979.48	490.75	26.96	4826762	Haptoglobin isoform I preproprotein	VGYSVGWGR	12772
081832	2	1202.62	602.32	31.34	4826762	Haptoglobin isoform I preproprotein	VTSIQDWVQK	12772
086085	2	1438.65	720.33	22.31	4826762	Haptoglobin isoform I preproprotein	TEGDGVYTLNNEK	12772
090404	2	1819.90	910.96	43.63	1942435	Chain A, Human C-Reactive Protein	YEVQGEVFTKPLWP	431

Cluster number refers to the number allocated to each peptide fragment in the Genedata software; Charge refers to the charge of the peptide; Mass indicates the mass of the peptide (Da); m/z indicates the mass to charge ratio; RT: Retention time (min), the time at which the peptide elutes off the LC column; Accession number refers to the corresponding protein from the NCBI human protein database; Protein indicates the protein that the peptide was matched to; Sequence is the sequence of amino acids; Protein score is a score assigned by MASCOT software to indicate the confidence with which the proteins identified match the NCBI human protein database. Only Protein scores greater than 40 were considered to match with sufficient confidence.

Discussion

This study found changes induced in the plasma proteome following CPB in children undergoing TOF repair. CPB predominantly results in a response characterised by the down-regulation of protein expression. The differences in expression were most apparent at 6 h and 12 h post CPB.

The current study confirms findings by Miller et al.,² investigating the proteomic response in children with congenital heart disease, who found that fibrinogen, ceruloplasmin and inter-alpha trypsin inhibitor were significantly lower during surgery.² These studies were also suggestive of a response, with acute onset and protein levels normalising quickly following CPB,² consistent with our findings of the proteomic profile returning to normal levels by 24 h.

Haptoglobin isoform 1 preproprotein was found to be down-regulated post CPB. Haptoglobin is an acute phase protein that is associated with ischaemia reperfusion injury. Its levels have been found to decrease during ischaemia reperfusion injury, with levels negligible 30 minutes after the initiation of CPB.¹⁰ This finding is consistent with that observed in the adult population undergoing coronary artery bypass graft surgery, where haptoglobin levels are reduced in the first 24 h post-surgery compared to baseline and in the paediatric setting of CPB.^{1,5} Haptoglobin scavenges free haemoglobin resulting from the haemolysis induced by CPB and removes it from the circulation in order to prevent renal damage and iron loss.¹ Previous studies have employed immune-depletion methods to fractionate the plasma, which specifically removed high abundance proteins, including haptoglobin, that account for a large proportion of the plasma proteome prior to analysis.²⁻⁴ This may explain why changes in haptoglobin in this setting have not been described previously.

Semaphorin 6C was down-regulated at the end of CPB as well as 6 h after CPB. Semaphorin 6C plays a role in organising neuronal structures, neuronal communication and guiding axons.¹¹ Down-regulation of this protein may interrupt normal neuronal function and predispose to neurological dysfunction following surgery with CPB.

Inter-alpha trypsin inhibitor is a serine protease that is produced in the liver and has been found in previous studies to be decreased following CPB, but returned to normal levels by 24 h post CPB.^{2,3} This is consistent with our findings and suggests a role in acute phase inflammation in response to CPB. The administration of inter-alpha trypsin inhibitors has improved survival among patients with sepsis and, therefore, may hold potential for exogenous administration to avoid the deleterious effects of CPB.¹²

Ceruloplasmin is a copper-containing protein that is important for iron metabolism. Ceruloplasmin regulates copper binding in plasma and regulates the peroxidation of iron and interaction with transferrin. Ceruloplasmin has a known role in iron homeostasis and the survival of neurons.¹³ Low levels of ceruloplasmin in the plasma may, therefore, enable the prediction for different pathologies arising from dysregulation of iron metabolism. These findings suggest that there is a potential role of ceruloplasmin in the pathogenesis of CPB-related injury and suggests a role for oxidative stress metabolism.³ Previous investigation has identified and described ceruloplasmin as being down-regulated following CPB - a finding confirmed by our results.³

Apolipoprotein B100 was found to be down-regulated 6 h after CPB. Apolipoproteins have been associated with functions over and above that of blood cholesterol metabolism. They have been determined as a biomarker

for cancer¹⁴ and may have anti-inflammatory properties by regulating T cell and macrophage interactions.¹⁵ Apolipoprotein precursors were found to be decreased in brain tissue of pigs undergoing hypothermic CPB, indicating differential regulation of these proteins in response to CPB.¹⁶

Fibrinogen was down-regulated at 6 h post CPB. These findings are similar to previous studies that reported differential expression over the first 24h post CPB in children undergoing cardiac surgery, with a decrease in the level of fibrinogen after commencement of CPB.^{6,17}

C-reactive protein (CRP) is an acute phase protein that is associated with inflammation following CPB. Not surprisingly, CRP levels were raised in the first 24 h post CPB. CRP is known to increase in the first 24 h post paediatric cardiac surgery.¹⁸ This increase is likely to be a result of activation of the inflammatory response and innate immunity in response to tissue damage induced by CPB and cardiac surgery.¹⁹

Studies have been conducted, examining the proteomic response of plasma biomarkers in paediatric CPB.³⁻⁶ Our results confirm findings from previous studies that found ceruloplasmin, c-reactive protein, fibrinogen, inter-alpha trypsin inhibitor and haptoglobin were differentially regulated over the first 24 hours post CPB.^{2,5} The strength of our study is the relatively large sample size and, therefore, statistical power compared with previous studies, the multiple time points examined and the powerful, yet sensitive, MS method employed which has not been applied to plasma proteomics in this setting.

Other studies have focused on acute kidney injury after cardiac surgery in children by examining the urinary proteome²⁰⁻²² and right atrial tissue after cardiac surgery.²³ These studies have found albumin, alpha 1 microglobulin and alpha 1 acid glycoprotein changing in the urinary proteome, using a mass spectrometry-based approach.²⁰ Another study of urinary proteomics in 66 patients, both two and six hours post CPB, failed to identify any proteins, instead describing the size of four urinary biomarkers that were significantly changing during these time points.²²

Investigating biomarkers may enable the identification of patients at risk of deleterious outcomes and allow treatments or preventative measures to be implemented promptly. Given the range of proteins identified, the response to CPB induces changes in a variety of biological processes and may represent biological processes that are initiated early after surgery. This study was not designed to assess proteomic differences in cyanotic compared to acyanotic children. Examination of the roles of the proteins that were identified in biological processes will be crucial for understanding the mechanisms behind CPB-induced inflammation and, therefore, provide insight into how potential complications may be averted.

A limitation of this study relates to the method of plasma fractionation. The method used to fractionate the plasma was focused on completely removing albumin from the samples as albumin is present in such a profound amount in plasma that even small residual amounts after the removal techniques poses a significant problem, significantly incapacitating the MS analysis. The ion exchange method used effectively removed the albumin and most of the IgG – the second most abundant protein in plasma, but it also involved removing other proteins with an isoelectric point between 5.2 and 6.8. It is possible some proteins of important biological significance were excluded from the analysis.

Cardiac surgery with CPB induces changes to the plasma proteome in children undergoing tetralogy of Fallot repair, with the differentially expressed proteins involved in processes such as inflammation and coagulation. The differences in protein expression were most apparent at 6h and 12 h post CPB.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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RESEARCH ARTICLE

Remote Ischemic Preconditioning (RIPC) Modifies the Plasma Proteome in Children Undergoing Repair of Tetralogy of Fallot: A Randomized Controlled Trial

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Abstract

Background

Remote ischemic preconditioning (RIPC) has been applied in paediatric cardiac surgery. We have demonstrated that RIPC induces a proteomic response in plasma of healthy volunteers. We tested the hypothesis that RIPC modifies the proteomic response in children undergoing Tetralogy of Fallot (TOF) repair.

Methods and Results

Children (n=40) were randomized to RIPC and control groups. Blood was sampled at baseline, after cardiopulmonary bypass (CPB) and 6, 12 and 24h post-CPB. Plasma was analysed by liquid chromatography mass spectrometry (LC-MS) in an untargeted approach. Peptides demonstrating differential expression ($p < 0.01$) were subjected to tandem LC-MS/MS and protein identification. Corresponding proteins were identified using the NCBI protein database. There was no difference in age (7.3 ± 3.5 vs 6.8 ± 3.6 months) ($p = 0.89$), weight (7.7 ± 1.8 vs 7.5 ± 1.9 kg) ($p = 0.71$), CPB time (104 ± 7 vs 94 ± 7 min) ($p = 0.98$) or aortic cross-clamp time (83 ± 22 vs 75 ± 20 min) ($p = 0.36$). No peptides were differentially expressed at baseline or immediately after CPB. There were 48 peptides with higher expression in the RIPC group 6h post-CPB. This was no longer evident at 12 or 24h, with one peptide down-regulated in the RIPC group. The proteins identified were: inter-alpha globulin inhibitor (42.0 ± 11.8 vs 820.8 ± 181.1 , $p = 0.006$), fibrinogen preproprotein (59.3 ± 11.2 vs 1192.6 ± 278.3 , $p = 0.007$), complement-C3 precursor (391.2 ± 160.9 vs 5385.1 ± 689.4 , $p = 0.0005$), complement C4B (151.5 ± 17.8 vs 4587.8 ± 799.2 , $p = 0.003$), apolipoprotein B100 (53.4 ± 8.3 vs 1364.5 ± 278.2 , $p = 0.005$) and urinary proteinase inhibitor (358.6 ± 74.9 vs 5758.1 ± 1343.1 , $p = 0.009$). These proteins are involved in metabolism, haemostasis, immunity and inflammation.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

We provided the first comprehensive analysis of RIPC-induced proteomic changes in children undergoing surgery. The proteomic changes peak 6h post-CPB and return to baseline within 24h of surgery.

Trial Registration

ACTR.org.au [ACTRN12610000496011](https://actr.org.au/actr12610000496011)

Introduction

First described in 1986 by Murray et al [1], ischemic preconditioning (IPC) is a phenomenon whereby brief periods of ischemia can provide protection against subsequent prolonged ischemia. The concept has since evolved into a clinically applicable protection known as remote IPC (RIPC) where ischemia of peripheral tissue, including skeletal muscle, could protect distant organs from ischemia-reperfusion (IR) injury [2]. This can be applied non-invasively in various clinical scenarios.

The first clinical application of RIPC in humans was reported in a randomized clinical trial (RCT) in children undergoing heart surgery and demonstrated cardiopulmonary protection with decreased inotrope requirement, decreased airway resistance and decreased troponin levels [3]. Subsequently, the RIPC-induced myocardial protection was demonstrated in other RCTs [4–8]. Recent meta-analysis of the RCTs also demonstrated significant myocardial protection by the RIPC against IR injury in patients undergoing heart surgery with cardioplegic arrest and cardiopulmonary bypass (CPB) [9].

In contrast to these studies with positive findings, the impact of RIPC on myocardial protection in the setting of chronic hypoxia in children is less clear. Our two recent RCTs demonstrate that in chronically hypoxic children with cyanotic heart disease, a high proportion of proteins are in phosphorylated form and RIPC does not further enhance phosphorylated protein signalling in myocardium or circulating leukocytes in children undergoing Tetralogy of Fallot (ToF) repair [10] or provide myocardial protection [11]. To dissect the molecular impact of the RIPC in children with cyanotic heart disease, we performed a further proteomic analysis of the RIPC-induced changes in plasma of children undergoing repair of ToF [10].

Methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see [S1 CONSORT Checklist](#) and [S1 Protocol](#).

This double blind RCT was approved by the Royal Children's Hospital Human Research Ethics committee and conducted in accordance with the National Health and Medical Research Council of Australia Statement on ethical conduct in human research. Informed, written consent was obtained from the parents of the patients involved in the study. The RCT was registered with the Australian and New Zealand Clinical Trials Registry. Registry number ACTRN12610000496011.

Patients

All patients who underwent elective repair of ToF from July 2010 to June 2012 were eligible to participate in the study if they were having their first surgery for complete ToF repair ([Fig. 1](#)). Eligible patients were enrolled by the surgeons.

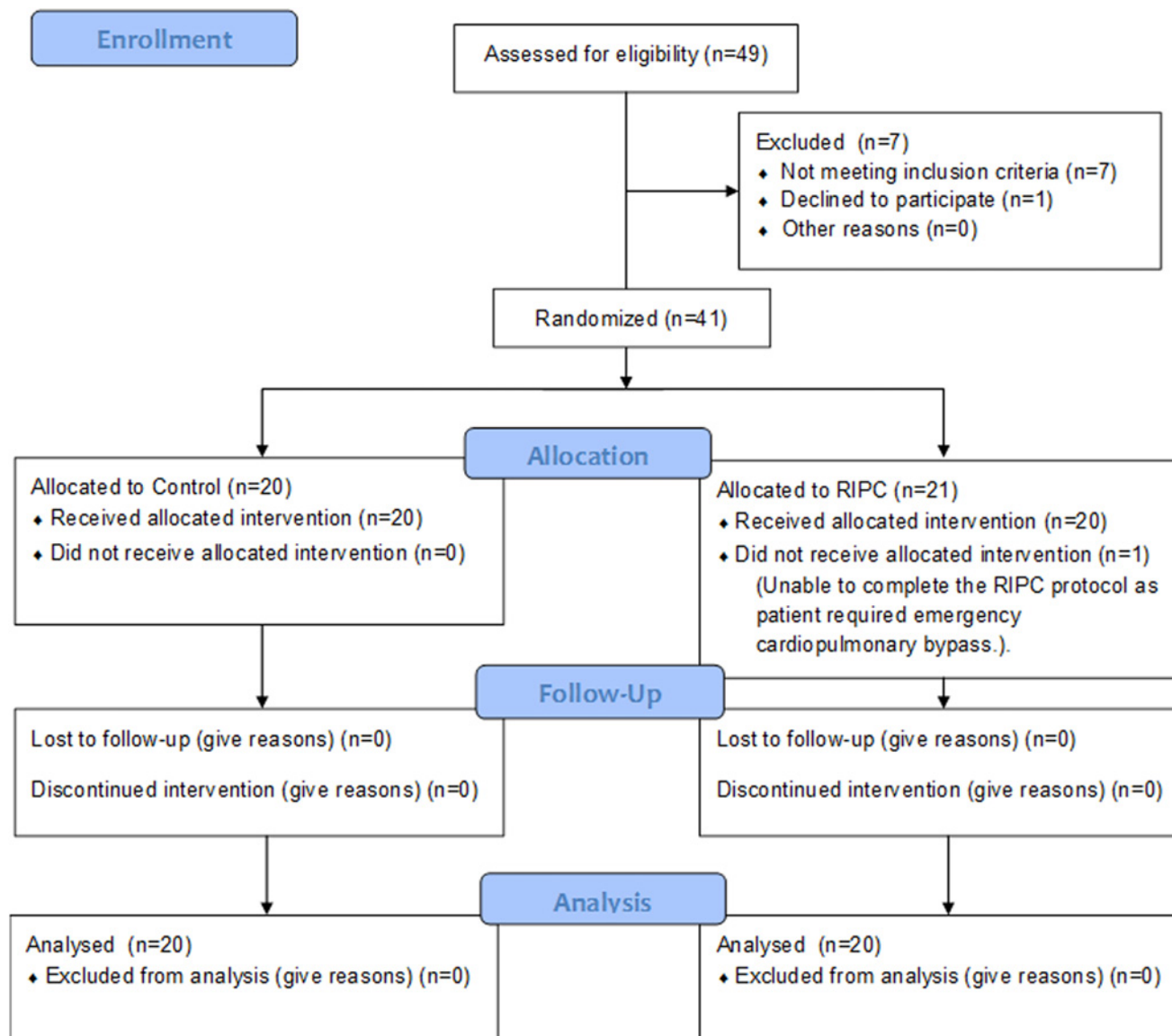


Fig 1. CONSORT diagram describing flow of the participants through the enrolment, allocation, follow up and analysis phases of the trial.

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Patients with chromosomal abnormalities, congenital lung malformations and haematological disorders were excluded. Patients (n = 40) aged between 1.8 months and 14 months (Table 1) were randomized into control group (n = 20) and RIPC group (n = 20).

Sample size was determined based on our successful study of RIPC in children which was amply powered to detect differences in clinical and molecular measures [3]. Injury was deemed to be diminished by RIPC on the basis of reduced troponin I release, thus for a power of 0.8, alpha of 0.05 and a sigma of 3 the sample size required is calculated as n = 16. Thus a sample size of n = 20 per group would yield ample statistical power.

Randomisation

Blocked randomisation (block size = 10) was used to allocate patients. The randomisation was generated by a member of the laboratory research staff using SPSS statistical software and

Table 1. Demographic data for the Control and RIPC groups.

	Control	RIPC	p value
Patients	20	20	
Gender	9M, 11F	11M, 9F	0.75
Weight (kg)	7.7 ± 1.8 (5.0–11.6)	7.5 ± 1.9 (5.1–10.8)	0.71
Age (months)	7.3 ± 3.5 (3.3–14.2)	6.8 ± 3.6 (1.8–15.1)	0.89
Cardiopulmonary bypass time (CPB)(mins)	104 ± 7 (80–158)	94 ± 7 (55–186)	0.98
Total aortic cross-clamp time (mins)	83 ± 22 (56–131)	75 ± 20 (43–108)	0.36
Preoperative oxygen saturations (%)	92.6 ± 5.6 (80–98)	87.3 ± 9.1 (70–100)	0.04
Total CPB prime volume (mL)	472 ± 12 (400–542)	461 ± 12 (356–556)	0.56

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concealed in sequentially numbered sealed envelopes. The envelopes were opened in numerical order immediately prior to the RIPC or sham intervention. The research co-ordinator assigned the interventions to patients.

Remote ischemic preconditioning protocol

The RIPC protocol consisted of 4 cycles of 5 minutes ischaemia, followed by 5 minutes of re-perfusion applied to the patient's leg in the operating theatre after induction of anaesthesia prior to sternotomy [3]. A standard blood pressure cuff (Welch Allen, Germany) was applied to the thigh and inflated to a pressure exceeding systolic pressure by 20 mmHg, determined by blood pressure monitored via an arterial line. This was followed by deflation of the cuff. Interruption and restoration of blood flow was confirmed by pulse oximetry applied distal to the blood pressure cuff. Control patients had sham placement of the blood pressure cuff on a lower limb without inflation. All clinical staff and patients' families were blinded to the allocation.

Cardiac surgery

Cardiac surgery via median sternotomy was performed using standard anaesthesia, cardioplegic arrest and CPB as described previously [10].

Blood sampling

A total of 5 blood samples were collected from each patient. Blood samples were collected prior to RIPC or sham, at the cessation of CPB and at six, 12 and 24 hours after CPB (Fig. 2A). Blood (1.4 mL) was collected in S-Monovette tubes (Sarstedt, Australia), containing one volume per nine volumes of blood of 0.106mol/L (3.2%) trisodium citrate anticoagulant. Plasma was obtained by centrifugation at 10°C for 10 min at 3000 rpm (Megafuge 1.0R, Heraeus) and stored at -80°C for batch testing.

Preparation of the plasma samples

For each of 5 time points, 40 samples (Control, n = 20 and RIPC, n = 20) were collected and the samples from each group were pooled into 5 samples, so that each of those pooled 5 samples was representative of 4 patients in that group (Fig. 2B). Prior to pooling, 100 uL of each individual sample was transferred into tubes containing 4 uL protease cocktail inhibitors (Sigma Aldrich) to stop degradation of the proteins by endogenous proteases. Samples were filtered through 45µm spin filters.

Ion exchange chromatography was employed to exclude albumin and IgG from the plasma based on the isoelectric point of these proteins. Plasma samples were passed through a strong

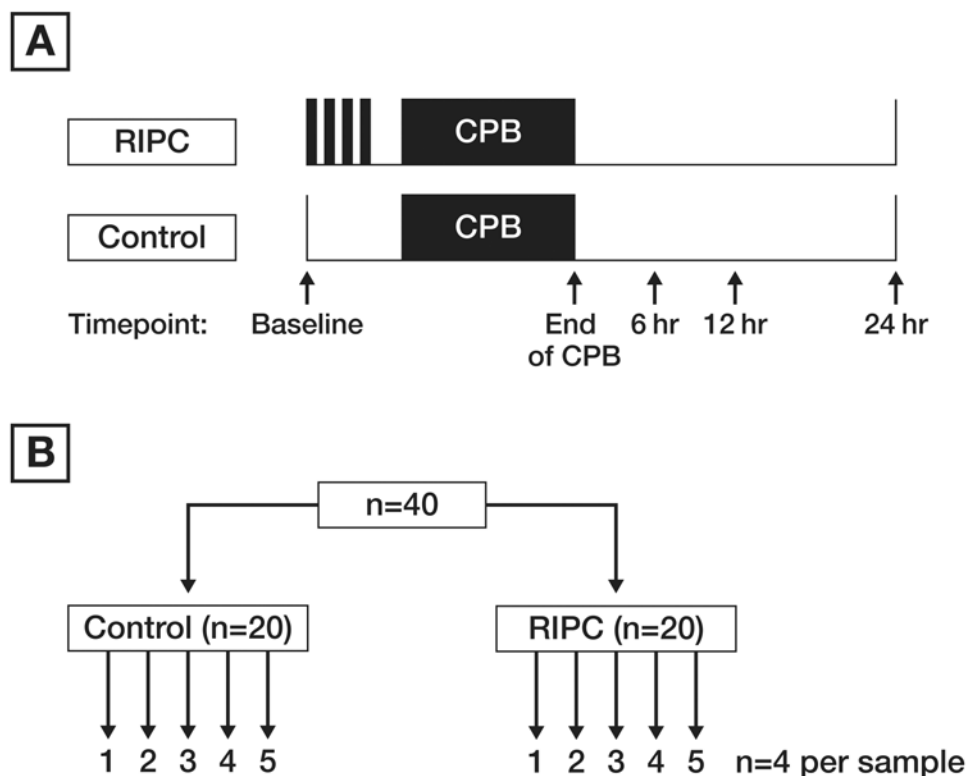


Fig 2. Time points of the sample collection in panel A. Pooling of the plasma samples at each time point in panel B. Plasma from all 20 patients of one group was pooled into 5 samples (each representing plasma of 4 patients).

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cation exchange column (50 mM Tris HCl pH 6.8) as well as a strong anion exchange column (50 mM sodium acetate at pH 5.2) (Pierce, Thermo Scientific) according to the manufacturer's instructions. The proteins from the strong cation exchange columns and the strong anion exchange columns were combined for analysis.

Quantification of protein content

The Bradford assay [12] (Bio-Rad, Hercules, CA, USA) was used to determine the protein content of each sample by comparing the absorbance at 595 nm against a standard curve of Bovine serum albumin dissolved in 50 mM Tris HCl, 50 mM sodium acetate 0.75 M NaCl, the same buffer that the samples were in.

Trichloroacetic acid (TCA) precipitation

Protein precipitation was carried out to concentrate the sample and dissolve the protein in a buffer suitable for trypsin digestion. The precipitation involved adding one volume of 100% (w/v) TCA to four volumes of protein sample.

The samples were incubated at 4°C for 10 minutes and pelleted by centrifuged at 14 000 g for five minutes. The supernatant was removed and the pellet washed with 200 µL cold acetone and centrifuged at 14 000 g for five minutes. The acetone wash was repeated. Pellets were dried by decanting off the acetone and evaporating the remaining acetone. The protein pellets were redissolved in 60 µL of 6 M Urea.

