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Letter to the Editors-in-Chief

Investigation of the *in vitro* effect of aspirin and tirofiban in children compared to adults

1. Introduction

Recent studies demonstrate that there are fundamental physiological differences in platelet phenotype, response to agonists as well as the proteome and releasate in platelets across the spectrum of age [1]. Despite these age-specific differences, to date there has never been an investigation of the effect of antiplatelet agents in children. In fact, all dosage guidelines for antiplatelet agents are extrapolated from adult data [2] and patient outcomes are suboptimal, especially in complex clinical situations [3]. In addition, there are currently no studies that demonstrate effective monitoring strategies for antiplatelet therapy in children using gold standard tests.

Aspirin and tirofiban are common antiplatelet agents used in paediatrics and function through interaction of canonical pathways of platelet function. Aspirin irreversibly binds to cyclo-oxygenase-1 (COX-1), preventing formation of Thromboxane A2 (TXA2), a thrombotic compound [4]. Thromboxane B2 (TXB2) release correlates to and can be used as a marker of platelet activation [5]. Tirofiban reversibly binds to the fibrinogen receptor GPIIb/IIIa on platelets, preventing platelet aggregation [6]. No clinical trials of aspirin have assessed the optimal dose for antithrombotic prophylaxis or treatment for children, while the package insert for Tirofiban states that safety or efficacy has not been established for children and does not recommend its use in paediatrics.

Our study investigated the age-specific differences in the *in vitro* platelet response to aspirin and tirofiban.

2. Methods

Blood samples were collected and processed according to established protocols [7]. All participants were healthy individuals without previous thromboembolic events and not subjected to any form of anticoagulant therapy.

Samples were obtained from healthy children attending the hospital for minor day-surgery. The paediatric cohort excluded adolescents taking oral contraceptive and smokers. Adult samples were obtained from healthy volunteers who were not taking any medication (including oral contraceptive), were not smokers and did not have a family history of coagulation disorders. Family history was assessed *via* a brief interview with, and written informed consent was obtained from, 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 32287).

Blood samples were collected *via* direct venous puncture in adults and