In-solution trypsin digestion

Trypsin digestion was performed on fifty micrograms of protein from each sample [13].

Sample preparation for mass spectrometry

Peptides were cleaned and concentrated by Solid Phase Extraction. Samples were passed through 1 mL (10 mg capacity) Oasis HLB extraction cartridges (Waters, Ireland). The columns were preconditioned with methanol and equilibrated with 3 mL 2% acetonitrile and 0.1% Trifluoroacetic acid (TFA). The sample was equilibrated 1/50 with 100% acetonitrile and 1/100 TFA then loaded onto the column. The column was washed with 3 mL 2% acetonitrile and 0.1% TFA. Bound peptides were eluted with 1 mL 80% acetonitrile and 0.1% TFA. Samples were frozen in liquid nitrogen and lyophilised by freeze drying overnight. Lyophilised peptides were then resuspended in 200 μ L 0.1% formic acid and sonicated for five minutes before being passed through 30 kD spin filters (Amicon Ultra 0.5 mL 30 kD) previously preconditioned with 0.1% formic acid and centrifuged at 14 000 rpm for 30 minutes. Sixty microliters of this solution was pipetted into glass vials in preparation for liquid chromatography, mass spectrometry (LC MS/MS).

Mass spectrometry

LC MS/MS was carried out on an LTQ Orbitrap Velos (Thermo Scientific, West Palm Beach, FL, USA) equipped with a nanoelectrospray interface coupled to an Ultimate 3000 RSLC nano-system (Dionex, Sunnyvale, CA, USA) using methodology we have previously described [13], with the following modifications. The 20 most intense peptide ions, with charge state ≥ 2 were isolated at a target value of 5000 for CID fragmentation. Dynamic exclusion was set at two repeat counts over 30 seconds and exclusion duration of 70 seconds. Raw data were collected using Thermo Xcalibur software (Thermo Scientific) and processed with Expressionist Refiner MS software (Genedata, Basel, Switzerland).

Data analysis

Initial data processing was carried out using Expressionist Refiner MS (Genedata, Basel Switzerland) to align MS data, carry out noise reduction and for peak extraction (clustering). The peak area intensity measurements of precursor ions were analysed for statistical relevance using Genedata Analyst (AG, Basel, Switzerland).

At each time point, the control group was compared against the RIPC group. An effect size analysis was conducted to compare samples based on the calculation of fold change ratios. The analysis was set to identify those peptides that demonstrated a fold change of greater than or equal to two.

An unpaired t-test was conducted to identify those peptides that were significantly different between the two groups. Bootstrapping was included to determine the accuracy of sample estimates. In addition, a permutation Q-test was conducted to indicate a permutation Q-value which calculates a false discovery rate. Only p-values less than the false discovery were accepted as significant.

The results from the effect size analysis and t test analysis for each time point were combined in the form of Venn diagrams. Those peptides intersecting and therefore demonstrating fulfillment of both criteria, that is a fold change and statistically significant change were included in a second pass targeted MSMS analysis and subsequent data mining and protein identification. In addition, a two way ANOVA was performed to assess differences between groups across time.

Targeted mass spectrometry

Three separate LC-MS/MS methods were implemented in an attempt to increase the chance of positive protein identification. For the targeted LC-MS/MS, the mass spectrometer was operated in data-dependent mode as described above using CID-LTQ but also in data-dependent mode using accurate mass, collision induced dissociation- Fourier transform (CID-FT) and higher-energy collisional dissociation- Fourier transform (HCD-FT) for MS/MS fragmentation at a resolution of 7500 in the Orbitrap. Independent of the MS/MS mode, the full MS scan was set at 60 000 resolution and the ten most intense peptide ions with charge states greater or equal to two were isolated at a minimum threshold value of 5000 from the assigned target list. A dynamic exclusion of two repeats over 30 seconds with an exclusion duration of 45 seconds was set.

Data mining

Data from Thermo Xcalibur (Thermo Scientific) were imported into Proteome Discoverer version 1.3 (Thermo Fisher Scientific, West Palm Beach, FL, USA,) and Mascot v.2.3.01 (Matrix Science, London, UK). Data were imported with the following settings. Retention time limits between eight and 60 minutes. Lowest ion charge state was set as 2 and highest charge state at 6 for those ions most likely to represent peptides.

A MASCOT search was carried out on the latest version of the NCBI human databases (National Centre for Biotechnology Information, Bethesda US) [13]. The identity of the target peptides was confirmed using the fragment information from the tandem MS in combination with matching retention time and m/z using the high resolution/accurate mass capabilities of the Orbitrap mass spectrometer. The characteristics of the peptides in the target lists, namely m/z , retention time, charge, and mass were compared against those from the Proteome Discoverer searches to identify matches within the databases. The fragmentation patterns from Proteome Discoverer and Genedata Analyst were compared to validate that the protein identifications were legitimate. Data were presented according to the CONSORT guidelines for reporting a RCT and the Paris guidelines for the presentation of proteomic data [14,15].

Validation of Complement C3 was conducted on the individual samples at all the time points using the Complement C3 human enzyme-linked immunosorbent assay (ELISA) kit (Abcam) According to the manufacturer's directions. A Student's t -test was performed at each time point to identify statistical differences between the groups with $p < 0.05$ considered statistically significant.

Results

Participant flow, through the enrolment, allocation and analysis phases of the trial is detailed in Fig. 1. There were no reported adverse events resulting from the RIPC or blood sampling protocols. There was no mortality.

Demographic data are presented in Table 1. With the exception of slightly lower preoperative saturations in the RIPC group, there were no differences in the baseline demographics between the two groups in terms of gender, weight, age, bypass time, total aortic cross clamp time or bypass circuit prime volume.

There were 335 964 clusters resulting from the MS analysis, of which 96 725 were selected for analysis based on a positive charge state ≥ 2 . This criterion was chosen to select those clusters most likely to represent peptides.

The results of the comparison between the groups at each time point are shown as Venn diagrams in Fig. 3. The results demonstrated that there were no peptides with significant differential expression between the RIPC and control groups at baseline or immediately after CPB. At 6 h after CPB, 48 peptides were significantly differentially expressed with all 48 peptides

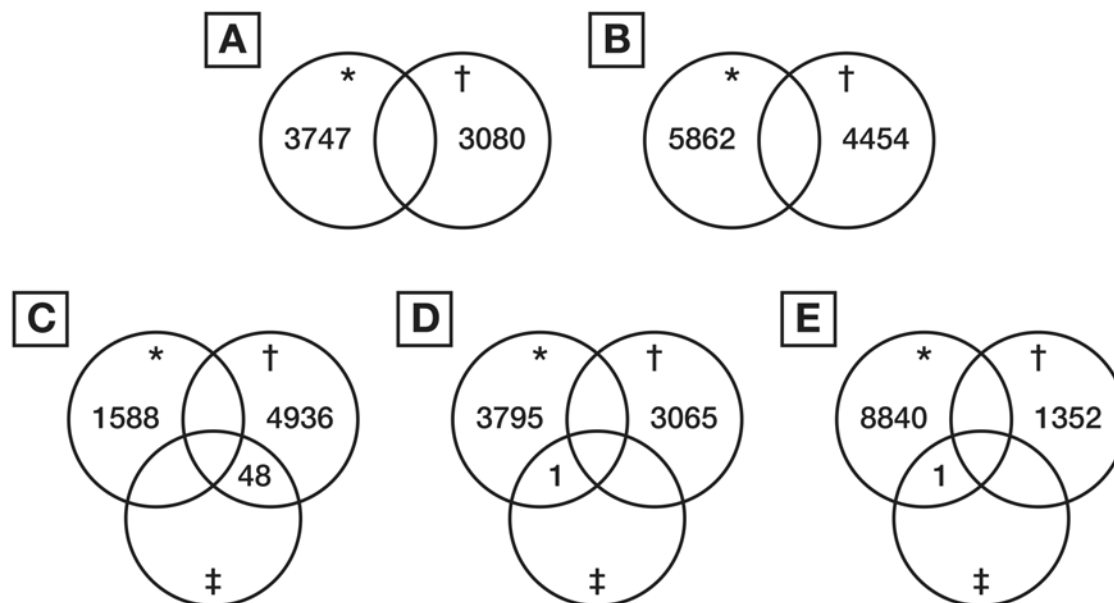


Fig 3. Venn diagram detailing the number of differentially expressed peptides in RIPC as compared to control samples at each time point. A. Baseline. **B.** End CPB. **C.** 6 hours post CPB sample. **D.** 12 hours post CPB. **E.** 24 h post CPB. *—down-regulated, †—up-regulated, ‡—significantly differentially expressed.

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being up regulated in the RIPC group. The response was less pronounced at 12 and 24 h after CPB, with only one peptide down regulated in the RIPC group compared to the control group at each time point.

The expression patterns for 48 peptides found to be up-regulated in the RIPC group at the 6 h time point are shown in [Fig 4](#).

These 48 peptides were matched to 6 proteins in the NCBI database as detailed in [Table 2](#). At 12 h post CPB and 24 h post CPB there was one down-regulated peptide, however we were unable to match these peptides to proteins with sufficient confidence.

The results of the validation study for human Complement C3 ([Fig 5](#)) confirmed significantly higher Complement C3 in the RIPC group at 6 h after CPB ($p = 0.048$). No difference was found between the groups at any other time point.

Discussion

RIPC is an intriguing phenomenon that has been applied successfully across a range of clinical settings. Although the mechanisms through which RIPC confers protection have not been fully elucidated, there is evidence that RIPC induces circulating humoral mediators [16,17]. We have previously demonstrated that RIPC induces global genomic changes in healthy adult volunteers and in children undergoing heart surgery [18,19]. We have also previously shown that RIPC modifies the proteomic profile in the plasma of healthy adult volunteers and gained the first clinical insights into the proteins that may be altered during the RIPC stimulus and at 15 min and 24 h after applying the RIPC stimulus [13]. We therefore extended this concept into the clinical setting to test the hypothesis that RIPC modifies the plasma proteomic response in children undergoing repair of Tetralogy of Fallot (TOF). This is the first study to describe the global proteomic response to RIPC in the plasma of children with congenital heart disease undergoing heart surgery. We have identified potential candidate proteins involved in the RIPC mechanism that require confirmation with further studies.

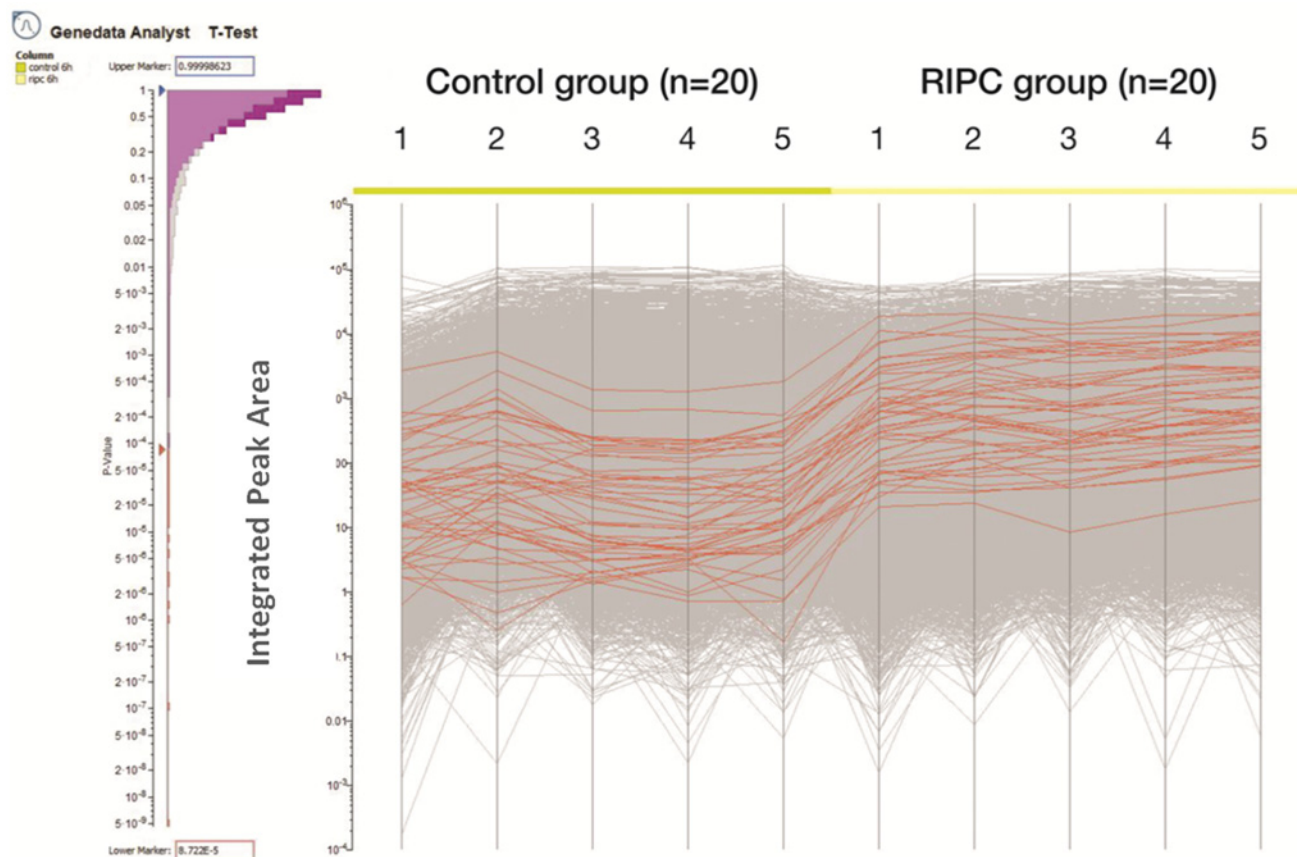


Fig 4. Integrated peak area of the peptides in the 5 pooled samples in each group. Expression of each peptide is depicted as a line with the lines in red each representing the significantly higher expressed peptides in the RIPC group compared to the control group. There were 48 up-regulated peptides in the RIPC group compared to the control group. The histogram on the left indicates the false discovery rate.

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There were no differences in clinical outcomes between the RIPC group and the control group [10], which may be attributed to the fact that chronic hypoxia may already activate the pathways involved in RIPC, so that further augmentation is not possible. This finding is supported by evidence suggesting that RIPC has little effect on the phosphorylation levels of pro-survival protein pathways that are involved in RIPC from the resected right ventricular outflow tract tissue of these patients [10]. Our results are in contrast to studies on the effect of ischemic post conditioning via invasive aortic clamping and de-clamping following reperfusion which showed clinical benefit including decreased morbidity, reduced duration of mechanical ventilation, intensive care unit stay and inotrope requirement in patients undergoing ToF repair

Table 2. Protein data for the 6 proteins that were up-regulated in RIPC group as compared to the control group at 6 hours after CPB.

Protein	Accession number	MASCOT Score	Peak integration area Control	Peak integration area RIPC	p value
Inter-alpha globulin inhibitor	55958063	9896.84	42.0 ± 11.8	820.8 ± 181.1	0.006
Fibrinogen preproprotein	11761629	22554	59.3 ± 11.2	1192.6 ± 278.3	0.007
Complement C3 precursor	115298678	52035	391.2 ± 160.9	5385.1 ± 689.4	0.0005
Complement C4B	310125237	76.5	151.5 ± 17.8	4587.8 ± 799.2	0.003
Apolipoprotein B100	306569733	542.04	53.4 ± 8.3	1364.5 ± 278.2	0.005
Urinary proteinase inhibitor	262428	45	358.6 ± 74.9	5758.1 ± 1343.1	0.009

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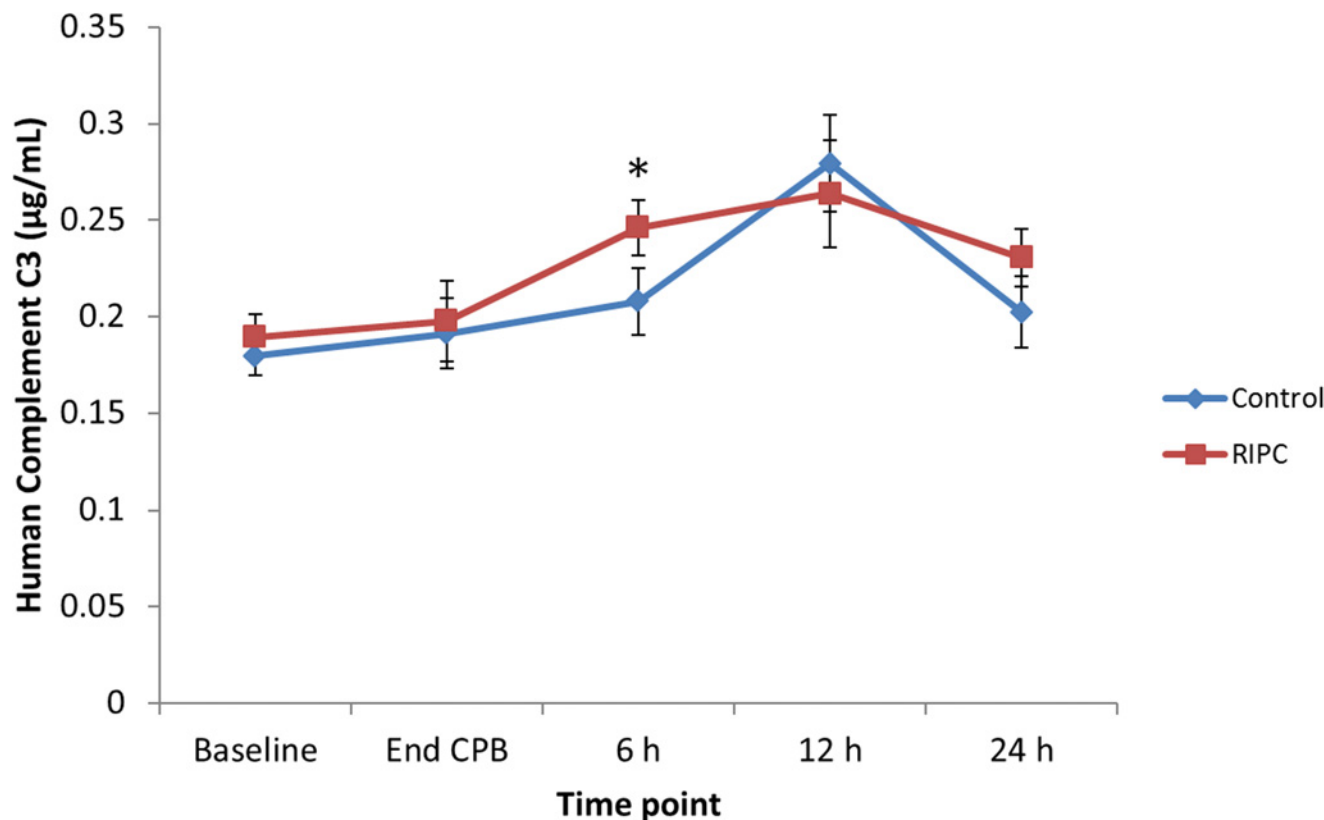


Fig 5. Complement C3 concentration for the control and RIPC groups at the time points in the study. Individual samples were assessed in this ELISA. There was significantly higher levels of Complement C3 in the RIPC group at 6 h after CPB ($p < 0.05$).

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[20–22]. Thus, clinical application of the RIPC and its variants still remains a controversial issue and further mechanistic insights are required.

In the current study, the most pronounced proteomic changes between control and RIPC groups occurred at 6 hours after CPB. The 48 peptides with significant higher levels in the RIPC group at 6 hours after CPB were matched to 6 proteins. Four of these 6 proteins were previously found to be differentially expressed in our study of the effect of the RIPC stimulus in healthy adult volunteers [13]. These 4 proteins were inter-alpha globulin inhibitor, fibrinogen preproprotein, complement C4B and apolipoprotein B. These findings consistent with our previous study suggest that there is a common mechanism of protection by RIPC. How these proteins could be linked to the RIPC specific protection is beyond the scope of this study and warrants further investigation. The functions of these proteins have previously been described and are as follows.

The protease inhibitors in blood control proteolysis during complement activation, coagulation, fibrinolysis and inflammation [23]. Inter-alpha globulin inhibitor is an acute phase protein known to be involved in inflammation. It increases in concentration in response to tissue injury. We found up-regulation of inter alpha globulin inhibitor during the RIPC and at 15 min after the RIPC and it is down-regulation at 24 h after the RIPC in our previous study in healthy adult volunteers [13]. This finding of early up-regulation has since been confirmed by Pang *et al.* [24] who reported a greater than 2-fold increase at 1 hour post RIPC stimulus in 60 healthy adult volunteers. In the current study, we found increased levels of inter-alpha globulin inhibitor at 6 h after the CPB, but not at the 12 h or 24 h after CPB. These findings suggested that the inter-alpha globulin inhibitor is involved in the early response to the RIPC stimulus,

but returns to the baseline within the first 24 hours. Inter-alpha globulin inhibitor regulates activation and adhesion of leucocytes during inflammation and it also has protease inhibitory activity [25]. Inter-alpha globulin inhibitor is also known to render tissue more resistant to damage from oxygen derived free radicals [26]. Higher levels in the RIPC group may indicate that RIPC-induced protection against oxidative stress.

Fibrinogen preproprotein has previously been demonstrated as being anti-inflammatory in local ischemic preconditioning during acute pancreatitis in rats [27]. The cleavage products of fibrinogen regulate cell adhesion and migration, have vasoconstrictor and chemotactic activities and are mitogens for several cell types [27]. Increased levels of fibrinogen preproprotein at 6 h post CPB may imply decreased cleavage products of fibrinogen as there were higher levels of its precursor. Thus, higher levels of fibrinogen may reflect decreased plasma protease activity and, as such, decreased activation of leucocytes. Furthermore, preconditioning ischemia attenuates platelet activation and aggregation characterized by a decrease in platelets aggregating with fibrinogen [28]. Thus, an anti-inflammatory property of fibrinogen preproprotein in clinical surgery deserves further investigation.

Similarly, the complement C3 precursor was found to be up-regulated at 6 h after CPB. This was confirmed by ELISA. In our preclinical study, we found complement C3 to be up regulated at 15 min post RIPC stimulus [13]. Complement factors play a key role in the inflammatory process. When cleaved, complement C3 precursor causes contraction of smooth muscle, increases vascular permeability and chemotaxis and causes histamine release from mast cells and basophilic leukocytes [29,30]. Complement C3 precursor has been implicated in a number of cellular pathways including the phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), protein kinase B (Akt) and mitogen activated protein kinase /extracellular signal- regulated kinases (MAPK/ERK) pathways, which have also been implicated in RIPC [31].

Complement C4B is important in controlling inflammation, where deficiency and low serum levels are associated with increased susceptibility to inflammation, including systemic lupus erythematosus [32,33]. Complement C4B has anti-inflammatory properties [34]. Increased levels of this protein in the RIPC group 6 h after CPB suggest that RIPC could have an anti-inflammatory effect. This suggests a role for involvement of the complement system in response to RIPC.

Apolipoprotein B levels were high in the RIPC group at 6 hours after CPB in the current study. The precise role of Apolipoprotein B in RIPC is unknown. We have shown apolipoprotein expression to be increased in adults in response to the RIPC stimulus [13]. Furthermore, subsequent studies have confirmed the upregulation of apolipoproteins in response to RIPC in an animal model [35] and have also shown that administration of apolipoprotein prior to prolonged coronary artery occlusion resulted in decreased myocardial infarct area that was as effective as RIPC [36].

Finally, increased level of urinary proteinase in the RIPC groups was an interesting observation. These inter alpha globulin inhibitors are degraded by neutrophils to form urinary proteinase inhibitors [37]. Urinary proteinase inhibitor confers anti-inflammatory effects by decreasing expression of pro-inflammatory cytokines, including TNF- α and interleukin 6, and chemokines [38]. The urinary proteinase inhibitors decrease lipopolysaccharide induced neutrophil activation [38] and have been used therapeutically for patients with inflammatory disease [39]. Administration of exogenous urinary proteinase inhibitors warrants testing in an animal model.

This is the first examination of RIPC induced proteomic changes in the plasma of children with cyanotic heart defects. The results of this study indicate that the RIPC stimulus in children undergoing ToF repair evokes a global proteomic response that peaks at 6 h post-CPB and returns to baseline within the first 24 h. This study provides preliminary insights into the proteins involved in RIPC mediated cellular protection. The observed proteomic changes are consistent with our previous observation in healthy volunteers and may play an integral role in the response to RIPC.

Limitations

Removal of albumin and Ig-G from plasma allowed for a more thorough analysis of the remaining proteins. This approach did, however, come at a sacrifice as other proteins with an isoelectric point between 5.2 and 6.8 were excluded from the analysis. There may be proteins that we excluded from our analysis that may be important in RIPC associated mechanisms that were not analysed in our study due to the fractionation process that we used.

This study was limited to measuring relative changes in the levels of peptides between the two groups at the various time points and as such involved denaturation and trypsin digestion of the proteins into peptides, therefore, we were unable to assess post translational modifications such as phosphorylation.

Anaesthetic drugs may impact on RIPC responses [40,41], and differentiating the impact of the drugs from RIPC can only be achieved through RCT's in which the anaesthetic techniques are identical and the only variable is the presence or absence of RIPC. Whether the RIPC leads to clinically important improvements in outcome, will then need to be considered across the range of surgeries and anaesthetics. This study was not powered to detect differences in echocardiogram assessment of diastolic dysfunction.

Conclusion

We provided the first comprehensive analysis of RIPC-induced proteomic changes in children undergoing heart surgery. The proteomic changes peak at 6 h post-CPB and return to baseline within the first 24 h of surgery.

Supporting Information

S1 CONSORT Checklist. CONSORT Checklist.
(DOC)

S1 Protocol. Trial Protocol.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: MH VI SB CA VK IEK PM YD. Performed the experiments: MH SB CA VK. Analyzed the data: MH VI SB CA VK YD PM IEK. Contributed reagents/materials/analysis tools: MH SB VI CA VK PM IEK YD. Wrote the paper: MH SB VI CA VK PM IEK YD.

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Platelets from children are hyper-responsive to activation by thrombin receptor activator peptide and adenosine diphosphate compared to platelets from adults

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Summary

Platelets are crucial subcellular elements of haemostasis at sites of vascular injury and are also known to be immune mediators in pathological thrombosis. Despite the integral role of platelets in many disease processes, there is very little information available on platelet function and response to agonists in healthy children. We recently reported important differences in the interaction of platelets with monocytes in the circulation, including increased formation of monocyte-platelet aggregates (MPAs) without concomitant increase in P-selectin expression. Our current study investigates parameters of platelet activation (PAC-1 binding and P-selectin expression) and MPA formation in response to a range of physiologically relevant platelet agonists in healthy children compared to healthy adults. All parameters were significantly higher in children in response to sub-maximal concentrations of thrombin receptor activator peptide and adenosine diphosphate, reflecting an age-specific difference in agonist-stimulated platelet reactivity in children. The results of our study challenge the general assumption that platelet reactivity in children is similar to adults. This finding is fundamental to investigating the role of platelets in diseases of childhood and pathogenesis of adult-based diseases that have their origins in childhood. Our findings underscore the need for age-specific reference ranges for platelet function in children rather than extrapolation from adult data.

Keywords: children, monocyte-platelet aggregates, platelet activation, agonists, flow cytometry.

Platelets are crucial protective cellular elements of haemostasis at sites of vascular injury and are also known to be immune mediators in pathological thrombosis. Despite the integral role of platelets in important physiological processes, such as thrombosis, inflammation and wound repair, knowledge of age-specific changes in platelet function is limited when compared to the reports of developmental changes in haemostatic proteins (Andrew *et al*, 1992; Monagle *et al*, 2006; Strauss *et al*, 2011; Attard *et al*, 2013). Such knowledge is critical in the management of sick children.

Previous studies assessing the physiological differences in platelet physiology and function have focused on differences between cord blood and adult peripheral blood (Rajasekhar *et al*, 1994, 1997; Kuhne *et al*, 1996; Simak *et al*, 1999; Sitaru *et al*, 2005). However, conclusions inferred from these

studies are hampered by multiple factors, including different sample types (cord blood *versus* peripheral blood) and the varying testing protocols used. Specific contradictions in results included the report of comparable expression of glycoprotein (GP)IIb/IIIa (also termed ITGAB), on resting platelets of healthy neonates to adults (Rajasekhar *et al*, 1994), whilst others reported lower expression of GPIIb/IIIa on neonatal platelets (Hezard *et al*, 2003). In addition, whilst some studies reported hyporeactivity of neonatal platelets to specific *in vitro* agonist stimuli that rapidly normalized to adult levels within first year of life (Rajasekhar *et al*, 1994; Gatti *et al*, 1996; Pietrucha *et al*, 2001), others reported persistent platelet hyporeactivity and lower GPIIb/IIIa expression on resting and activated platelets in older children until the age of 15 years (Hezard *et al*, 2003). This demonstrated that

platelet studies with standardized protocols are needed in order to inform the role of platelets and enable multiple studies comparison (Schmitz *et al*, 1998).

There are multiple overlapping pathways that contribute to platelet activation. Platelets are activated by thrombin or thrombin receptor activator peptide (TRAP), which is a potent and direct platelet activator, signalling through protease-activated receptors. Endogenous arachidonic acid (AA) liberated from platelet phospholipids is metabolized through a cyclo-oxygenase-1 (COX1) dependent pathway to produce thromboxane A₂, which propagates platelet activation through the thromboxane prostanoid receptor (Angiolillo *et al*, 2010). With dense granule exocytosis, mediators such as adenosine diphosphate (ADP), are released *in situ*, further propagating platelet activation and recruitment through P2Y receptors. Where sufficient platelet activation signalling occurs, the GPIIb/IIIa receptor undergoes a conformational change from a low affinity to a high affinity state, facilitating platelet aggregation that leads to the formation of a platelet-rich thrombus. Where granule exocytosis occurs, P-selectin expressed on the platelet surface facilitates the formation of monocyte-platelet aggregates (MPAs). Antiplatelet agents, such as aspirin and clopidogrel inhibit AA and ADP-stimulated platelet activation pathway, respectively. Reduction in MPAs in patients after receiving antiplatelet treatments has been reported (May *et al*, 1997; Braun *et al*, 2008). Therefore, in order to assess and characterize platelet response, each of these pathways must be separately investigated using specific physiologically relevant agonists.

We recently reported that circulating MPA are elevated in healthy children compared to adults (Yip *et al*, 2013). To date, there is limited data of *in vitro* platelet response to various agonists at sub-maximal concentrations in healthy children. The use of sub-maximal rather than supra-maximal agonist concentration enables a sensitive determination and differentiation of inter-individual differences in platelet reactivity. Our current study aimed to characterize platelet activation (as measured by PAC-1 [first procaspase activating compound] binding to GPIIb/IIIa in its high affinity state), granule exocytosis (as measured by platelet surface P-selectin expression) and MPA formation in response to sub-maximal concentrations of TRAP, ADP and AA in platelets from children compared to adults.

Materials and methods

Blood collection

Whole blood samples were obtained from healthy children and adults according to a previously established protocol and selection criteria (Ignjatovic *et al*, 2006, 2007; Monagle *et al*, 2006; Attard *et al*, 2013). All blood samples were collected and stored in one volume of 0.106 mol/l citrate per nine volumes of blood. Paediatric samples were collected from healthy children attending the hospital for minor elective

surgery who did not have a family history of coagulation disorders. Participants had not received any antiplatelet medication within 10 d of blood draw. Family history was assessed via a brief interview with the parents of the children and the adult volunteers themselves. In order to minimize pre-analytical variables, cannula needle gauge and blood handling processing time (period from blood collection to initiation of testing) were identical for children and adults. This study was approved by the Melbourne Royal Children's Hospital Ethics in Human Research Committee, reference number 20031. Informed consent was obtained from the parents of the children and from the adult participants themselves.

Determination of sub-maximal concentration of agonists

Sub-maximal concentration of agonist is defined as the concentration of an agonist that triggers at least three-fold response higher than its baseline (Panzer *et al*, 2006). For each agonist, platelet reactivity ($n = 3$ each for children and adults) was tested with serial diluted concentration of agonists. As wide individual variability was observed in the preliminary experiments, two concentrations of agonists ('high' and 'low') were selected for subsequent testing in adult and paediatric samples. TRAP (Castle Hills, NSW, Australia) and ADP (Chronolog, Havertown, PA, USA) dilutions were prepared fresh or from frozen aliquots according to the manufacturer's instructions. AA (Chronolog) was prepared, stored at -80°C and used within 10 min of thawing.

Detection of MPA formation in response to agonists

Flow cytometric determination of MPA was performed according to a previously described protocol (Linden, 2013; Yip *et al*, 2013). Briefly, within 20 min of collection, whole blood was incubated with an antibody cocktail containing 1.0 $\mu\text{g/ml}$ fluorescein isothiocyanate (FITC)-conjugated anti-CD42a monoclonal antibody (clone ALMA.16, Becton Dickinson, San Diego, CA, USA) or 1.67 $\mu\text{g/ml}$ FITC conjugated mouse IgG1 κ isotypic control and 0.5 $\mu\text{g/ml}$ phycoerythrin-cyanin 5.1 (PC5)-conjugated anti-CD14 monoclonal antibody (clone RMO52, Beckman Coulter Immunotech, Marseille, France) with predetermined concentrations of TRAP, ADP and AA. All reactions were carried out at room temperature except for AA, which was incubated at 37°C . After a 15-min incubation, the reaction was stopped with 800 μl of FACS Lysing solution (Becton Dickinson) and stored at $2-8^{\circ}\text{C}$ in the dark until analysis.

Detection of surface expression of P-selectin and PAC-1 binding in response to agonists

Flow cytometric measurements of PAC-1 binding and P-selectin expression were performed according to a previously described protocol (Linden, 2013). Briefly, whole blood was diluted 1:5 with HEPES saline (10 mmol/l HEPES, 0.15 mol/l

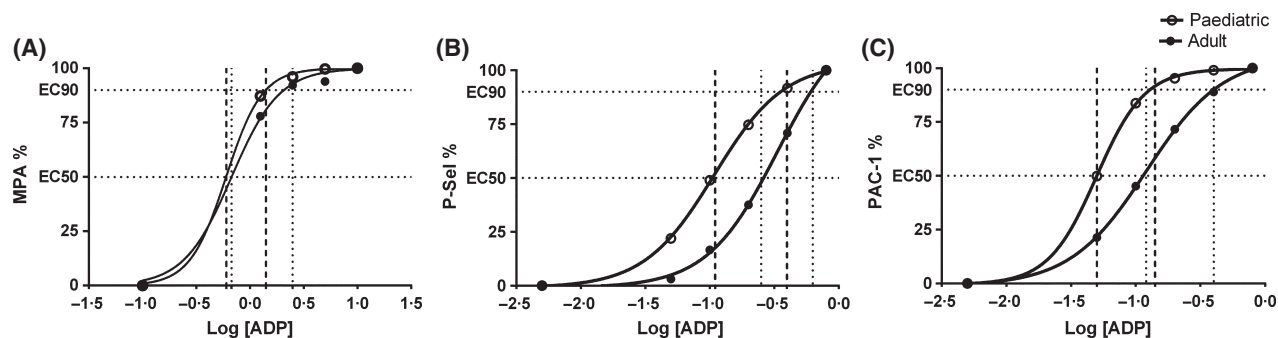


Fig 1. A representation of normalized logarithmic concentration-response curves for ADP. Open circles indicate the paediatric sample and filled circles indicate the adult sample. (A) Effective concentration giving 50% (EC50) and 90% (EC90) positive for monocyte-platelet aggregate (MPA) in the paediatric sample are 0.60 $\mu\text{mol/l}$ and 1.41 $\mu\text{mol/l}$; EC50 and EC90 in the adult sample are 0.67 $\mu\text{mol/l}$ and 2.50 $\mu\text{mol/l}$. (B) EC50 and EC90 for P-selectin (P-Sel) for the paediatric sample are 0.11 $\mu\text{mol/l}$ and 0.40 $\mu\text{mol/l}$; EC50 and EC90 for the adult sample are 0.25 $\mu\text{mol/l}$ and 0.63 $\mu\text{mol/l}$. (C) EC50 and EC90 for PAC-1 binding for children are 0.05 $\mu\text{mol/l}$ and 0.14 $\mu\text{mol/l}$, EC50 and EC90 for adults are 0.12 $\mu\text{mol/l}$ and 0.40 $\mu\text{mol/l}$. MPA, monocyte-platelet aggregate; PAC-1, first procaspase activating compound; TRAP, thrombin receptor activator peptide; ADP, adenosine diphosphate; AA, arachidonic acid.

NaCl, pH 7.3–7.4) and incubated with an antibody cocktail containing 1.0 $\mu\text{g/ml}$ PC5-conjugated anti-CD42b monoclonal antibody (clone HIP1, Becton Dickinson Pharmingen, San Jose, CA, USA), 6.25 $\mu\text{g/ml}$ FITC conjugated PAC-1 (Becton Dickinson) with or without blockade by 5 $\mu\text{g/ml}$ eptifibatide (Schering-Plough, Whitehouse Station, NJ, USA), and phycoerythrin (PE)-conjugated anti-CD62P monoclonal antibody (clone AK4, Becton Dickinson) or 1 $\mu\text{g/ml}$ PE-conjugated mouse IgG1 κ isotypic control (clone MOPC-21, Becton Dickinson). The reactions were carried out with predetermined concentration of TRAP and ADP at room temperature and AA at 37°C. The reaction was stopped with addition of 800 μl of 1% formaldehyde (Sigma Aldrich, St. Louis, MO, USA) in HEPES-Saline.

Flow cytometry analysis

Samples were analysed on the BD FACS Calibur or BD LSR II flow cytometers using 488 nm excitation. FITC emission was collected with a 530/30 nm bandpass (BP) filter, PE with 585/42 nm BP and PC5 with a 670 longpass (LP) filter. Photomultiplier tube voltages were determined using unstained controls and calibrated for each experiment using eight peak rainbow calibration particles. Compensation was performed using single stained controls. A minimum of 1000 monocytes in whole blood was identified by characteristic laser scatter and differential expression of CD14. Monocyte-platelet aggregates were identified by co-expression of CD14 and CD42a on a monocyte event, using an isotype control to determine gates. A minimum of 10 000 single platelets in whole blood were identified by characteristic log laser scatter and expression of CD42b. PAC-1 binding or CD62P expression was measured relative to small molecule blocked or isotype control, respectively.

Statistical analysis

Statistical analysis was performed using GRAPHPAD PRISM version 6 (GraphPad Software Incorporation, San Diego, CA,

USA). Comparison of platelet reactivity between children and adults were analysed using the Mann–Whitney's *U* test where *P*-values of <0.05 were considered as significant. Spearman's correlation was performed to explore potential correlation of age and platelet reactivity as well as influence of pre-existent platelet reactivity to subsequent *in vitro* platelet activation. The variables were considered well correlated when the coefficient was above 0.4 ($n = 22$) and above 0.6 ($n = 10$), estimated from Spearman's Rho table.

Results

Blood samples were obtained from 22 healthy children (median age 8 years; range 1–14 years of age) and 10 healthy adults (median age 32 years; range 22–43 years of age).

Sub-maximal concentration of agonists used for platelet stimulation

Representative concentration-response curves for ADP are shown in Fig 1. The final concentrations of agonists determined

Table I. Final concentrations of agonists used to induce MPA formation and platelet response.

	MPA Assay (Final concentrations, $\mu\text{mol/l}$)		P-selectin/PAC-1 Assay (Final concentrations, $\mu\text{mol/l}$)	
	High	Low	High	Low
TRAP	5.0	1.25	2.5	1.25
ADP	5.0	1.25	0.1	0.05
AA	500	62.5	500	125

MPA, monocyte-platelet aggregate; PAC-1, first procaspase activating compound; TRAP, thrombin receptor activator peptide; ADP, adenosine diphosphate; AA, arachidonic acid.

to be appropriate for the main experiments are summarized in Table I. Effective concentration (EC) of ADP that gives 50% and 90% of platelet reactivity in children and adults are indicated in Fig 1.

Monocyte-platelet aggregate formation and surface expression of P-selectin and PAC-1 binding in response to agonists

Formation of MPAs was higher upon activation by both high and low concentrations of TRAP, ADP and low concentration of AA in children compared to adults (Fig 2A). Platelet surface expressions of P-selectin activated by TRAP and ADP were higher in children compared to adults, whilst there was no difference in response to AA (Fig 2B). PAC-1 binding of platelets activated by TRAP, ADP and high concentration of AA was increased in children compared to adults (Fig 2C).

Correlation analysis of pre-existent or circulating MPA with *in vitro* agonist-induced platelet activation

Analysis of the effect of pre-existent platelet activation on subsequent platelet response to agonists demonstrated a significant correlation between the pre-existent MPA levels with *in vitro* agonists-stimulated MPA formation in children (Fig 3A–C). This trend was not observed in adults (Fig 3D–F).

Correlation analysis of age and agonist-induced changes in MPA formation and platelet reactivity

There was an inverse correlation between age and agonist-stimulated MPA formation in children for TRAP, ADP and AA at high concentrations, with the correlation being statistically significant for TRAP-stimulated MPA formation (Fig 4A). On the other hand, there were no statistically significant correlations between age and agonist-stimulated MPA in adults (Fig 4D–F).

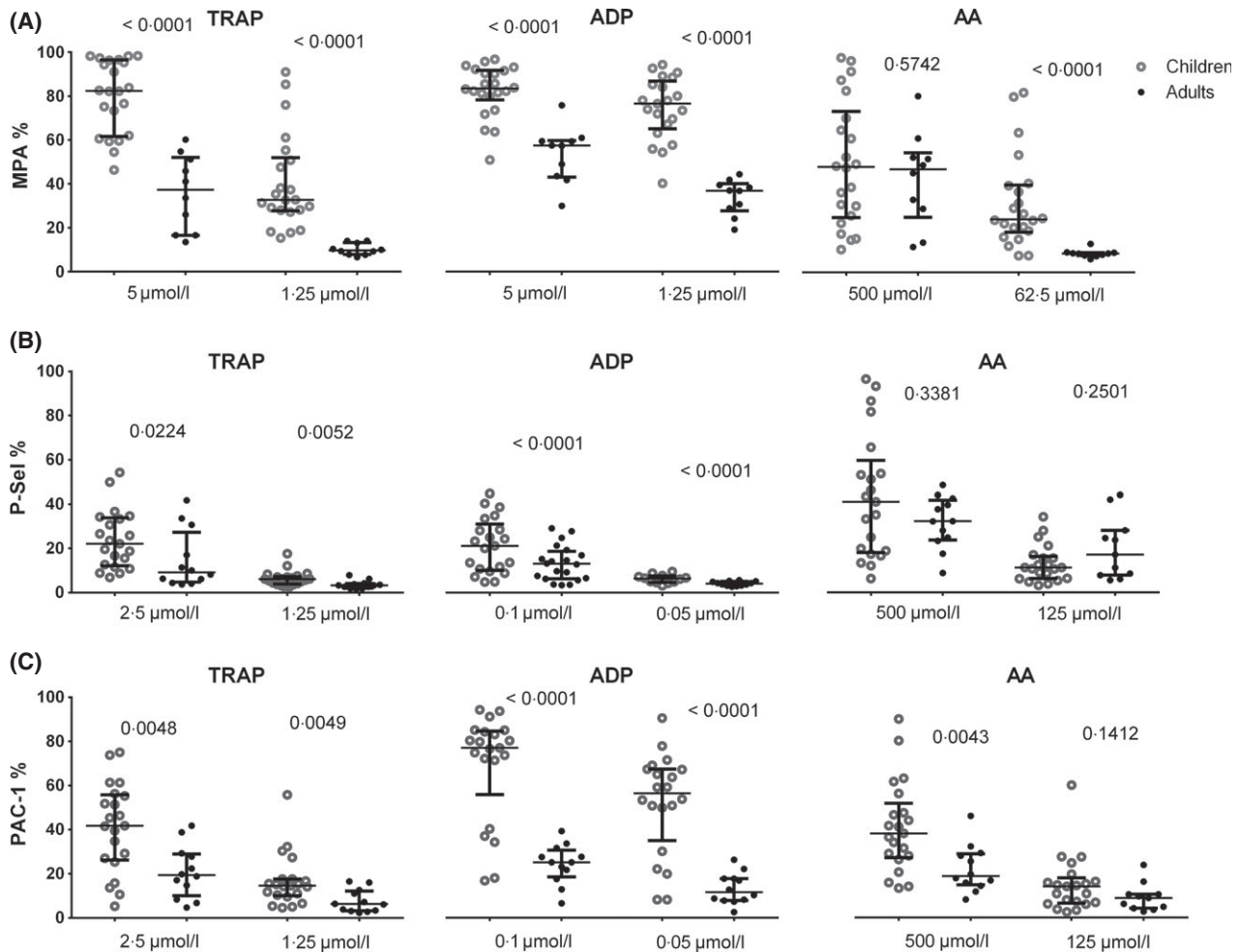


Fig 2. Comparison of MPA formation, platelet surface P-selectin expression and PAC-1 binding between children and adults in the presence of TRAP, ADP and AA. The median and inter-quartile range for percentage MPA formation (A), P-selectin (B) and PAC-1 (C) in response to sub-maximal concentrations of TRAP, ADP and AA are shown. The comparison was performed using Mann–Whitney *U* test and *P*-values are indicated in each figure respectively. MPA, monocyte-platelet aggregate; P-Sel, P-selectin; PAC-1, first procaspase activating compound; TRAP, thrombin receptor activator peptide; ADP, adenosine diphosphate; AA, arachidonic acid.

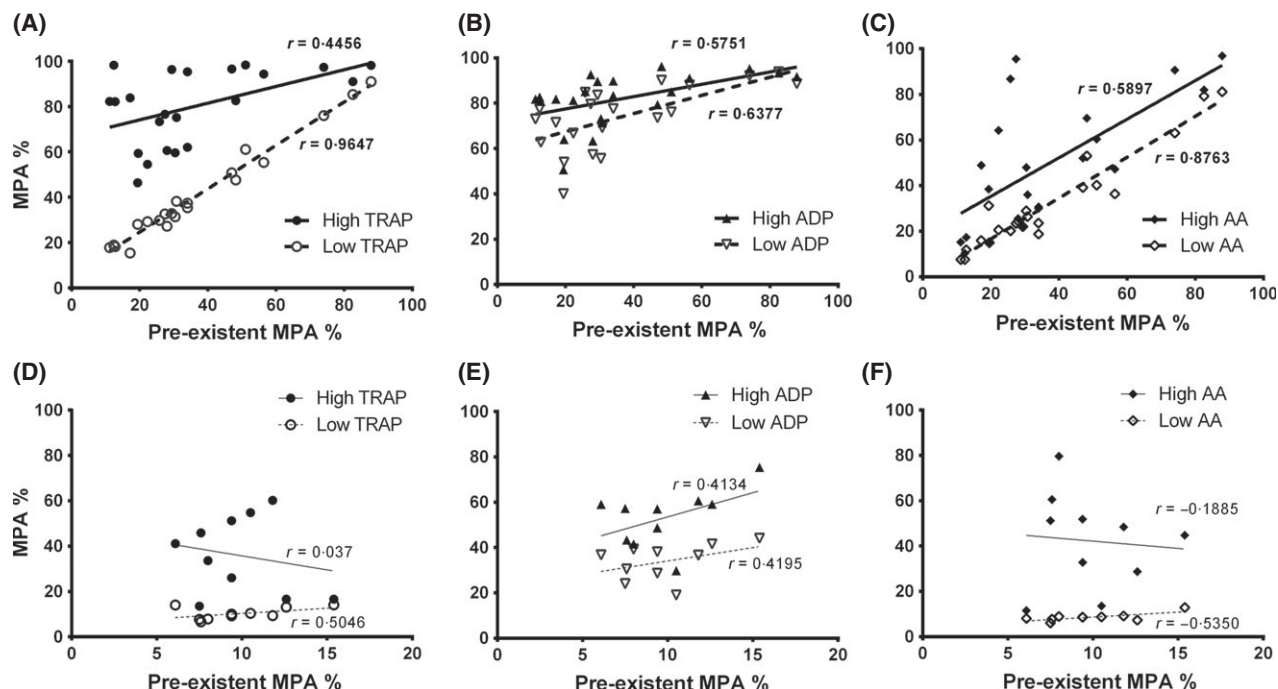


Fig 3. Increased agonist-stimulated MPA formation in children correlates significantly with pre-existent MPA. The correlation between pre-existent percentage of MPA and agonist-stimulated MPA in children (A–C) and adults (D–F) are indicated. The correlation lines of pre-existent MPA and the level of MPA when stimulated by high (solid lines) and low (dotted lines) concentration of agonists. Spearman's correlation coefficients for each line are indicated in the figures and significant correlation lines are indicated as bold print face. Significant correlations ($P < 0.05$) were found in children ($n = 22$) but not in adults ($n = 10$). MPA, monocyte-platelet aggregate; TRAP, thrombin receptor activator peptide; ADP, adenosine diphosphate; AA, arachidonic acid.

Discussion

Developmental primary haemostasis is an important area of research that has specific implications in the diagnosis and treatment of platelet-related disorders in children. Our objective was to determine if there is any age-dependent change in platelet physiology. This is the first study to measure platelet reactivity using flow cytometry, including *in vitro* agonist-stimulated MPA formation, in healthy children. The results indicate that platelets from children respond to lower threshold concentrations [lower 50% effective concentration (EC₅₀)] of agonists when compared to adults. Platelet hyper-responsiveness in children, as measured by P-selectin expression, PAC-1 binding, and MPA formation, was observed across all of the platelet agonists tested in this study, although the differences were not statistically significant in the high concentration of AA for MPA formation and low concentration of AA for PAC-1 binding (Fig 2). This is in contrast to an earlier study (Hezard *et al*, 2003), which reported platelet hyporeactivity, as measured by P-selectin and PAC-1 binding, in children that persisted beyond the neonatal period. However, our strategy was different from the experimental approach used by Hezard *et al* (2003). We measured the percentage of P-selectin and PAC-1 using sub-maximal concentrations of TRAP (final concentration 2.5 $\mu\text{mol/l}$ and 1.25 $\mu\text{mol/l}$) as opposed to the supra-maximal

concentration (60 $\mu\text{mol/l}$ of TRAP) used by Hezard *et al* (2003). Furthermore, unlike Hezard *et al* (2003) who reported lower expression of GPIIb/IIIa in children, we did not measure the absolute number of platelets with glycoprotein expression. Instead, we reported the proportion of platelets expressing P-selectin and PAC-1 binding, which were higher when stimulated with platelet agonists.

Consistent with previous studies of agonist-induced P-selectin expression and PAC-1 binding in healthy adults (Hezard *et al*, 2003; Panzer *et al*, 2006), we observed a wide inter-individual variability. Pre-analytical sampling variation can contribute to inter-individual variability. However, in our study this was carefully excluded as we standardized the blood collection methods and followed the exact time duration from sample collection to test initiation. Genetic variability, including polymorphisms in loci involved in platelet function can also contribute to inter-individual variability in platelet function (Johnson *et al*, 2010; Guerrero *et al*, 2011). Genome-wide association studies reported that specific variants, such as LPAR1 and MYO5B, are associated with increased platelet reactivity among healthy children (Guerrero *et al*, 2011). Children might be expected to have lower variability in parameters of platelet function than adults, given the greater occurrence of confounders, such as acquired and environmental factors (e.g., smoking, hormone intake and other clinically

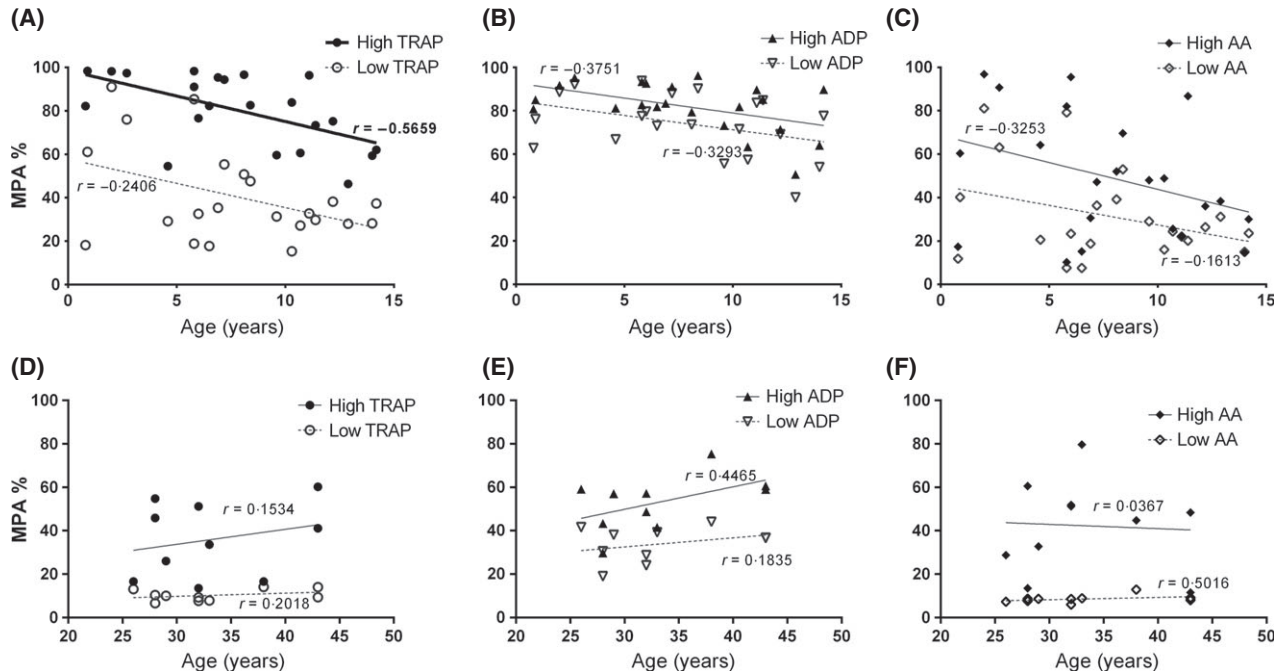


Fig 4. Correlation between age and agonist-stimulated MPA. The correlation between age and agonist-stimulated MPA in children (A–C) and adults (D–F) are indicated. The solid and dotted lines represent correlations of age of participants to MPA as stimulated by high and low concentration of agonists respectively. Spearman's correlation coefficients for each line are indicated in the figures and significant correlation lines are indicated as bold print face. A significant inverse correlation was observed in high concentration of TRAP-stimulated MPA in children (Spearman's correlation, $r = -0.5659$, $n = 22$, $P < 0.05$). MPA, monocyte-platelet aggregate; TRAP, thrombin receptor activator peptide; ADP, adenosine diphosphate; AA, arachidonic acid.

silent co-morbid conditions) that affect platelet function in adults.

Despite inter-individual variability, the circulating (i.e. unstimulated) MPA levels in children correlated significantly with platelet response following *in vitro* agonist stimulation (Fig 3). This finding suggests that pre-existent platelet reactivity may predict the difference in sensitivity to various platelet activation pathways in children. Moreover, another important finding of this study is that agonist-stimulated MPA formation in children inversely correlates with age (Fig 4A). While this might suggest the potential for an age-dependent sensitivity to antiplatelet agents, there is limited other evidence to support this and further investigation is warranted. Findings from the Platelet Inhibition in Children On cLOpidogrel (PICOLO) trial determined that clopidogrel at a dose of 0.20 mg/kg/d in children (0–24 months) was sufficient to achieve a platelet aggregation inhibition level similar to that in adults taking 1 mg/kg/d or 70 mg/d (Li *et al*, 2008). Another single-centre retrospective study evaluating the safety and efficacy of clopidogrel showed that children (mean age 4.9 years old) were sufficiently protected when administered at 0.1–0.7 mg/kg/d as compared to typical adult dose of 1 mg/kg/d (Mertens *et al*, 2008). These reports highlighted the need for understanding the potential difference in pharmacokinetic and platelet physiological in children compared to

adults. While platelet activation markers as measured by flow cytometry are not used in routine clinical practice, it provides important molecular information about the mechanism of platelet interactions (Michelson *et al*, 2007). Our previous study (Yip *et al*, 2013), together with this current study highlights the need to establish age-specific reference ranges for agonist-stimulated platelet activation for interpreting platelet reactivity in children.

In conclusion, our study challenges the assumption that the platelets of children are the same as of adults. We demonstrated that platelets in children showed hyper-responsiveness to TRAP and ADP at sub-maximal concentrations. There is a significant inverse correlation of age with agonist-stimulated MPA in children. This finding is fundamental to investigating the role of platelets in diseases of childhood and the pathogenesis of adult-based diseases, such as cardiovascular disease and diabetes, that have their origins in childhood (Osmond *et al*, 2007; Lohse *et al*, 2010; de Mutsert *et al*, 2014). Further investigation with a larger age-stratified cohort of healthy children may inform the specific age range when agonist-induced parameters of platelet activation approach adult levels. In addition, *in vitro* assays with specific platelet inhibitory drugs, such as aspirin and P2Y₁₂ receptor antagonists, could determine the potential difference in children's response to antiplatelet therapy.

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Authorship

C. Yip, M. Linden, P. Monagle and V. Ignjatovic designed the study; C. Yip, M. Linden and V. Ignjatovic analysed and interpreted data; C. Attard recruited participants for the study. C. Yip performed the experiments, collected data and wrote the manuscript. V. Ignjatovic, M. Linden, C. Attard, and P. Monagle, revised and approved the manuscript.

Disclosure of conflicts of interest

All authors state no conflict of interest.

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Plasma Levels of Soluble Interleukin 1 Receptor Accessory Protein Are Reduced in Obesity

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Context: Adipokines actuate chronic, low-grade inflammation through a complex network of immune markers, but the current understanding of these networks is incomplete. The soluble isoform of the IL-1 receptor accessory protein (sIL1RAP) occupies an important position in the inflammatory pathways involved in obesity. The pathogenetic and clinical influences of sIL1RAP are unknown.

Objective: The objective of the study was to elucidate whether plasma levels of sIL1RAP are reduced in obesity, using affluent clinical, biochemical, and genetic data from two diverse cohorts.

Design, Setting, and Participants: The study was conducted in two cohorts: the San Antonio Family Heart Study (n = 1397 individuals from 42 families) and South Asians living in Mauritius, n = 230).

Main Outcome Measures: Plasma sIL1RAP levels were measured using an ELISA. The genetic basis of sIL1RAP levels were investigated using both a large-scale gene expression profiling study and a genome-wide association study.

Results: A significant decrease in plasma sIL1RAP levels were observed in obese subjects, even after adjustment for age and sex. The sIL1RAP levels demonstrated a strong inverse association with obesity measures in both populations. All associations were more significant in females. Plasma sIL1RAP levels were significantly heritable, correlated with IL1RAP transcript levels (NM_134470), showed evidence for shared genetic influences with obesity measures and were significantly associated with the rs2885373 single-nucleotide polymorphism ($P = 6.7 \times 10^{-23}$) within the *IL1RAP* gene.

Conclusions: Plasma sIL1RAP levels are reduced in obesity and can potentially act as biomarkers of obesity. Mechanistic studies are required to understand the exact contribution of sIL1RAP to the pathogenesis of obesity. (*J Clin Endocrinol Metab* 99: 3435–3443, 2014)

Chronic inflammation is a characteristic feature of obesity. Local accumulation of immunologically active cells in the adipose tissue (1, 2) combined with enhanced

cytokine production (3, 4) and subsequent cellular responses leading to systemic inflammation (5) together characterize this process. Although the complete immu-

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Abbreviations: AVI, abdominal volume index; BMI, body mass index; Col, conicity index; CV, coefficient of variation; IL1RAP, IL-1 receptor accessory protein; mIL1RAP, membrane bound form of IL1RAP; SAFHS, San Antonio Family Heart Study; sIL1RAP soluble, circulating form of IL1RAP; SNP, single-nucleotide polymorphism; WC, waist circumference; WHR, waist to hip ratio; WHtR, waist to height ratio.

nological jigsaw of obesity is far from resolved, the roles of individual components of the immunological responses are better understood. The IL-1 signaling pathway is considered a critical component of the immunological ensemble involved in the pathogenesis of obesity (6, 7).

The IL-1 pathway is triggered by the IL-1 receptor and a coreceptor, the IL-1 receptor accessory protein (IL1RAP) (8, 9). IL1RAP also partakes in the IL-33/ST2 (suppression of tumorigenicity 2; the IL-33 receptor) signaling pathway involved in chronic allergies, atherosclerosis, and rheumatoid arthritis (10–12). Thus, IL1RAP assumes a theoretically crucial position in the immunological axis implicated in obesity development and complications. However, whether this molecule plays a clinically and epidemiologically significant role in obesity is unknown. Another feature of IL1RAP biology that beckons an investigation is that there are two isoforms of IL1RAP consequent to an alternative splicing event (13): a membrane bound form (mIL1RAP) that is involved in signaling pathways and a soluble, circulating form (sIL1RAP) (14–16) that acts as a decoy receptor. This circulating receptor can mop up excess IL-1 β and IL-33 and thereby regulate the ambient levels of these circulating proinflammatory cytokines. Arguably, reduced levels of sIL1RAP would therefore be associated with an increased proinflammatory immune response that can enhance the risk or facilitate the progression to obesity and its complications. However, whether sIL1RAP levels in plasma correlate with the clinical features of obesity is currently unknown.

We hypothesized that reduced levels of sIL1RAP are associated with phenotypic traits characterizing obesity. We tested this conjecture in the large and extended families of Mexican Americans recruited in the San Antonio Family Heart Study (SAFHS) (17–19). This cohort has been extensively characterized with respect to obesity, has been genetically analyzed, and has full genome-wide gene expression profiles available on study participants (20). Therefore, we wanted to determine whether plasma sIL1RAP levels correlate with the phenotypic traits of obesity and whether there was a genetic basis for the association. In addition, we sought to replicate our findings in another large family cohort recruited in Mauritius. Here we show consistent epidemiological associations between plasma sIL1RAP and obesity features in two diverse cohorts and provide leads into the genetic basis of plasma sIL1RAP variability.

Materials and Methods

Study subjects

Samples were from two independent studies; the SAFHS and the Mauritius Family Study. The SAFHS is a large, ongoing in-

vestigation of extended Mexican American families in San Antonio (17, 21). Details of enrolment procedures, inclusion and exclusion criteria, and phenotypic assessment of the study subjects have been detailed elsewhere (5, 6). Data and samples were collected during the first visit of the participants (from 1992 to 1996).

In the Mauritius cohort, individuals were drawn from a large longitudinal South Asian noncommunicable diseases survey in which participants were examined sequentially at either two or three time points over an 11-year period from 1987 to 1998 (22–24). sIL1RAP levels were measured in plasma drawn in 1992 when all individuals were normal glucose tolerant ($n = 230$). The clinical characteristics of the study participants from both cohorts are shown in Table 1.

Ethics statement

All participants provided written informed consent. The SAFHS was approved by the Institutional Review Board at the University of Texas Health Science Centre (San Antonio, Texas). The Mauritius Family Study was approved by ethics committees in Australia and Mauritius, according to the Council for International Organizations of Medical Sciences and World Health Organization guidelines for International Biomedical Research Involving Human Subjects (Geneva 1993).

sIL1RAP enzyme-linked immunosorbent assay

A sandwich ELISA was developed to measure sIL1RAP in the plasma of participants from both cohorts. The ELISA was highly sensitive with a lower limit of detection of 0.4 ng/mL and showed robust intra- and interassay reproducibility. The ELISA used commercially available unlabeled and biotinylated polyclonal antihuman IL1RAP antibodies (R&D Systems). A standard curve was established using recombinant human IL1RAP protein (R&D Systems). The interassay coefficient of variation (CV) was 5.6% and the within-assay CV was 3.9%. The linear working range of the ELISA assay was 0.4–50 ng/mL, and the mid-range of the assay was 5 ng/mL. The mean CV among duplicates of measured human samples was 3.2%. The plasma sIL1RAP analyte was tested for stability across eight freeze-thaw cycles and found to be highly stable.

Obesity-related phenotypic traits

Data on waist circumference (WC), weight, height, body mass index (BMI), hip circumference, waist to hip ratio (WHR), waist to height ratio (WHtR), conicity index (CoI), abdominal volume index (AVI), and total body fat were collected as previously described (18, 25–28). Total body fat (percentage) was measured using bioimpedance as described previously (29, 30). Serum levels of leptin, adiponectin, and chemerin were also measured by an ELISA as previously described (31, 32). The association of plasma sIL1RAP levels with prevalent obesity was investigated. For these analyses, obesity was defined as a BMI of 30 kg/m² or greater.

Quantification of IL1RAP gene expression

Genome-wide quantitative transcriptional profiles previously obtained (20) from lymphocyte samples of 1240 participants were used to quantify the IL1RAP gene expression. A total of 19 648 autosomal transcripts were reliably detected, which contained two isoforms related to the *IL1RAP* gene: NM_002182 and NM_134470 (Entrez gene identification

Table 1. Clinical Characteristics of the Study Subjects

Characteristic	SAFHS	Mauritius
Age, y, mean (SD)	39.18 (16.74)	46.52 (11.58)
Females, n, %	837 (59.9)	120 (52.2)
Fasting glucose, mmol/L, mean (SD)	5.57 (2.46)	5.41 (0.33)
Two-hour postglucose load glucose, mmol/L, mean (SD)	7.24 (4.96)	6.08 (1.05)
Fasting insulin, μ U/mL, mean (SD)	15.97 (19.33)	13.29 (8.63)
Two-hour postglucose load insulin, μ U/mL, mean (SD)	75.32 (73.88)	74.30 (50.06)
Homeostasis model of assessment-insulin resistance, mean (SD)	4.36 (7.13)	1.46 (0.27)
Participants with T2D, n, %	202 (14.46)	Not determined
Weight, kg, mean (SD)	76.55 (18.36)	64.80 (12.45)
Height, m, mean (SD) m]	1.62 (0.09)	1.57 (0.10)
BMI, kg/m ² , mean (SD)	29.25 (6.60)	26.33 (4.40)
WC, cm, mean (SD)	94.8 (171.7)	87.9 (9.0)
Hip circumference, cm, mean (SD)	105.8 (15.9)	98.5 (8.8)
WHR, mean (SD)	0.90 (0.09)	0.89 (0.06)
WHtR, mean (SD)	0.59 (0.11)	0.56 (0.06)
Col, mean (SD)	1.27 (0.13)	1.26 (0.07)
AVI, mean (SD)	18.73 (7.07)	15.72 (3.12)
Systolic blood pressure, mm Hg, mean (SD)	120.35 (18.68)	124.66 (20.76)
Diastolic blood pressure, mm Hg, mean (SD)	70.67 (10.33)	76.36 (12.45)
Total serum cholesterol, mg/dL, mean (SD)	189.31 (39.60)	182.64 (40.91)
Serum triglycerides, mg/dL, mean (SD)	149.81 (128.04)	154.06 (102.74)
HDL cholesterol, mg/dL, mean (SD)	50.21 (12.83)	47.40 (11.04)
sIL1RAP, ng/dL, mean (SD)	131.24 (40.77)	54.57 (12.42)

Abbreviations: HDL, high-density lipoprotein; T2D, type 2 diabetes.

3556). Quality control measures, data normalization procedures, and analytical approaches for the transcript data have been previously described (20).

Single-nucleotide polymorphism (SNP) genotyping

The genotyping was performed previously using the Illumina HumanHap550 Genotyping BeadChip. in 1202 individuals, using the Infinium II assay and Illumina's standard protocols on a Tecan Freedom Evo Robot. After staining, each BeadChip was imaged on the Illumina BeadArray 500GX Reader using the Illumina BeadScan image data acquisition software (version 3.2.6). Genotype data were assessed using Illumina's BeadStudio software (version 2.0).

Statistical analysis

Heritability analysis was performed in the SAFHS participants under the classical approach decomposing the phenotypic variance into independent genetic and environmental components, assuming an additive model of gene action (narrow sense heritability) and expected kinship coefficients based on the observed intrafamilial relationships. Because of the assumption of multivariate normality, direct inverse Gaussian transformation was performed on both IL1RAP measures and obesity traits prior to all analyses.

Association analyses were done using polygenic regression models for SAFHS participants (to account for the kinship structure) and linear regression in the Mauritius cohort (because the study participants were not related to one another). In all regression analyses, potential confounding covariates including sex, age, and their interactions as well as the use of antidiabetic, antihypertension, and lipid-lowering medications were also included in all analyses. All hypothesis testing was performed using likelihood ratio statistics and used their expected asymptotic distributions to obtain *P* values. The SOLAR software package

(version 7.2) (33) was used for all statistical analysis on related individuals using the variance component-based approach. Because we tested the association of sIL1RAP methods with multiple correlated phenotypic traits related to obesity, we adopted the method suggested by Suo et al (34) to avoid overinterpretation due to multiple testing. Using this procedure, we derived the first principal component based on the multiple phenotypes and generated each individual's principal component score. We then used this score as a phenotypic trait for association testing. We used Stata 12.0 (Stata Corp) to conduct the principal components analysis.

For the genome-wide association study, SOLAR was used to perform tests of Hardy-Weinberg equilibrium allowing for non-independence among pedigree members. To eliminate missing genotypic data, we used likelihood-based imputation with the MERLIN computer package (35) to rapidly impute SNP data in pedigrees. Under this procedure, imputation is performed and the posterior genotypic probabilities for each missing genotype are stored for each pedigree. These posterior probabilities are used to construct an appropriately weighted covariate for each SNP that is then used in association analyses. Maximum likelihood marker allele frequencies were calculated using the modified genetic map developed by deCODE genetics (20).

We used measured genotype analysis (36) to test the association of SNPs with sIL1RAP gene and protein expression. The measured genotype model is embedded in a variance components-based linkage model, assuming an additive model of allelic effect (ie, the SNP genotypes AA, AB, and BB were coded as -1 , 0 , and 1 , respectively, and used as a linear predictor of the phenotype) (37). To correct for the effect of multiple testing for a given phenotype, we estimated the effective number of SNPs using the method of Li and Ji (38). To eliminate the potential for hidden population stratification, we also utilized the Quantitative trait linkage disequilibrium test approach to association test-

ing implemented in SOLAR (37). The Manhattan plot of genome wide association data was generated using HaploView (39). The localized view of the *IL1RAP* gene region showing SNP association data was generated using LocusZoom (40).

Lastly, we used the bivariate trait analysis methods to estimate the genetic correlation of the plasma sIL1RAP levels with obesity-related traits. The bivariate trait analyses permit the further partition of the phenotypic variance of a trait into genetic and environmental correlations. Specifically, the phenotypic covariance (ρ_p^2) is regarded as a function composed of the additive genetic (ρ_g^2) and environmental (ρ_e^2) correlations between two traits (denoted in the equation as i and j).

$$\rho_p(i,j) = \rho_G(i,j) \sqrt{h_i^2 h_j^2} + \rho_E(i,j) \sqrt{(1 - h_i^2)(1 - h_j^2)}$$

Significant genetic correlation ($\rho_G(i,j)$) between two phenotypes indicates the shared pleiotropic effect of causal genes. We used this approach to estimate the genetic correlation between plasma sIL1RAP levels and several obesity-related traits. The bivariate trait analyses were also conducted using the SOLAR software.

Results

sIL1RAP levels in Mexican American and Mauritius subjects

The SAFHS component of this study represents Mexican American families at a high risk of obesity. We measured sIL1RAP in 1397 participants of the SAFHS where levels ranged from 29.8 ng/mL to 356.6 ng/mL (mean 131.2 ng/mL; SD 40.8 ng/mL). In males (n = 560), the sIL1RAP levels ranged from 35.6 ng/mL to 356.6 ng/mL (mean 130.06 ng/mL; SD 40.80 ng/mL), whereas in females (n = 837), the sIL1RAP levels ranged from 29.8 ng/mL to 356.6 ng/mL (mean 132.03 ng/mL; SD 40.78

ng/mL). Interestingly, the estimated plasma sIL1RAP in subjects from the Mauritius cohort were strikingly low as compared with the SAFHS cohort (Table 1) and ranged from 30.22 ng/mL to 134.94 ng/mL, but the mean plasma sIL1RAP levels were marginally lower in males as compared with females (53.77 ng/mL vs 55.30 ng/mL), although not statistically significant ($P = .3517$). Detailed clinical characteristics of the study participants are described in Table 1.

Relationship of plasma sIL1RAP with age and sex

There is strong evidence for sex differentials with regard to the circulating levels as well as potential interactions of sIL1RAP with sex hormones. For example, IL1RAP is constitutively expressed in the uterus (41, 42) and can bind and interact with estradiol as well as T through different pathways. Although we did not have data on T and estradiol levels in the SAFHS subjects, there was a significant association between plasma levels of sIL1RAP and dehydroepiandrosterone sulfate in females [polygenic regression coefficient (ρ) = 0.0806 (0.0077)] but not in males [−0.0312 (0.2210)]. Considering these sex-based differences, we conducted all the ensuing analyses separately in males and females as well as in all subjects together.

When we studied the combined and interactive association of age and sex with circulating levels of sIL1RAP, the sIL1RAP levels were quadratically related to age and the age × sex interaction was also statistically significant (Figure 1A). Thus, younger males had substantially lower circulating levels of sIL1RAP than comparatively aged females, but this difference rapidly narrowed with increasing age. These observations together indicated that age, age², and sex could confound the association of sIL1RAP with metabolic syndrome risk because they can also act as risk factors of obesity.

Plasma sIL1RAP levels are reduced in obesity

In the SAFHS participants, the mean plasma sIL1RAP levels were significantly lower in obese subjects compared with nonobese subjects whether examined separately in males (125.7 ng/mL vs 132.4 ng/mL, $P = .074$), females (127.2 ng/mL vs 135.2 ng/mL, $P = .004$), or combined together (127.0 ng/mL vs 133.6 ng/mL, $P = .003$; (Figure 1B). These differences remained signifi-

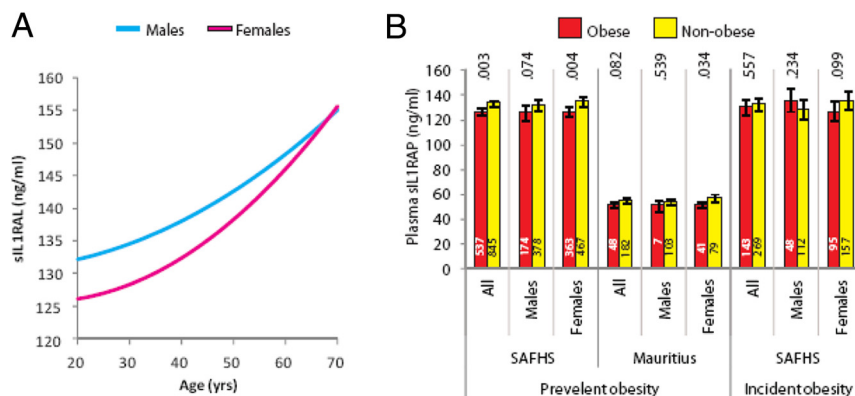


Figure 1. Relationship of plasma sIL1RAP levels with age, sex, and obesity. A, Predicted relationship with age and sex. Results are predictions based on the best fitting polygenic regression model that accounted for the kinship structure. Blue curve is for females and red curve is for males. Values estimated from a polygenic regression model were $P = .0080$ for age, $P = .0177$ for age², and $P = .0572$ for age × sex interaction. B, Mean plasma sIL1RAP levels in obese (red bars) and nonobese (yellow bars) subjects. Mean levels in the SAFHS participants were estimated using polygenic regression accounting for the kinship structure. Error bars indicate 95% confidence intervals, and the rotated numbers at the top show the statistical significance of the difference of means using Student's *t* test. Numbers of subjects included in the analyses are shown inside the bars.

cant, even after adjustment for serum dehydroepiandrosterone sulfate levels ($P = .0985$ for males, $P = .0451$ for females, and $P = .0376$ for all subjects). In the Mauritius cohort, obese subjects had reduced mean plasma sIL1RAP levels as compared with nonobese subjects: males (51.0 ng/mL vs 54.0 ng/mL, $P = .539$), females (51.9 ng/mL vs 57.1 ng/mL, $P = .034$), or all combined (51.8 ng/mL vs 55.3 ng/mL, $P = .082$). In both cohorts the statistical significance of the studied association was greater for females than for males.

Association of plasma sIL1RAP with obesity-related traits

We next conducted a series of regression analyses in both the SAFHS and Mauritius cohorts with an aim to uncover potential consistency of the association of the plasma sIL1RAP levels, with several traits characterizing obesity (Table 2). In the SAFHS participants, we observed the strongest associations of inverse-normalized plasma sIL1RAP levels with WC, BMI, WHtR, AVI, and total body fat but not with WHR and CoI. The associations were also more significant in females than males. Furthermore, we were able to replicate these findings in the Mauritius cohort such that WC, BMI, WHtR, and AVI again showed statistical significance or near significance, and these observations were also more significant in females compared with males (Table 2).

To investigate the potential role of sIL1RAP in the immune component of obesity, we studied the association of

inverse-normalized plasma sIL1RAP levels with three well-recognized adipokines: adiponectin and leptin in the SAFHS participants and chemerin in the Mauritius cohort. In the SAFHS cohort, plasma sIL1RAP levels were associated inversely with serum leptin but not with serum adiponectin (Table 2), in which the significance was higher in females compared with males. Notably, although an overall negative association of plasma sIL1RAP levels with serum chemerin in the Mauritius cohort was observed, this observation was more significant in males rather than females. Together these findings indicated that sIL1RAP levels associate somewhat specifically with serum leptin levels in females.

Lastly, to avoid overinterpretation due to multiple testing, we tested the association of plasma sIL1RAP levels with the first component derived from obesity-related traits listed in Table 2. We found that this first principal component explained 76.35% and 66.69% of the inter-subject variability in the SAFHS and Mauritius cohorts, respectively. This principal component (labeled as obesity principal component in Table 2) was significantly associated with reduced plasma sIL1RAP levels more strongly in females as compared with males in the SAFHS cohort. In the Mauritius cohort, however, statistical significance for association of plasma sIL1RAP and obesity principal component was observed in females only.

sIL1RAP levels are heritable and share genetic influences with obesity traits

Given that plasma sIL1RAP levels were measured in the SAFHS family-based cohort, we were able to estimate the

Table 2. Association of sIL1RAP Levels With Obesity-Related Traits*

Trait (Abbreviation)	All		Males		Females	
	β	P Value	β	P Value	β	P Value
SAFHS participants						
WC	-.0986	2.0×10^{-5}	-.0715	0.0172	-.1263	5.3×10^{-5}
BMI	-.1071	5.6×10^{-6}	-.0707	0.0252	-.1408	7.5×10^{-6}
WHR	-.0443	.0240	-.0474	0.0505	-.0517	.0527
WHtR	-.0875	.0001	-.0728	0.0248	-.1058	.0007
CoI	-.0474	.0431	-.0468	0.0954	-.0514	.1318
AVI	-.0904	.0002	-.0774	0.0226	-.1022	.0020
Total body fat	-.0699	.0014	-.0644	0.0716	-.0776	.0044
Serum adiponectin	.0112	.7454	.0242	0.6572	.0174	.6954
Serum leptin	-.0631	.0034	-.0272	0.4291	-.0878	.0014
Obesity principal component	-.1268	.0005	-.1029	0.0497	-.1927	.0016
Mauritius cohort						
WC	-.1415	.0278	-.0919	0.2184	-.2015	.0594
BMI	-.1051	.0849	-.0491	0.4801	-.1728	.0911
WHR	-.1161	.0430	-.1252	0.0581	-.1050	.2730
WHtR	-.1720	.0018	-.1480	0.0305	-.2010	.0222
CoI	-.1303	.0196	-.1283	0.0382	-.1327	.1625
AVI	-.1359	.0357	-.0831	0.2664	-.1996	.0638
Total body fat	-.0921	.1292	-.0501	0.5467	-.1589	.0781
Serum chemerin	-.1175	.0057	-.1675	0.0033	-.0568	.3748
Obesity Principal Component	-.1734	.0044	-.1243	0.0855	-.2232	.0189

Abbreviation: β , regression coefficient. Bold values denote significance at $P < .05$.

proportion of variance due to additive genetic factors. We observed a strong heritability ($h^2 = 0.40$) that was highly statistically significant ($P = 3.9 \times 10^{-23}$). However, the estimate of heritability was larger in females (0.50 , $P = 1.7 \times 10^{-14}$) compared with males (0.45 , $P = 1.7 \times 10^{-6}$). The potential correlation of *IL1RAP* gene expression and plasma levels of sIL1RAP was also investigated, and we found that the transcript NM_134470 was associated with the sIL1RAP protein differentially across sexes. In males, this transcript showed a negative nonsignificant association with sIL1RAP levels (polygenic regression coefficient -0.057 , $P = .186$), but in females it showed a significant positive association (polygenic regression coefficient -0.087 , $P = .013$).

Furthermore, bivariate trait analyses were conducted to estimate the genetic and environmental correlation between sIL1RAP levels and measures of obesity and we observed stronger correlations in females than males, even after adjusting for age, age², sex, and their interactions (Table 3). In females, the genetic correlation coefficients were statistically significant for BMI ($P = .0081$), AVI ($P = .0292$), and total body fat ($P = .0198$); and with an association nearing significance with WC ($P = .0780$). In contrast, BMI was the only trait that showed a trend toward a genetic correlation with plasma sIL1RAP in males

($P = .0818$). Interestingly, with the exception of CoI and WHR, there was a statistically significant inverse phenotypic correlation between plasma sIL1RAP and each of the remaining obesity-related traits in both males and females (Table 3).

In the Mauritius cohort, the total phenotypic correlation was estimated. We found that WHR and CoI were the only variables that failed to show a significant inverse association with plasma sIL1RAP levels. This pattern was not observed in males where the only significant phenotypic correlations of sIL1RAP levels were with WHR and WHtR. Considered in totality, these patterns of correlation demonstrated a consistency of direction and strength of evidence in females (in both cohorts). Thus, plasma sIL1RAP levels are not only significantly heritable but also likely share similar genetic influences as those of features of obesity, especially in females. Therefore, to gain insight into the biological mechanisms of control, we sought to identify genetic factors that may influence variation in plasma sIL1RAP levels.

Genome-wide association analysis identifies strongly associated genetic variation within sIL1RAP

We further evaluated genetic factors influencing sIL1RAP by performing genome-wide association using

Table 3. Bivariate Trait Analyses of sIL1RAP Levels in Plasma and Obesity-Related Traits

Trait	Phenotypic Correlation				Genetic and Environmental Correlations			
	Mauritius		SAFHS					
	ρ_P	P Value	ρ_P	P Value	ρ_G	P Value	ρ_E	P Value
All participants								
WC, cm	−0.1782	.0068	−0.1262	1.4×10^{-5}	−0.2463	.0107	−0.0232	.7205
BMI, kg/m ²	−0.1303	.0484	−0.1445	7.1×10^{-9}	−0.2921	.0021	−0.0126	.8365
WHR	−0.1529	.0203	−0.0444	0.1153	−0.0404	.7409	−0.0471	.3833
WHtR	−0.1628	.0134	−0.1193	4.3×10^{-5}	−0.2205	.0205	−0.0298	.6509
CoI	−0.1189	.0718	−0.0554	0.0535	−0.0674	.5309	−0.0472	.4207
AVI	−0.1718	.0090	−0.1241	2.0×10^{-5}	−0.2651	.0056	−0.0009	.9889
TBF	−0.0817	.3121	−0.1092	0.0004	−0.3420	.0122	0.0423	.8445
Males only								
WC, cm)	−0.1812	.0582	−0.1107	.0156	−0.2300	.2061	0.0196	.9058
BMI, kg/m ²	−0.1501	.1176	−0.1100	.0149	−0.3065	.0818	0.0901	.5477
WHR	−0.1912	.0454	−0.0805	.0763	−0.0656	.7439	−0.0937	.5259
WHtR	−0.2324	.0145	−0.1049	.0237	−0.1850	.3130	−0.0108	.9527
CoI	−0.1452	.1302	−0.0595	.1835	0.1129	.6441	−0.1728	.2148
AVI	−0.1704	.0751	−0.1104	.0158	−0.2327	.2027	0.0211	.8976
TBF	−0.1151	.3064	−0.0909	.0582	−0.2498	.3098	0.0009	.9941
Females only								
WC, cm	−0.1713	.0614	−0.1321	.0005	−0.2051	.0780	−0.0310	.8092
BMI, kg/m ²	−0.1779	.0518	−0.1692	7.3×10^{-6}	−0.3059	.0081	0.0257	.8440
WHR	−0.0758	.4108	−0.0175	.6321	0.1819	.2079	−0.1749	.0640
WHtR	−0.1818	.0469	−0.1243	.0010	−0.1649	.1493	−0.0685	.6025
CoI	−0.0726	.4304	−0.0429	.1487	0.0216	.8619	−0.1141	.2872
AVI	−0.1706	.0628	−0.1298	.0006	−0.2510	.0292	0.0463	.7235
TBF	−0.2270	.0518	−0.1265	.0014	−0.3650	.0198	0.0670	.5382

Abbreviation: TBF, total body fat. Bold values denote significance at $P < .05$.

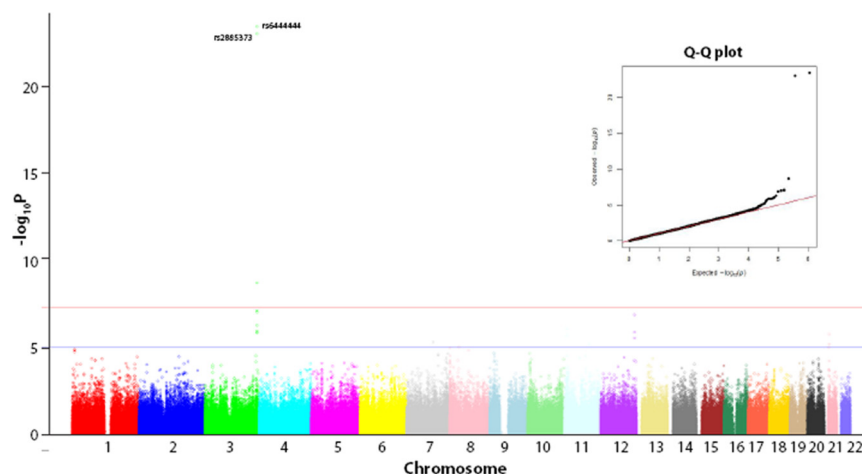


Figure 2. Genome-wide association study of sIL1RAP. Manhattan plot of genome wide association analysis results for plasma sIL1RAP levels. Chromosome position is on the x-axis, and the $-\log P$ value of the association result is shown on the Y-axis. The horizontal line at $-\log P = 7.0$ indicates the Bonferroni adjusted P value for multiple testing ($P = 9.2 \times 10^{-8}$) above which association is significant on a genome-wide scale. Black arrow indicates the two most significant associations. Inset, Genome-wide association results of regions containing IL1RAP in which the strongest association signals were observed. The expected $-\log P$ value of the association result is shown on the x-axis and the observed $-\log P$ value of the association result is shown on the y-axis.

542 944 SNPs previously genotyped in 1222 participants in the SAFHS cohort. After allowing for nonindependence, population admixture, and adjustment for sex and age, association analysis revealed two very strongly associated SNPs within intron 9 of the *IL1RAP* gene on chromosome 3q28 (Figure 2). These were rs6444444 ($P = 2.6 \times 10^{-23}$) and rs2885373 ($P = 6.7 \times 10^{-23}$). Because the two SNPs were in high linkage disequilibrium ($D' = 0.90$), we believe that they do not represent independent findings (Figure 2). The association result far exceeded the conservative Bonferroni-corrected threshold of $P = 9.2 \times 10^{-8}$ after an adjustment for multiple testing. This result was consistently observed in both males and females. In males, the most significantly associated SNP was rs2885373 ($\beta = .90$, $P = 2.9 \times 10^{-13}$) followed by rs6444444 ($\beta = .80$, $P = 2.4 \times 10^{-11}$), whereas in females the most significantly associated SNP was rs6444444 ($\beta = .89$, $P = 2.2 \times 10^{-23}$) followed closely by that for rs2885373 ($\beta = .87$, $P = 1.1 \times 10^{-22}$; Figure 2). **Supplemental Figure 1** shows the position of rs2885373 relative to the alternatively spliced isoforms of *IL1RAP*. Interestingly, when we investigated the association between rs2885373 and *IL1RAP* gene expression, we observed that this variant was significantly associated with the transcript NM_134470 in females ($\beta = .22$, $P = .0269$) but not in males ($\beta = -.01$, $P = .9308$).

Discussion

Using data and samples from individuals who were clinically evaluated, we demonstrated that sIL1RAP levels

were lower in plasma of obese compared with nonobese subjects. Our study has identified a novel, easy-to-measure, and consistent biomarker of obesity. The consistency of our observations across geoepidemiologically diverse cohorts further highlights the novelty of our findings. Moreover, demonstration of a strong genetic involvement and identification of novel polymorphisms through a genome-wide approach lends further credence to the candidature of plasma sIL1RAP levels as a marker of inflammation in obesity.

The consistent pattern of all associations in females across both cohorts is an important observation that demands a closer look. Our results indicate that the direction of association of *IL1RAP* gene expression and the sIL1RAP protein was

inverted in males and females, but these associations and the genetic correlation coefficients were stronger in females. Even if one considers that the larger number of females in the SAFHS may work in favor of a better statistical significance, these observations raise the possibility of differential post-translational modifications, protein-protein interactions, and/or epigenetic regulatory mechanisms of sIL1RAP regulation across sexes. Despite these differentials, however, the association of the circulating levels of sIL1RAP with disease outcomes was directionally comparable in males as well as in females. Therefore, we believe that the potential disparity in mechanisms of genetic control of sIL1RAP levels will not impact the use of sIL1RAP protein as a biomarker of obesity-induced inflammation.

The concept that sIL1RAP is likely involved in the obesity-related inflammation is supported by its specific association with leptin but not with adiponectin in SAFHS participants. Leptin structurally resembles IL-6 and is known to be engaged in a cross talk with IL-1 family of cytokines in the context of breast cancer (43, 44). It is conceivable that the decoy activity of sIL1RAP may also be providing a sink effect for circulating the leptin levels also through unknown cross talk. Although data in support of this hypothesis are currently not available, our study provides a lead in this direction and warrants further investigation.

Our genetic analysis revealed that sIL1RAP was significantly heritable ($h^2 = 0.40$). Genome-wide association analysis revealed a single, very strongly associated locus

directly within the *IL1RAP* gene near the boundary of intron 8 and exon 9 (rs6444444 and rs2885373). The primary splicing event determining membrane and soluble forms occurs at exon 9 and may be influenced directly by either rs6444444 or rs2885373 lying juxtaposed in intron 8, only 908 or 671 bp, respectively, from the start of exon 9. The 356-amino acid sIL1RAP protein is encoded by the first 9 exons of *IL1RAP* and lacks the transmembrane and intracellular domains of the longer 570-amino acid membrane bound isoform (mIL1RAP). Although mIL1RAP is recruited after binding of IL-1 α and IL-1 β to the IL-1R type I receptor and enhances IL-1 signaling, sIL1RAP has the opposite effect and is thought to inhibit IL-1 α - and IL-1 β -mediated signaling (45). Therefore, it is conceivable that the rs2885373 SNP may affect splicing directly (or is in linkage disequilibrium with another causative variant) and influences relative proportions of mIL1RAP to sIL1RAP that in turn could impact upon the body's response to inflammatory signals.

Two other issues from this study deserve closer scrutiny. First, our results show that sIL1RAP levels are significantly influenced by age. However, it is conceivable that population-specific differences in the relationship of age with sIL1RAP levels may exist. Therefore, future longitudinal studies are required to establish whether there are individual-level sIL1RAP trajectories with respect to age such that a deviation from the expected trajectory of sIL1RAP levels can signal the impending risk of obesity. Second, we observed overall lower levels of plasma sIL1RAP in the Mauritius cohort than in the Mexican American cohort. Factors contributing to this difference may include natural genetic and environmental variation in the cohorts with differing ancestries. However, the sample-specific z-scores in the obese subjects from both cohorts were highly comparable, indicating that a similar degree of relative reduction in sIL1RAP across populations may be associated with an increased risk of obesity (data not shown).

In summary, this study demonstrates the validity of combined genome wide genetic variation and gene expression data in a large human cohort for the discovery of biomarkers of obesity. The observations reported here also raise the possibility that a recombinant sIL1RAP protein or related therapeutic agent may enhance existing anti-IL-1 β therapies and provide clinical utility in modulation or reversal of inflammation induced by the adipose tissue and complications thereof. Future studies need to investigate these possibilities in more controlled and direct research settings.

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Letter to the Editors-in-Chief

Personalised anticoagulation approach to improve the prevention and treatment of thrombosis

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Dear Editors,

Thrombosis Prevention and Treatment Using Anticoagulants

The most widely used anticoagulants for prophylaxis and treatment of thrombosis are unfractionated heparin (UFH) and low molecular weight heparin (LMWH), with UFH remaining the most commonly used parenteral anticoagulant [1]. Despite the frequency of use, extensive experience and numerous studies with these drugs, the treatment failure rate and risk of clinical bleeding remains problematic. There is also significant variation in the dose of UFH required to achieve the therapeutic range, leading to frequent dose modifications. For adults, the inter-individual variation in drug effect for fixed dose of UFH and LMWH is 50% and 35%, respectively, as measured by the Anti-Xa assay [2]. This large variation in dose effect, combined with the risk of sub- and supra-anticoagulation is a major reason why these drugs need to be regularly monitored.

New Oral Anticoagulants (NOACs) such as rivaroxaban and dabigatran have been developed and introduced into the adult clinical setting. Benefits of these NOACs are that they are synthetic, administered orally, specifically target haemostatic proteins, have a shorter half-life with a more predictable response compared to conventional anticoagulants, fixed dosing and no requirement for monitoring. Despite these benefits, NOACs are known to exhibit high inter-individual variability in dose response. Rivaroxaban demonstrates up to 40% inter-individual variability, while dabigatran reaches up to 81%, as measured by standard coagulation tests [3].

There is a Significant Inter-individual Variation in Quantity and Function of Haemostatic Proteins

We have previously demonstrated a wide inter-individual variation in the functional measurement of haemostatic proteins [4] and we recently completed the first comprehensive study for the quantitative (antigen based) measurement of haemostatic proteins [5]. We demonstrated up to a 50-fold difference in the concentration of haemostatic proteins in specific age groups [5]. There was significant inter-individual variation in both Prothrombin (Factor II) and Factor X, the precursors for Thrombin (Factor IIa) and activated Factor X (Factor Xa), which are in turn the drug targets of UFH, LMWH, rivaroxaban and dabigatran. Historically, the focus on functional assessment of coagulation proteins is derived from the management of inherited and acquired bleeding and clotting disorders, where the functional level of the proteins are thought to be more critical in predicting clinical phenotype than the quantitative concentration of the proteins, because they will reflect both type I and type II disorders. Despite the relevance of functional assays for disease management, protein concentration is likely to be extremely important when predicting drug response in an individual, particularly with NOACs, which target specific haemostatic proteins, as the anticoagulant effect will be directly influenced by changes in the concentration of the target protein.

Personalised Anticoagulation Approach

The wide inter-individual variation in the concentration of haemostatic proteins in healthy individuals has lead us to hypothesize that this intrinsic variability may be responsible for the wide variation in dosing requirements that are observed clinically.

We propose that it may be possible to correlate the haemostatic profile of an individual with the anticoagulant effect, a concept of *Personalised Anticoagulation* that may be applicable to a wide range of anticoagulants. The question is whether the variation in inter-individual dose response for anticoagulants is directly related to the inter-individual variation in antigenic levels of specific haemostatic proteins. This approach has never been tested, as quantitative levels of coagulation proteins have never previously been used to determine anticoagulant dosage strategies and the association between the concentration of haemostatic factors and the resulting anticoagulant effect has not been made to date.

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Previous personalised approaches to anticoagulation have focused on genetic factors (i.e. polymorphisms) that influence the metabolism of Warfarin and coumarin derivatives such as Acenocoumarol and Phenprocoumon. Hence, the *Personalised Anticoagulation* approach based on the quantity of haemostatic proteins provides a novel direction in guiding the dosing strategies for patients requiring anticoagulation.

Testing the Personalised Anticoagulation Approach

We performed this study in plasma samples from 10 healthy adults, median age 26 years, range 21 to 46 years. Blood samples were collected and processed according to a standard protocol [5–7]. Factor II (Affinity Biologicals, Ontario, Canada) and Factor X (Diagnostica Stago, Asnieres sur Seine, France) antigen levels were measured in individual samples [10] prior to spiking with clinically relevant low and high dose of UFH (0.3 IU/ml and 0.7 IU/ml; final concentration of 1% saline) [7] as well as Rivaroxaban (100 ng/ml and 200 ng/ml; final concentration of 1% DMSO) [6]. Rivaroxaban (Bayer HealthCare, Wuppertal, Germany) was dissolved in 100% dimethyl sulfoxide (DMSO) as per previously published methods to a concentration of 50 µg/ml prior to spiking into plasma [6,8]. Two control samples for each individual were created and contained 1% saline and 1% DMSO to act as baseline levels for UFH and Rivaroxaban respectively. Activated Partial Thromboplastin Time (APTT) was measured using the PTT-A assay kit (Diagnostica Stago, Asnieres sur Seine, France) at baseline and in samples spiked with UFH; while Prothrombin Time (PT) was measured using the Neoplastine-R assay kit (Diagnostica Stago, Asnieres sur Seine, France) at baseline and in samples spiked with Rivaroxaban. APTT and PT were performed on the STA-R analyser (Diagnostica Stago, Asnieres sur Seine, France) and were selected as tests that have previously shown to correlate well with the dose of UFH and Rivaroxaban, respectively [6,8]. In addition, the APTT is also the most frequently clinically used test for monitoring UFH. Correlation between the functional and antigen based assays was calculated using linear regression modelling (Graphpad Prism version 6.0, California, USA).

Results demonstrate a 70% and 57% inter-individual variation in starting Factor II and Factor X levels (Fig. 1A), with a corresponding 20%, 52% and 51% variation in APTT in unspiked samples (0 IU/ml), and samples spiked with 0.3 IU/ml and 0.7 IU/ml UFH, respectively (Fig. 1B). There was a corresponding 13%, 37% and 40% variation in PT in unspiked samples (0 ng/ml), and samples spiked with 100 ng/ml and 200 ng/ml Rivaroxaban, respectively (Fig. 1C).

As part of this study we also demonstrated that there was a significant negative correlation between baseline Factor II antigen levels and UFH effect at 0.3 IU/ml (Fig. 2A); while this correlation was low at 0.7 IU/ml UFH (Fig. 2B). The correlation between baseline Factor X antigen levels and the effect of Rivaroxaban at 100 ng/ml was moderate (Fig. 2C); while this correlation was low at 200 ng/ml Rivaroxaban (Fig. 2D).

These results demonstrate not only significant variation in Factor II and Factor X levels at baseline, but also a large inter-individual variation in response to UFH and Rivaroxaban that increased with increasing dose of anticoagulants in this group of healthy individuals. This concept may be more clinically relevant in sick patients, where coagulation protein quantities may change significantly with the underlying disease process, potentially leading to wide variation in response to anticoagulants over time even within the same patient.

Implications of the Personalised Anticoagulation Approach

Heparin, warfarin and LMWH are currently monitored using a number of specific assays, however current strategies are not personalised, as they use standard initial doses and then incremental changes based on empiric responses.

The knowledge generated through future testing of our personalised anticoagulation approach will improve the current strategy for

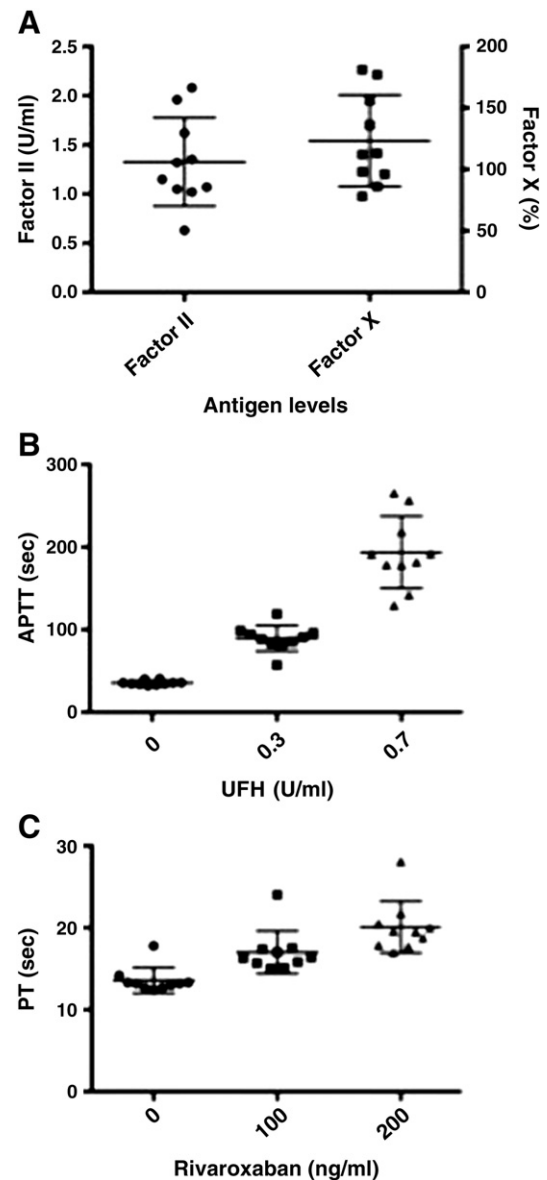


Fig. 1. Inter-individual variation in healthy individuals. (A) Factor II and Factor X antigen levels at baseline; (B) APTT at baseline and samples spiked with 0.3 IU/ml and 0.7 IU/ml UFH; (C) PT at baseline and samples spiked with 100 ng/ml and 200 ng/ml Rivaroxaban. Results are presented as mean and standard deviation.

anticoagulation that is based on populations rather than individuals. The potential to personalise anticoagulation based on the predicted drug effect with measured physiological proteins would be an enormous step forward and has the potential to significantly reduce the mortality and morbidity of thrombosis and anticoagulant therapy by improving time to achieve and maintain therapeutic range. At the very least, this approach may also lead to more effective predictions of dose response to anticoagulants in patients with specific disease processes based on knowledge of the usual impact of the specific disease on coagulation protein amount. Developing an understanding of the typical impact of specific disease processes on the concentration of haemostatic proteins would enable safe predictive changes in therapy to be made.

The widespread clinical use of NOACs in specific clinical populations (e.g. paediatrics) is being hampered by our lack of understanding as to why such wide inter-individual dose response occurs. Defining the mechanism for dose response variation will enable improved application of NOACs, again through a personalised approach to dose determination.

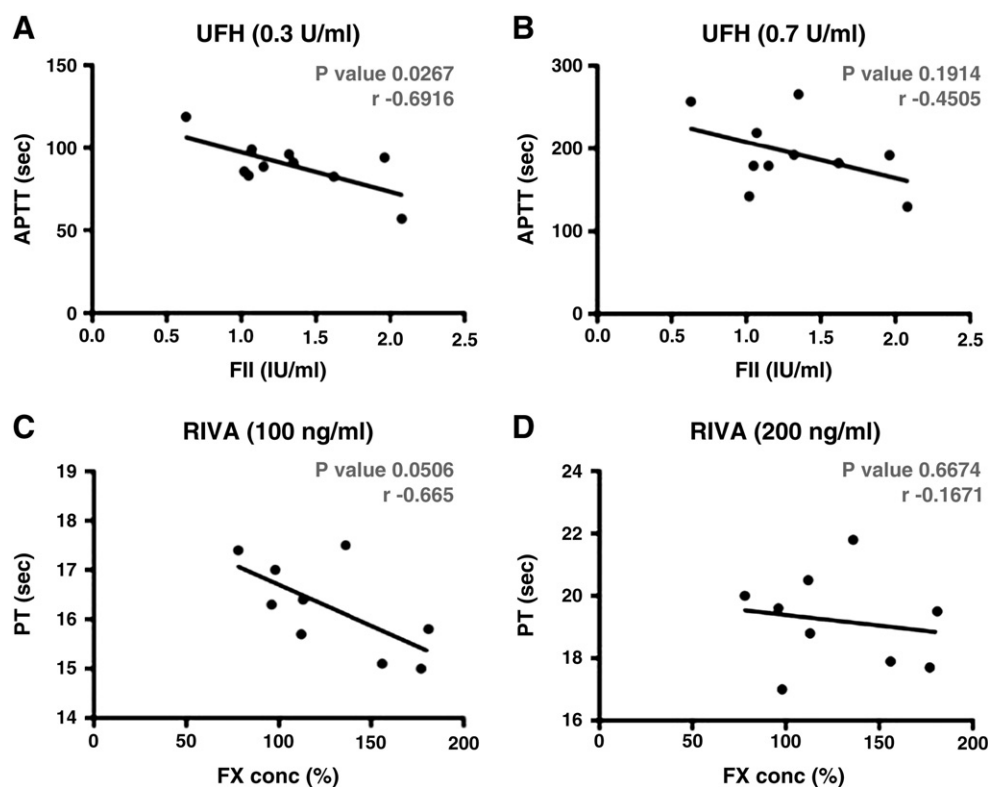


Fig. 2. Correlation between Factor II antigen level and the effect of unfractionated heparin (UFH) as measured by the Activated Partial Thromboplastin Time (APTT) (A) 0.3 IU/ml UFH; (B) 0.7 IU/ml UFH; Correlation between Factor X antigen level and the effect of Rivaroxaban as measured by the Prothrombin Time (PT) (C) 100 ng/ml Rivaroxaban; (D) 200 ng/ml Rivaroxaban. 'r' represents the correlation coefficient.

This new concept of personalised anticoagulation and hence our proposed approach should be assessed in large cohorts of both healthy and clinical populations.

Authors Contribution

VI and PM designed the study, supervised laboratory and data analysis and interpretation and wrote the manuscript. VK and CA executed the study, including sample collection, laboratory and data analysis, and contributed to the revisions of the manuscript.

Conflict of Interest Statement

The authors report no potential conflict of interest.

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The in-vitro anticoagulant effect of rivaroxaban in neonates

Chantal Attard^a, Paul Monagle^{a,b,c}, Dagmar Kubitz^d and Vera Ignjatovic^{a,b}

The use of anticoagulants in neonates is increasing because of the medical advances improving the long-term survival of very sick infants who are at risk of venous thromboembolism (VTE). Current anticoagulation therapy in neonates is less than ideal, because of the physiological differences compared to children and adults regarding the pathophysiology of thrombosis and pharmacology of the drug. Limitations associated with conventional anticoagulants have prompted the development of novel drugs that specifically target the key proteins in the coagulation system. Rivaroxaban is the first oral, direct Factor Xa inhibitor available for the prevention of VTE in adults. Its predictable pharmacokinetic profile, high oral bioavailability and once-daily dosing make rivaroxaban an optimal anticoagulant that warrants investigation in neonates. This study was designed to determine whether there are age-related differences in the pharmacodynamic effects of rivaroxaban *in vitro* amongst neonates. Neonatal and adult plasma pools were created and spiked with increasing concentrations of rivaroxaban (0–500 ng/ml). Commercially available prothrombin time (PT), activated partial thromboplastin time (aPTT) and anti-Factor Xa assays as well as a sub-sampling thrombin generation assay were used to measure the rivaroxaban effect. A

dose-dependent response was observed for PT, aPTT and lag time in both the age groups. Rivaroxaban caused a significant increase in the clotting time for PT and aPTT as well as an increase in lag time (as measured by thrombin generation) in neonates when compared with adults. In-vivo studies are required to confirm the consistency of dose-response in neonates. *Blood Coagul Fibrinolysis* 25:000–000 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: anticoagulant, developmental haemostasis, direct Factor Xa inhibitor, neonate, rivaroxaban, vitamin K antagonist

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The use of anticoagulants in neonates is increasing because of the medical advances improving the long-term survival of very sick infants who are at risk of venous thromboembolism (VTE) [1]. An increasing number of neonates require long-term anticoagulation for conditions such as congenital heart disease and their associated surgical repairs [2]. Long-term anticoagulation is achieved using warfarin and the heparins, unfractionated heparin (UFH) and low molecular weight heparin (LMWH). The use of each anticoagulant has its own set of clinical challenges. These challenges are further pronounced in neonates because of the physiological differences compared with children and adults regarding the pathophysiology of thrombosis and pharmacology of anticoagulant drugs. For example, warfarin management in neonates is complicated by the differences in vitamin K levels of breast milk and infant formula. Furthermore, there is no suspension/liquid preparation for vitamin K antagonists available, rather the tablets are crushed, which may lead to inconsistent dosing [3]. LMWH is administered subcutaneously which is difficult to manage in the home as LMWH is most readily available in predosed syringes which are based on adult weights [1]. Additionally, in-dwelling subcutaneous catheters (i.e. insuflon) which are used to reduce the pain caused by frequent subcutaneous administration of drugs (such

as LMWH or UFH) are not recommended for use in infants weighing less than 3 kg. Another concern is reduced bone density when administering long-term warfarin, LMWH and UFH [4,5].

Further compounding the issues with current anticoagulation therapy is the effect of developmental haemostasis. This concept recognizes the fundamental differences in the haemostatic system of neonates, children and adults. The effect of developmental haemostasis on anticoagulant therapy with warfarin, UFH, LMWH, the direct thrombin inhibitors and rivaroxaban have been assessed in the paediatric population [6–11]. In-vitro studies have demonstrated age-related effects with some of these drugs. In younger populations, UFH demonstrated exaggerated anticoagulation compared with adults which is in contrast to LMWH and rivaroxaban, in which age-related differences were not observed [6,8,11]. These differences are thought to be because of the differences in specific inhibitory activity of the drugs. Whereas UFH inhibits thrombin as well as Factor Xa, LMWH predominantly inhibits Factor Xa and rivaroxaban directly inhibits Factor Xa.

Rivaroxaban is a highly specific inhibitor of Factor Xa which prevents the formation of the thrombin burst by inhibiting coagulation at the point of amplification [12].

Rivaroxaban is the first oral, direct Factor Xa inhibitor available for clinical use. This drug is currently approved for use in the adult population for the treatment and prevention of VTE in patients undergoing elective hip or knee replacement surgery as well as stroke prevention in patients with atrial fibrillation [13]. Rivaroxaban has also been approved for secondary prevention of VTE as well as pulmonary embolism and deep-vein thrombosis [13–16]. The in-vitro paediatric study (28 days to 16 years) recently published by our team has demonstrated that there are no age-related differences in the anticoagulant effect of this drug [11]. Rivaroxaban's high oral bioavailability, once-daily dosing, tablet or liquid formulation and limited requirement for monitoring make rivaroxaban an easier to use anticoagulant and its applicability in neonates should be investigated.

This study was designed to determine whether there are age-related differences in the pharmacodynamic effects of rivaroxaban *in vitro* amongst neonates.

Materials and methods

Plasma samples were obtained from healthy individuals according to the previously established protocol [6–8, 17–19]. The inclusion criteria were healthy neonates (up to 72 h after birth) who were more than 37 weeks, birth weight greater than 2.5 kg, Apgar score at least 7 at 5 min, absence of systemic abnormalities and who did not have a family history of coagulation disorders (e.g. thrombosis). Vitamin K (1 mg) was administered intramuscularly as per normal clinical practice. Adult samples were from healthy volunteers who were not taking medication and did not have a family history of coagulation disorders. This study was approved by the Royal Children's Hospital, Melbourne Ethics in Human Research Committee (#24124) and by the Royal Women's Hospital, Melbourne Ethics Committee (HREC #02/08). Informed consent was obtained from the parents of neonates and from the adult participants themselves.

Blood samples were collected in tubes containing 0.105 mol/l (i.e. 3.2%) trisodium citrate anticoagulant in a ratio of nine volumes of whole blood to one volume of anticoagulant. Citrated samples were centrifuged at 3000 rpm for 10 min at 10°C (Megafuge 1.0R, Heraeus) and platelet-poor plasma was frozen at –70°C for batch testing.

Plasma samples were pooled into age-specific pools and included two neonatal plasma pools ($n = 20$ per pool) and three adult pools (pools A and B, $n = 20$ per pool; pool C, $n = 11$). Each pool contained separate groups of donors.

Rivaroxaban (Bayer Healthcare, Wuppertal, Germany) was dissolved in 100% dimethyl sulfoxide (DMSO) as per a previously published method to a concentration of 50 µg/ml prior to spiking into plasma [20]. The final concentration of DMSO in each sample was 1%.

Each pool was spiked with six different concentrations of rivaroxaban (25–500 ng/ml) to match the therapeutic levels of this drug (100–300 ng/ml of plasma) [12]. Two controls for each pool were created by spiking with 1% saline (control 1) or 1% DMSO (control 2).

Rivaroxaban effect was assessed using a number of validated assays [11]. Activated partial thromboplastin time (aPTT) (STA PTT-A; Diagnostica STAGO, Asnières sur Seine, France), prothrombin time (PT) (Neoplastine CI; Diagnostica STAGO) and anti-Xa assays [(Coamatic Heparin; Chromogenix Instrumentation Laboratory Company, Milan, Italy) (Rotachrom Heparin; Diagnostica STAGO)] were performed on the automated STA analyser (Diagnostica STAGO). Spiked concentrations of rivaroxaban below 100 ng/ml were not measured using the Rotachrom assay owing to the detection limits of this assay.

Endogenous thrombin potential (ETP) was quantitated using a nonautomated microplate method, which activates the clotting cascade using a tissue-factor-based reagent (Thromborel S) [11,21].

Statistical analysis

The results are expressed as mean $\pm$ standard deviation (SD) of the combined age-specific pools. All data was normalized by logarithmic transformation prior to the statistical analysis.

Results from the neonates were compared to those observed for adults using a Student's *t*-test. When unequal variance was determined, an Aspin–Welch test was used. Correction for multiple testing was conducted using randomization tests and the Monte-Carlo approach based on 10 000 samples.

Results

Automated assays

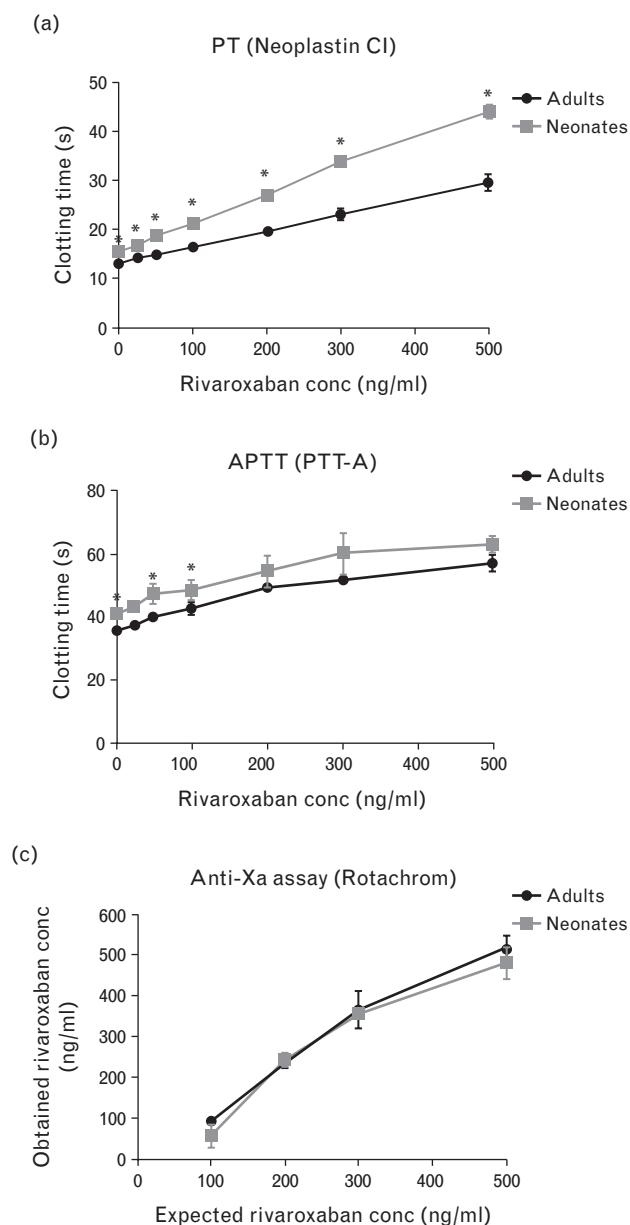
PT, aPTT (Fig. 1) and anti-Xa assays all demonstrated a dose-dependent response in both the age groups to increasing rivaroxaban concentrations. Neonates demonstrated a significantly increased clotting time for the PT assay at all concentrations of rivaroxaban and the controls. The aPTT assay also demonstrated a significantly increased clotting time when there was an absence of or low concentration of the drug (<25 ng/ml).

No age-related differences were observed for the anti-Xa assays.

Thrombin generation

Measured parameters of thrombin generation included ETP, lag time, time to peak and peak-free thrombin. Lag time was the only parameter affected by rivaroxaban in an age-dependent manner. Neonates demonstrated a significantly prolonged lag time with increasing concentration of rivaroxaban when compared with adults (Fig. 2) [22].

Fig. 1



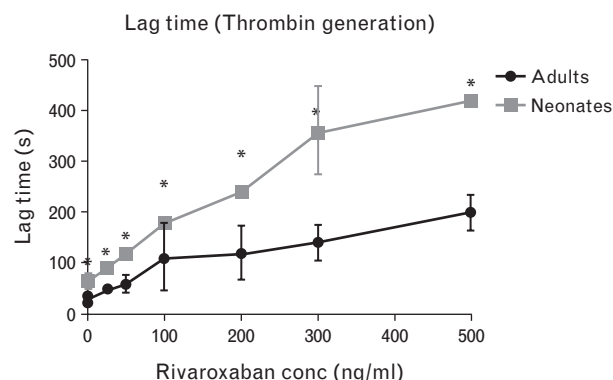
Age-specific response to rivaroxaban on clotting assays. Prothrombin time (a), activated partial thromboplastin time (b) and anti-Xa assay (c). Each point represents the mean $\pm$ SD of the age-specific plasma pools. * represents statistically significant differences between the age groups. SD, standard deviation.

Discussion

This is the first study to measure the in-vitro effect of rivaroxaban in neonates. Age-related differences were observed between neonates and adults with some of the assays used to monitor the drug; however, rivaroxaban demonstrated a predictable, dose-dependent pharmacokinetic profile in both groups.

The differences in the responses observed in the neonates compared with the adults appear to be less dramatic

Fig. 2



Age-specific response to rivaroxaban on lag time. Each point represents the mean $\pm$ SD of the age-specific plasma pools. * represents statistically significant differences between the age groups. SD, standard deviation.

than the differences observed previously with UFH in children less than 1 year of age [6]. Anticoagulants such as UFH and lepirudin are known to exhibit age-related differences in their pharmacokinetics, which have been attributed to the interaction of these drugs with thrombin [6,7]. In contrast, in-vitro studies of LMWH have demonstrated that there are no age-specific differences in drug response [8]. Although both UFH and LMWH inhibit thrombin as well as Factor Xa, LMWH predominantly inhibits Factor Xa, which has led to the hypothesis that the age-related differences in effect are mediated by thrombin [8].

Although LMWH has also been previously reported to respond similarly in children and adults [8], the ability to give rivaroxaban via the oral route makes it considerably more attractive as an agent for use at home, especially in the neonatal population.

This study was performed *in vitro* and as such cannot provide recommendations as to the dosing strategies in neonates. Clinical studies are required. However, the results of this study suggest that the use of rivaroxaban could be extended down into the neonatal age group with similar initial dosing strategies that have been used for determining dosing in infants and children, which were linked to a similar in-vitro study [11]. This study would confirm that the PT (using Neoplastine CI) may be an appropriate assay for monitoring rivaroxaban in neonates, as there is a good linear dose-response curve across the therapeutic range. Although the aPTT also gave a linear response, the curve was considerably less steep, and so the measurement of dose responsiveness may be more appropriate with the PT. In terms of the anti-Xa assay, although no differences were observed between neonates and adults, the curve for the assay was linear and this assay could also be used to monitor rivaroxaban in the

neonatal population. Studies in adults support the use of anti-Xa chromogenic assays using specific rivaroxaban calibrators and controls to monitor the levels of rivaroxaban, as these methods appear to be more sensitive and specific compared with PT-based methods [23,24].

This study was performed using plasma from well term neonates and further studies would be required clinically to assess the response in sick and premature neonates.

The results of the thrombin generation assays are highly consistent with our previous study performed in children and adults, in which rivaroxaban caused a prolongation in lag time. Although lag time was prolonged in both adult and neonatal groups, neonates demonstrated a significant increase in lag time when compared with adults. This difference in increase in lag time was not observed when children and adults were compared. The implications of the differences in lag time response when assessing thrombin generation presumably reflect the differences in the initiation process of clotting between neonates and children.

In summary, rivaroxaban is an oral direct Factor Xa inhibitor that has a number of features which make it attractive for long-term use in neonates compared with warfarin or LMWH. This study confirms a linear dose-response curve for rivaroxaban *in vitro* and provides some baseline data that will be useful when considering initial dosing strategies in clinical studies. Although the dose-response curve in neonates differs to that in adults, the differences are not as marked as previously noted with other anticoagulants and appear predictable. Clinical trials of rivaroxaban in neonates are warranted.

Acknowledgements

Conflicts of interest

D.K. is an employee of Bayer Healthcare, Germany.

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Epidemiology of venous thrombosis in children with cancer

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Summary

There has been an extensive body of research focusing on the epidemiology of thrombosis in adult cancer populations; however, there is significantly less knowledge about thrombosis in paediatric cancer populations. Thrombosis is diagnosed with increasing frequency in children being treated for cancer, and there is an urgent need to increase our understanding of the epidemiology of thrombosis in this population. Currently, there are no guidelines for identification of high-risk groups, prophylaxis or management of thrombotic complications in paediatric cancer patients. We reviewed the available literature regarding the epidemiology, mechanisms, risk factors, prophylaxis and outcomes of thrombosis in children with cancer and identified areas that require further research. The reported incidence of symptomatic venous thromboembolism (VTE) in children with cancer ranges between 2.1% and 16%, while the incidence of asymptomatic events is approximately 40%. Approximately 30% of VTE in this population is

associated with central venous lines (CVL). The most common location of VTE is upper and lower extremity deep venous thrombosis (43 to 50% of events, respectively), while 50% of events in ALL patients occur in the central nervous system. Key characteristics that increase the risk of thrombosis include the type of cancer, age of the patient, the presence of a CVL, presence of pulmonary/intra thoracic disease, as well as the type of chemotherapy. Outcomes for paediatric cancer patients with VTE include post-thrombotic syndrome, pulmonary embolism, recurrent thromboembolism, destruction of upper venous system and death. Prospective studies aimed at enabling risk stratification of patients are required to facilitate development of paediatric specific recommendations related to thromboprophylaxis in this population.

Keywords

Cancer, child, epidemiology, incidence, thrombosis, paediatrics

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Introduction

The majority of studies investigating epidemiology of thrombosis in the setting of cancer have focused on adult populations, with thromboembolic complications being the second leading cause of death in cancer patients (1). In comparison, little is known about the pathophysiology and epidemiology of thromboembolism in the paediatric cancer populations.

The incidence of venous thromboembolism (VTE) is approximately 0.7 to 1.4 events per 100,000 children and 53 events per 100,000 hospital admissions (2–4). This is significantly lower compared to adults, with the incidence of VTE ranging between 71 to 117 events per 100,000 individuals (2). In addition, 95% of VTE events that occur in children are secondary to underlying diseases such as cancer, trauma, surgery and congenital heart disease (2, 5).

This paper reviews the literature available from January 1990 to November 2013 regarding the epidemiology, mechanisms, risk factors, prophylaxis and outcomes of thrombosis in children with cancer and critical areas where more research is needed. The ultimate aim of this review is to provide the basis for prospective studies in this population that will facilitate the establishment of

guidelines for identification of high-risk groups, prophylaxis and management of thrombotic complications in children with cancer.

Method

A PubMed search was performed by one author and verified by a second author using key words: cancer, children, epidemiology, paediatric, thrombosis, and thromboembolism. The search was limited to humans, English and papers published between January 1990 and November 2013. Case series were excluded from this study.

Characteristics of published studies

This review included 47 studies, 14 (30%) of which were retrospective and 25 (53%) were prospective, with one meta-analysis and seven reviews, accounting for the remaining 17% of the studies (► Figure 1). There was a significant heterogeneity in the studies identified. Despite this, the majority of the studies were prospec-

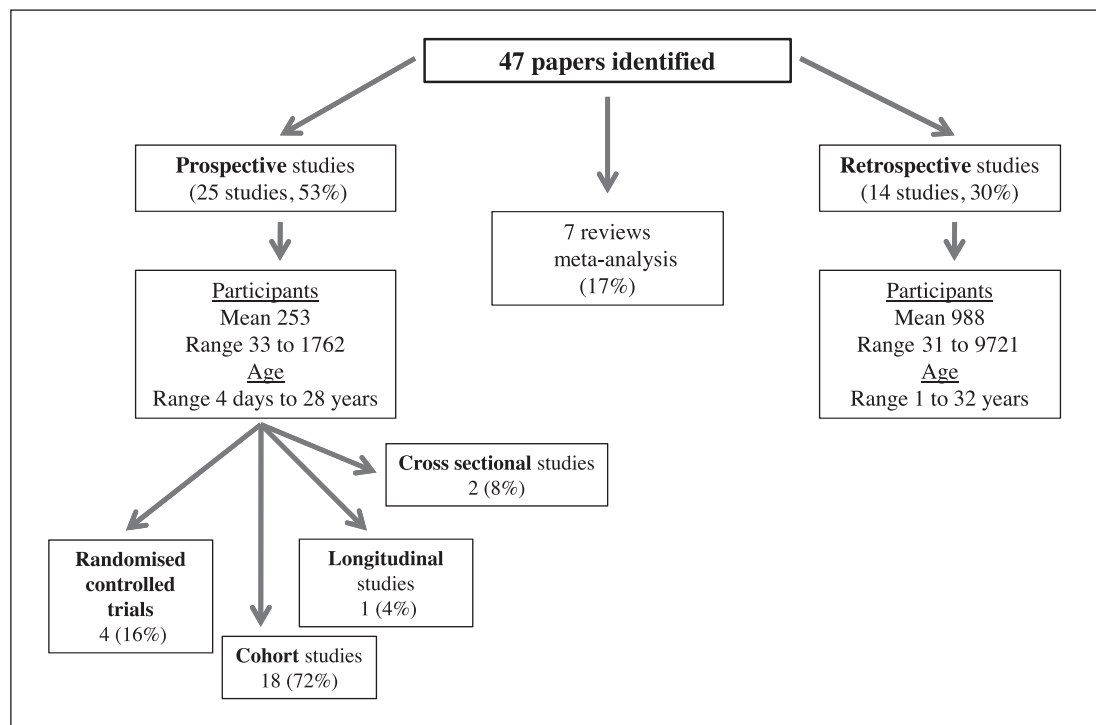


Figure 1: Summary of the studies identified in this review.

tive. The mean number of participants for all studies was 567 (range 31 to 9,721). For retrospective studies, there was a mean of 988 participants (range 31 to 9,721); while for prospective studies the mean number of participants was 253 (range 33 to 1,762). The participants for all studies were aged between four days to 32 years (1 to 32 years for retrospective; 4 days to 28 years for prospective). The majority of the studies included children only, with two studies including some adult data up to 32 years of age. Overall, 43% of the studies focused on paediatric patients with acute lymphoblastic leukemia (ALL), 11% on solid tumours or sarcomas, 5% on paediatric haematological malignancies, while 41% were based on paediatric patients with all types of cancer.

It is important to note that only one publication referred to arterial thrombosis, with the incidence of thrombotic events being between 2% and 3.6% (6). Hence, this review will focus on venous thrombosis.

Incidence of VTE in children with cancer

The overall incidence of symptomatic VTE in children with cancer was 2.1 to 16% (6-9), while the incidence of asymptomatic VTE was significantly higher, at approximately 40% (9, 10). The incidence of symptomatic VTE is based on a number of both retrospective and prospective studies where the number of participants for specific studies ranged from 44 to 9,721. The methods for the diagnosis of VTE also varied, with studies utilising radiographic tests, clinical symptoms, laboratory tests or a combination of these. The variability in the number of patients, differences in study design and the way in which VTE was detected could contribute to

the variation in the incidence of symptomatic VTE. The most common location of VTE in paediatric cancer patients is upper and lower extremity deep venous thrombosis (DVT), accounting for 43 to 50% of events (2, 8). In addition, 75% of the VTE in children with cancer are central venous line (CVL)-associated (7). The incidence of pulmonary embolism (PE) is not well defined, with reports varying between 2% and almost 20% of all VTEs in children with cancer (6).

Risk factors involved in developing TE
Cancer type

The summary of the effect of cancer type on the risk of thrombosis is presented in ►Table 1. Athale et al. demonstrated that the prevalence of TE in paediatric patients across all cancer types was 7.9% (8). Interestingly, while adult studies have shown that individuals with brain tumours have a high incidence of TE, such events are highly uncommon in paediatric patients with brain tumours (11). Therefore, excluding brain tumours, the prevalence of TE across all other cancer types in children was found to be significantly higher at 10.7% (8). In paediatric cancers excluding ALL, the overall incidence of TE is 16% (12). In patients with ALL, the rate of asymptomatic VTE is 30–70% and the rate of symptomatic VTE is 5%, with 50% of these events located in the upper venous system (13). Central nervous system (CNS) thrombosis is a common occurrence in children with ALL in approximately 50% of children with symptomatic thrombosis (14). Approximately 52% of ALL patients with CNS thrombosis had sinovenous thrombosis (14). In children with ALL treated on BFM ALL 90/95 proto-

Table 1: Effect of type of cancer on risk of thrombosis in children.

Cancer type	Incidence of TE	Location of TE	Chemotherapy increased risk	CVL increased risk	Other risk factors
ALL	Symptomatic: 1–5.2% [30, 43] Asymptomatic: 30–70% [30]	CNS (up to 53%) [16, 30] Upper and lower deep venous system 42.8% [16]	Yes, concomitant use of L-asparaginase and corticosteroids [17, 26–28] 90% of VTE's occur during induction therapy [30]	37% of asymptomatic TE associated with CVL [17] Symptomatic TE unknown	T-immunophenotype associated with higher risk [44]
Sarcoma	Symptomatic: 14% Asymptomatic: ~8% [45]	43% DVT, 22% PE, 17% inferior vena cava, 35% tumour compression [45]	Not applicable	30–50% of TE associated with CVL [45]	Incidence of TE: rhabdo- myosarcoma (15%), Ewing sarcoma (13%) and osteosarcoma (10%) [45]
Lymphoma	Symptomatic: 4.1–12% [7, 18] Asymptomatic: Not available	Upper venous DVT (75%) [17] PE (2.6%)[18]	No, study suggests devel- opment of TE related to mechanical factors rather than biology of disease and chemotherapy [18]	~68% of TE associated with CVLs [18] All patients with CVL-re- lated TE had mediastinal mass lymphadenopathy [18]	Older children (>12 years) and advanced stage dis- ease associated with higher risk [18]
AML/APL	AML: Symptomatic 6% [7] Asymptomatic: Not available APL: Symptomatic 14.3% [7] Asymptomatic: Not available	Not available	AML: Yes APL: Same as ALL [7]	Not available	Not available

ALL, acute lymphoblastic lymphoma; AML, acute myeloid leukaemia; APL, acute promyelocytic leukaemia; CVL, central venous line; DVT, deep venous thrombosis; PE, pulmonary embolism; VTE, venous thromboembolism.

col, there was a 6% incidence of cerebral sinus venous thrombosis (SVT) (15).

Age

Older paediatric cancer patients have a higher risk of developing TE (8, 16), with the median age of patients with VTE being 13.5 compared to the median age of patients without VTE being 9.2 years old (7), while the highest risk of VTE for children receiving chemotherapy observed in 10- to 17-year-olds (8, 16). In a retrospective review, however, with cancer patients aged 15 to 24 years, the increased risk of VTE was highest in the range of 18 to 24 years, where 236 (46.18%) had non-superficial VTE (6). Unfortunately, this study did not include children below the age of 15, and thus further studies that encompass a broader age range need to be done in order to confirm the age range at which VTE risk is highest. The current hypothesis is that post-pubescent children/adolescents in this setting may be more prone to VTE due to differences in the epidemiology of cancer with respect to different age groups, as well as the more aggressive therapy used in older paediatric patients, particularly in the setting of ALL (17). Another hypothesis is that the higher risk in adolescents is due to the fact that their haemostatic system may more closely resemble that of adults, who are more prone to thrombosis.

Central venous lines (CVLs)

A significant number of individuals who developed TE also had CVLs, indicating that the presence of a CVL is a confounding factor to the role of cancer type in children (7, 8, 18). Catheter-related thrombosis has been demonstrated as prevalent among all cancer types (8). The presence of a CVL is an important factor that has been strongly associated with causing upper venous thrombosis (8, 9, 15, 19–23) with one study identifying 43% of catheter-related DVT as asymptomatic (24). The incidence of CVL occlusion in children with cancer is estimated at 1.35 per 1,000 catheter days, while the incidence of CVL-DVT is 0.13 per 1,000 catheter days (23). These incidences were found to increase significantly if the catheter was inserted in an angiography suite rather than surgically (23). It was also demonstrated that paediatric cancer patients who developed DVT (7%) had on average more catheters placed than those without DVT (25). In the setting of ALL, 30% of symptomatic VTE events are associated with CVLs (10). The risk of thrombosis increases significantly when CVLs are present in conjunction with L-asparaginase treatment, a scenario that results in 25% to 100% vessel occlusion (13).

In terms of CVL-related VTE and other associated complications, Journeycake et al. found that 73% of total catheters placed in the children who developed DVT were associated with at least one episode of occlusion or infection (6.4 times higher risk), with

90% of the patients with DVT experiencing at least one catheter occlusion and 47% experiencing CVL-related bacteraemia (25). Additionally, having multiple episodes of occlusion and infection were associated with unidentified DVT (25).

Pulmonary/ intra-thoracic disease

There is a high incidence of TE in paediatric patients with concurrent pulmonary or intra-thoracic disease. Athale et al. concluded that 26.1% of paediatric sarcoma patients with pulmonary disease had TE as compared to 8.5% of those without pulmonary disease. (21). The same study observed that five of the six patients with upper venous system DVT had pulmonary disease (21).

Chemotherapy

One of the major risk factors for children with cancer is the potential for the chemotherapy itself to induce a prothrombotic state and cause thrombosis. This may be caused by the direct effect on proteins involved in haemostasis, endothelial cell damage or by complications such as infections and immunosuppression (17).

Chemotherapy also plays a significant role in increasing the risk of TE, particularly in the setting of paediatric patients with ALL, where the concomitant administration of L-asparaginase and corticosteroids increases the risk of thrombosis (17, 26–28). This is because L-asparaginase causes suppression of natural anticoagulants such as Antithrombin, while corticosteroids increase the levels of procoagulant factors (Factor II, VIII) and induce a hypofibrinolytic state (increased plasminogen activator inhibitor type 1, reduced alpha-2-macroglobulin and tissue type plasminogen activator) (17, 29). Specifically, the risk of thrombosis in paediatric patients with ALL receiving both L-asparaginase and corticosteroids increases by up to 10-fold, with 90% of events occurring during induction therapy, with the remaining 10% occurring during consolidation therapy (16, 26, 30). Paediatric patients with ALL reach a hypercoagulable state just four weeks after the start of dexamethasone treatment (26), with VTE events occurring at approximately the 8th week of treatment of ALL (7).

Other treatment options such as growth factors (i.e. anthracyclines) have also been observed to increase the risk of TE in children in the setting of ALL, acute myeloid leukaemia (AML) and lymphomas (7). In addition, in ALL patients that there is an increased risk of VTE in patients being treated with anthracyclines (6.1%), compared to those without (2.7%) (16).

The PARKAA study, with a population of 60 children with ALL who had CVLs and were undergoing chemotherapy with L-asparaginase (13), demonstrated that the prevalence of TE was high at 36.7%, that 40% of collaterals were categorised as major and that the majority of TEs were located in the upper central venous system (95.5%). In this study, of the 22 patients with TEs, three patients had TEs in the right atrium placing them at risk for PE, while three patients were found to be clinically symptomatic (13). From this study, it can be concluded that the presence of both risk factors of chemotherapy and the presence of CVLs in a patient population, present a significantly high risk and should be targeted for further investigation.

Inherited prothrombotic defects

While some studies have determined a correlation between inherited prothrombotic defects and thrombosis in paediatric oncology patients, this link is not very clear (13, 31, 32).

One study demonstrated that while prothrombotic defects were prevalent among the paediatric population with malignancies, only 3.2% actually developed thrombosis. It should be noted that, the study population consisted of only 31 native Cretan children and therefore no major conclusions can be drawn from this study (33). The PARKAA prospective cohort study demonstrated that there was no association between VTE and thrombophilia in children with ALL where no children with TEs were positive for FV G1691A mutation or the prothrombin G20210A mutation (13).

However, Knofler et al. demonstrated that prothrombotic risk factors (such as hyperlipoproteinaemia, heterozygous FV G1691A mutation, prothrombin G20210A mutation and protein C deficiency type I, protein S deficiency) were found in 17 (23%) patients in a study population of 77 paediatric oncologic patients with CVLs (22). Of seven of the 11 patients that had CVL-related thrombosis, four of the children had a single defect and three patients had combined prothrombotic defects suggesting that inherited thrombophilia may play a significant role in the development of thrombosis in paediatric oncologic patients with CVLs (22). This conclusion was also supported in a multicentre, prospective study that evaluated the risk of TE in children with ALL that had at least one inherited prothrombotic defect (34). With regard to prophylactic use of LMWH in paediatric ALL patients with prothrombotic defects, a study by Harlev et al. demonstrated that those with prothrombin gene mutation may have a higher risk of TE than those with FV Leiden mutation (35).

The prevalence of prothrombotic defects in children with: haematological malignancies, particularly ALL, with FV Leiden mutation G1691A ranges between 3.3–18.5% (13, 15, 34, 36, 37); prothrombin G20210A mutation and MTHR T677T mutation are 2–11.1% and 7.6–18.5%, respectively (13, 15, 34, 36, 37). While the prevalence of prothrombotic defects in children diagnosed with ALL appeared similar to that of the general population, Nowak-Gottl et al. demonstrated that a significantly higher proportion of children with ALL and prothrombotic defects developed VTE (46.5%), compared to children with ALL but without prothrombotic defects (2.2%), and additionally, 29.6% of the 27 patients with VTE and inherited prothrombotic defects had combined defects (34). In addition, patients with ALL with at least one biological abnormality had lower “thrombosis-free survival” compared to patients without that abnormality (34). This study indicates that while the overall incidence of VTE in paediatric cancer patients is low, the incidence of VTE in the setting of ALL is much higher and that inherited prothrombotic defects may be a significant contributing factor in VTE development. However, amongst the studies investigating the role of prothrombotic defects in paediatric cancer patients, only the study by Nowak-Gottl et al. demonstrated that there was a significant correlation between the incidence of TE and the presence of at least one prothrombotic defect (34).

Patients with ALL that have at least one prothrombotic defect, exhibit increased levels of thrombin and fibrin formation, fibrinolysis inhibition, decreased inflammatory cytokine levels as well as endothelial activation (28). These results become more pronounced during steroid administration (26), suggesting that a prothrombotic condition may not be an independent risk factor for the development of TE but may interact with other risk factors such as the specific chemotherapy used in ALL patients.

It can be suggested from the above data that before or shortly after CVL insertion and/or steroid administration, particularly in ALL patients, testing should occur for the presence of factor V G1691A mutation, and prothrombin G20210A variants.

Biomarkers and haemostatic alterations in paediatric cancer patients

A recent study by Giordano et al. suggested that certain biomarkers might be able to predict the tendency for a hypercoagulable state in paediatric patients with cancer (28). Specifically, biomarkers such as D-dimer and thrombin-antithrombin complex (TAT) were high at diagnosis with ALL (indicative of increased thrombin generation and fibrin formation), but decreased during induction therapy towards normal values. This trend was also observed in the inflammatory cytokines, tumour necrosis factor- α and interleukin 6, suggesting that the reduction of the tumour correlates with the normalisation of the hypercoagulable state (28). Plasminogen activator inhibitor 1, von Willebrand factor antigen and P-selectin levels were also high at diagnosis (indicative of fibrinolysis inhibition and endothelial activation, respectively). These markers, however, increased significantly during induction therapy, suggesting that chemotherapy plays a role in causing injury to the vascular endothelium. While two symptomatic VTE episodes occurred during induction therapy in the study by Giordano et al., there was no conclusion of a specific association between a prothrombotic marker and the thrombotic outcome (28). Therefore, the clinical relevance of prothrombotic biomarkers remains to be established and should be investigated further, as they may be able to provide an accurate prediction of the risk of TE in the paediatric cancer population (28).

While the pathogenesis of VTE in adult cancer patients is well understood, the exact pathogenesis and molecular mechanisms involved in the development of VTE in paediatric cancer patients is not clearly defined (12). It has been documented in children with ALL that there is an increased thrombin activation and thrombin generation, inferring that this may be an important factor in the pathogenesis of VTE in ALL patients (12, 38).

In addition, mechanical factors such as venous stasis are considered important factors in the development of VTE and large tumours are likely to compress the vessels which consequently alter venous flow (8, 12). However, this hypothesis is yet to be confirmed and more research is needed in this area.

Prophylaxis

Catheter maintenance

CVLs increase the risk of TE through various mechanical and biochemical effects including changes in the venous flow, trauma to the endothelium or by hyperosmolar substances infused through the CVL (10, 39). As a result, it is of utmost importance that the patency of the CVL is maintained. When comparing the efficacy of standard unfractionated heparin (UFH) and saline, it was found that UFH was superior at maintaining catheter patency and reducing infection in CVLs (17).

Primary prophylaxis

It has been suggested that patients with inherited prothrombotic defects undergoing intensive therapy such as L-asparaginase, adolescent patients undergoing major surgery and patients with a prior episode of TE should be considered for primary thromboprophylaxis. However, there have been no clear guidelines about the use of thromboprophylaxis in paediatric cancer patients.

LMWH vs UFH

There have been several prospective studies that investigate primary prophylaxis in the general paediatric setting. LMWH was compared to the standard of care, which was UFH flush, or infusion (<3 U/kg/hour) in children with CVLs, demonstrating that there was no difference in the rate of VTEs between the two treatment arms (40). However, while this study included 186 participants, only 51.1% and 50% of children had cancer in the LMWH and UFH treatment arms, respectively (40). In paediatric sarcoma patients undergoing major surgery LMWH has been shown to be effective for primary prophylaxis (41). LMWH also seems to be an effective prophylactic agent in children with ALL that are undergoing L-asparaginase therapy who also have inherited prothrombotic defects; however, the study only provides pilot data for future randomised trials investigating LMWH as a primary prophylactic agent during ALL therapy (37).

Antithrombin (AT) concentrate

The use of AT concentrate for primary prophylaxis in paediatric ALL cancer patients undergoing L-asparaginase therapy has increased and demonstrated a trend for a beneficial effect; however, the result was not statistically significant, potentially due to the low sample size (13).

Warfarin

The use of low-dose oral warfarin for primary thromboprophylaxis in a study population of 62 paediatric oncologic patients with CVLs was investigated in a randomised controlled study (35). In this study, 80% of the children had international normalised ratios (INRs) in the target range (INR 1.3–1.9) for more than 50% of the study period. However, the dose to achieve this fluctuated a lot due

to significant inter- and intra-individual variations (35). Additionally, none of the patients had the intended level of INR for the whole study period (35). They found that incidences of CVL-related VTE that occurred in the jugular vein (where CVLs were placed) were equally as frequent in the children on low-dose warfarin compared to those who were not (35). Therefore, there were no benefits observed of warfarin in children with malignancies to prevent asymptomatic VTE associated with CVL use.

Outcomes of thrombosis in children with cancer

Outcomes for paediatric cancer patients who have suffered TEs are highly variable (► Table 2) and could reflect the heterogeneity of the population. As a consequence of these TE-related complications, chemotherapy is often interrupted which is known to reduce the chances of treating the cancer (9, 42). In patients with ALL and thrombosis, over 50% of TEs are reported in potentially life-threatening sites such as the CNS, right atrium and in the lungs (PE) (14). In children with lymphoma, 44.4% have recurrent TE while 33.3% have signs and symptoms of post-thrombotic syndrome (18). In paediatric patients with sarcoma, 60% of children that had a TE had an adverse outcome (21).

Areas that require further research

There are many different aspects related to TEs in paediatric cancer patients that require further research. One of the major areas that need to be explored further is the pathogenesis of TE in the paediatric cancer population. Gaining knowledge in this area will assist in the development of risk stratification of patients and thus improve the outcomes of paediatric cancer patients at risk of thrombosis. These insights can also then facilitate further areas of research such as thromboprophylaxis and treatment of TEs within this population. Currently, the guidelines for thromboprophylaxis in paediatric cancer patients are unclear and thus determining the efficacy and safety of anticoagulants in sub-populations and/or specific risk groups is essential. There have also been no prospective studies that successfully evaluate the relationship between TE

and infection and this is therefore another important area that needs to be investigated.

After detailed review of the available studies that investigate TE in children with cancer, the two groups of patients that are at the highest risk of developing thrombosis are ALL patients undergoing concomitant L-asparaginase and corticosteroid therapy and the overall use of CVLs. Therefore, other treatment options for paediatric patients with ALL may need to be further investigated due to the high incidence of TEs that occur in patients subjected to this type of chemotherapy.

Other areas of research that should be explored further include studies that compare the survival rates of paediatric cancer patients who have had a TE to those who have not had a TE. This kind of information is important because it is currently not known how TE events impact the outcomes of this population. The notion that biomarkers could facilitate prediction of TEs in paediatric cancer patients is also interesting and potentially beneficial. Hence, directed studies should investigate potential biomarkers that could be used in this setting. Most of the retrospective studies examined in this review focus on TEs in children across all types of cancers whereas prospective studies have focused largely on TEs in ALL paediatric patients; therefore, there is a need for more large randomised trials that focus on the whole paediatric cancer population.

Conclusion

TE is a common complication in the paediatric oncology population, with a number of biochemical and mechanistic risk factors playing a role in development of thrombosis in this population. Accurate identification of children at high risk of thrombosis in this setting remains problematic, and prospective cohort studies and clinical trials are required to better understand the aetiology and define the risk factors. This will in turn allow for development of age-specific guidelines for risk stratification of patients and hence better directed individualised prophylaxis and treatment that will improve the outcomes of children with cancer who are at risk of thrombosis.

Conflicts of interest

None declared.

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Table 2: Outcomes for paediatric patients with VTE.

CVL-associated TE outcomes	Incidence
Infection	22–40% [8, 9]
Post-thrombotic syndrome	15–35% [8, 9]
Patients with ALL and CVLs VTE outcomes	Incidence
Recurrent VTE	4–19% [30, 46, 47]
Post-thrombotic syndrome	5–25% [30, 46, 47]
PE	8–15% [30, 46, 47]
Death	2–4.8% [30, 46, 47]

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Letter to the Editor

The microparticle-specific procoagulant phospholipid activity changes with age

Sir, Procoagulant microparticles (MPs) are small membrane vesicles shed from the plasma membrane surface and which express a variety of phospholipids and proteins specific to the cell they originate from [1]. MPs are released by any cell type into the vascular compartment during either cell activation or apoptosis [2]. Anionic phospholipids present in the microparticle membranes have procoagulant activity *in vivo* through their role as catalytic sites for factor Xa and thrombin formation [3].

Increased procoagulant phospholipid activity (PPA) of MPs is associated with many pathological states, including cancer, cardiovascular disease, infection and inflammatory diseases, as well as other clinical states associated with platelet activation. [4, 5]. Therefore, detection and measurement of MPs have a potential application in the study of different pathologies, as well as a prognostic role and could serve as a tool in monitoring effectiveness of therapy in the setting of major diseases.

The procoagulant Phospholipid kit, STA-Procoag PPL (Asnières-sur-Seine, Diagnostica Stago, France), determines the functional haemostatic effect of MPs. Specifically, the STA-Procoag PPL kit measures the clotting time of a plasma sample that is added to human phospholipid depleted plasma in the presence of excess exogenous factor Xa and calcium. In this assay system, the phospholipid-dependent acceleration of prothrombin activation by the factor Xa/factor Va complex directly impacts the test result, recorded as clotting time. Therefore, shorter clotting times are associated with increased phospholipid content and are an indication of increased concentration of MPs.

This study aimed to measure the age-specific differences in circulating MPs in healthy neonates, children and adults.

All blood samples were collected in S-Monovette® tubes (Sarstedt, Nümbrecht, Germany), containing 1 volume of citrate per 9 volumes of blood. Samples were collected, processed and stored according to an established protocol [6]. Informed consent was obtained from the

parents of the neonates and children and from the adult participants themselves. All participants were healthy individuals, were without previous thromboembolic events, had family history of bleeding or thrombosis and were not subjected to any form of anticoagulant therapy.

For paediatric samples, the inclusion criteria were healthy children attending the hospital for minor day surgery and who did not have a family history of coagulation disorders (e.g. thrombosis) nor were they taking any medications. Adult samples were from healthy volunteers aged over 18 years who were not taking medication and did not have a family history of coagulation disorders. Family history was assessed via a brief interview with the parents of the children and the adult volunteers themselves. This study was approved by the Melbourne Royal Children's Hospital Ethics in Human Research Committee, reference number 20031.

Neonatal samples were collected from healthy term neonates from the postnatal wards at the Royal Women's Hospital, Melbourne. Selection criteria included gestational age of more than 37 weeks, birth-weight over 2500 g, APGAR score of at least 7 at 5 min and absence of systemic abnormalities as well as no family history of coagulation disorders. One milligram of vitamin K was administered intramuscularly in the delivery room, as per normal clinical practice. Samples were collected on day one or day three postbirth, via clean venepuncture using the 'broken needle technique' from the dorsum of the hand. This study was approved by the Royal Women's Hospital Research Ethics Committee (EHRC 02/08).

Plasma samples were tested individually and grouped for analysis into the following age groups: Day 1 and Day 3 neonates, 1 month to 1 year, 1–5 year olds, 6–10 year olds, 11–16 year olds and adults based on seminal studies performed by Andrew *et al.* [7, 8].

Plasma samples were thawed at 37 °C for 10 min and assayed using the Procoagulant Phospholipid kit on the STA-R analyser (Diagnostica Stago) as per manufacturer's instructions.

Statistical analysis was performed using the statistical software package STATA (STATA Corporation, College

Table 1. Age-specific procoagulant phospholipid content of plasma

	Day 1 Neonates	Day 3 Neonates	<1 years (0.17–0.99)	1–5 years (1.1–4.8)	6–10 years (5.1–9.5)	11–16 years (10.4–15.9)	Adults (20–40)
Age-group (Range)	<i>N</i> = 10 (5M/5F)	<i>N</i> = 10 (5M/5F)	<i>N</i> = 20 (10M/10F)	<i>N</i> = 20 (10M/10F)	<i>N</i> = 20 (10M/10F)	<i>N</i> = 20 (10M/10F)	<i>N</i> = 20 (10M/10F)
PPL assay (s)	41.4 (32.8–48.2)*	40.8 (34.4–46.3)	31.6 (20.6–40.7)	27.7 (22.8–35.4)**	26.1 (20.0–33.2)**	27.6 (20.7–36.2)**	36.4 (29.6–43.2)

The first row denotes the mean value and the second the boundaries including 95% of the population.

*Denotes values that are significantly different from adult values ($P < 0.05$).

**Denotes values that are significantly different from adult values ($P < 0.0001$).

Station, TX, USA). Results are expressed as mean and boundaries including 95% of the population (between 2.5th and 97.5th centiles). Differences between the age groups were evaluated using the *t*-test, with a *P*-value of less than 0.05 considered statistically significant.

The results demonstrate significant age-specific differences in the Procoagulant phospholipid content of plasma (Table 1).

The PPL clotting times were increased in neonates and decreased in children aged 1–16 years, indicative of decreased and increased concentrations of MPs in these populations, respectively.

This is the first study that measured PPL clotting times in neonates and therefore the first report of a potential decrease in the number of MPs in the neonatal population. The significance of this finding is currently not known and should be investigated in future. However, our results in the paediatric population are consistent with published evidence showing increased levels of MPs in healthy individuals aged less than 18 years compared with adults [9].

The main limitation of this study is that the PPL assay provides a surrogate measure for the number of MPs. In addition, this assay does not provide information about the cellular origin of the microparticles. Future studies should focus on age-specific differences in the number of MPs using direct measures such as flow cytometry, as

this knowledge is important for the understanding of developmental physiology and disease development.

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Developmental haemostasis: age-specific differences in the quantity of hemostatic proteins: reply to a rebuttal

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The authors would like to thank George M. Rodgers for the letter in response to our recent article [1].

We would like to highlight our earlier publication as a comprehensive study of standard laboratory coagulation tests, including prothrombin time, activated partial thromboplastin time, fibrinogen, and antithrombin, among others [2].

We are in agreement with Rodgers that the differences observed between the studies may be due to disparities in instrument-reagent combinations, types of assays, sample size, and population differences. To support this, we would like to highlight the ISTH position paper by Ignjatovic *et al.* [3], which specifically addresses issues concerning the impact of developmental hemostasis on laboratory tests used to diagnose coagulation disorders and to monitor anticoagulants.

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Disclosure of Conflict of Interests

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BRIEF REPORT

Beta (β)-antithrombin activity in children and adults: implications for heparin therapy in infants and children

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Summary. *Background:* Antithrombin, a hemostatic protein and naturally occurring anticoagulant, is a major thrombin inhibitor. The capacity of antithrombin to inhibit thrombin is known to increase a 1000-fold whilst in the presence of unfractionated heparin. β -antithrombin is an isoform of antithrombin with a high affinity for unfractionated heparin. This study aimed to determine the differences in the anticoagulant activity of the β -antithrombin isoform in children compared with adults. *Methods:* Plasma samples were obtained from 105 healthy individuals from the following age groups: neonates (day 1 and day 3), 28 days to 1 year, 1–5 years, 6–10 years, 11–16 years and adults. The method utilized to measure the activity of β -antithrombin in plasma is a modified version of the total antithrombin assay routinely used in diagnostic laboratories. The modified version of this assay allows for the specific quantification of the β -antithrombin glycoform anticoagulant activity alone, as the β -antithrombin molecule is activated under a high salt concentration, which in turn does not allow activation of other antithrombin isoforms. *Conclusions:* This study demonstrated that there are no age-specific differences in the activity of β -antithrombin. However, considering that the total AT activity is significantly reduced in neonates, our results suggest that in this population β -antithrombin activity is a major contributor to the overall activity of AT.

Keywords: anticoagulants; antithrombin III; blood coagulation factors; heparin; pediatrics.

Introduction

Thrombosis is a significant problem in the unhealthy pediatric population, with 95% of venous thromboembolism (VTE) events that occur in children being secondary to underlying diseases such as cancer, trauma, surgery and congenital heart disease. Whilst thrombosis is rare in healthy children, the annual incidence of VTE in hospitalized children exceeds 58 per 10 000 admissions, making thrombosis a major cause of secondary morbidity and mortality in seriously ill children [1].

Heparin, the most commonly used anticoagulant in pediatric clinical practice, is used for the treatment and prophylaxis of thromboembolic events, with unfractionated heparin (UFH) being the most common heparin used, as recommended by The College of American Pathologists and the American College of Chest Physicians guidelines [2]. Whilst there are new oral anticoagulants available, they are not regulated for use in children. In children, approximately 50% of all drugs used are unlicensed or off-label, reflecting the small number of specific trials in this young population [3]. UFH is the only anticoagulant with evidence from a randomized-control trial for its use in children [4]. For this reason, UFH remains the most used anticoagulant, particularly in the settings of cardiac disease and cardiac surgery.

At our pediatric tertiary center, The Royal Children's Hospital, Melbourne, up to 11.2% of all in-patients receive UFH, and within the Pediatric Intensive Care Unit (PICU) the frequency of UFH exposure is almost 100% [5,6]. The PICU population represents infants and children with complex and chronic diseases, as well as critically ill children who are now surviving due to improvements in intensive care practices.

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Antithrombin (AT) is a major physiological inhibitor of thrombin that increases its anticoagulant capacity up to 1000-fold by activation with UFH [7]. Different factors, including mutations affecting the heparin binding site and post-translational modifications, may impair the heparin affinity of AT with pathological consequences [8]. The presence or absence of an N-glycan at Asn135, a physiological difference that differentiates the two main plasma AT isoforms, α -AT and β -AT, also has relevant consequences for the heparin affinity of AT. Thus, the β -AT isoform is able to bind UFH with greater affinity compared with α -AT [9]. It has been previously hypothesized that increased β -AT content may provide a protective effect due to its hypothesized more efficient anticoagulation properties [10].

Previous reports from our groups demonstrate age-specific differences in the activity of whole AT, whereby the activity of AT is significantly lower in neonates compared with children and adults [11]. In addition, in a mouse animal model, significant post-translational modifications of AT in neonatal mice are observed [12]. Our present study aimed to determine the activity of the β -AT isoform in healthy infants, children and adults, in order to provide knowledge to guide future clinical use of UFH in the pediatric population.

Methods

Citrated, platelet-poor plasma samples from neonates, children and adults were collected, processed and stored using a previously established collection protocol [11,13,14]. All participants were healthy individuals, without previous thromboembolic events, family history of bleeding or thrombosis, and not subjected to any form of anticoagulant therapy. The pediatric cohort is representative of the general population as they were healthy children about to undergo minor elective day surgery (e.g. circumcision and tonsillectomy) at the Royal Children's Hospital, Melbourne. Other than the need for elective surgery, these children were healthy and were not receiving any medications. This protocol was approved by the Royal Children's Hospital, Melbourne, Ethics in Human Research Committee (EHRC 20031).

Neonatal samples were collected from healthy, term neonates from the postnatal wards at the Royal Women's Hospital, Melbourne. Selection criteria included gestational age of more than 37 weeks, birth weight over 2500 g, APGAR score of at least seven at 5 min and absence of systemic abnormalities, as well as no family history of coagulation disorders. One milligram of vitamin K was administered intramuscularly in the delivery room, as per normal clinical practice. Samples were collected on day one or day three post-birth, via clean venipuncture using a 21 or 23 gauge straight needle with the hub removed ('broken needle technique') from the dorsum of the hand [15]. This study was approved by the

Royal Women's Hospital Research Ethics Committee (EHRC 02/08).

Plasma samples were tested individually and grouped for analysis into the following age groups: neonates ($n = 5$), < 1 year ($n = 20$), 1–5 years ($n = 20$), 6–10 years ($n = 20$), 11–16 years ($n = 20$) and adults ($n = 20$).

The method utilized to measure the activity of β -AT in plasma is a modified version of the assay that is routinely used in diagnostic laboratories to measure the activity of total AT. This method was recently presented at the International Society of Thrombosis and Haemostasis Congress in 2013 [16]. Briefly, the plasma being tested is incubated with thrombin in the presence of UFH, and the residual thrombin is quantitated using a chromogenic substrate (reagents from the Stachrom AT III Kit, Diagnostica Stago, Asnières sur Seine, France) at 405 nm on the Power Wave XS plate reader (BioTek, Winooski, VT, USA). The β -AT specific method allows for the quantification of the anticoagulant activity of the β -AT glycoform alone, as the β -AT molecule is activated with UFH under a high salt concentration (1.1 M NaCl), which in turn does not allow α -AT activation. As there is a lower concentration of β -AT in plasma, compared with α -AT, four times the amount of plasma was used as compared with the standard total AT activity assay.

Statistical analysis was performed using the software package GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). An overall comparison across the age-groups was performed with a one-way ANOVA followed by Bonferroni's *post-hoc* test. P values < 0.05 ($P < 0.05$) were considered significant. Neonatal and pediatric age groups were compared with adults by use of a two-sample *t*-test to compare the activity of β -AT, with $P < 0.05$ considered to be statistically significant.

Results and discussion

The results demonstrate that the activity of β -AT does not change with age (Table 1). However, considering that

Table 1 Age-specific β -AT activity

	β -AT activity (% adult value)	Standard deviation	P -value (compared with adult)
Neonates $n = 5$	78	45.8	0.63
< 1 year old $n = 20$	114	38.4	0.54
1–5 years $n = 20$	101	35.4	0.95
6–10 years $n = 20$	116	41.4	0.53
11–16 years $n = 20$	105	41.1	0.86
Adults $n = 20$	100	36.3	N/A

the function of total AT is significantly reduced in neonates [17], we suggest that significant AT activity in neonates can be attributed to β -AT, the isoform with greater UFH affinity, as compared with older children and adults.

This is consistent with previously published data demonstrating that the amount of AT that binds to UFH is highest in the neonatal population as compared with other pediatric age groups, as well as adults [14]. It is well established that neonates require increased doses of UFH to achieve the same therapeutic targets as adults [18]. This difference in dosing has been attributed to the increased clearance of UFH observed in neonates, as demonstrated in the pharmacokinetic study of UFH, with the half-life of UFH in neonates being 35.5 min, compared with 63.3 min in adults [19].

In addition, β -AT in rabbits has previously been demonstrated to have a higher affinity for both injured and intact vessels both *in vitro* and *in vivo* compared with the other AT isoforms [20,21]. Furthermore, present on the endothelial cell surfaces of rabbits is heparin sulphate, a glycosaminoglycan that is structurally similar to UFH and is able to complex with AT to inhibit thrombin [22]. Whilst these findings are yet to be demonstrated in humans, it could be hypothesized that the increased β -AT in neonates may lead to increased thrombin inhibition on vessel walls, in turn leading to reduced fibrin formation and subsequently contributing to the decreased incidence of thrombosis that is observed in healthy neonates.

The results of this study demonstrate high inter-individual variability of β -AT activity within each specific age group. This variability further emphasizes the need for personalized anticoagulation treatment that will in turn account for variability in AT isoforms.

In conclusion, the results of this study further emphasize the importance of characterization of developmental differences of AT and provide avenues for future investigation into why these differences occur.

Addendum

V. Karlaftis collected the samples, performed the laboratory assay, analyzed the data and wrote the manuscript. G. Sritharan performed the laboratory assay, contributed to the data analysis and contributed to the drafting of the manuscript. C. Attard collected the samples and contributed to the drafting of the manuscript. J. Corral contributed to the laboratory methods utilized and contributed to the drafting of the manuscript. P. Monagle and V. Ignjatovic designed the research study and contributed to the drafting of the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

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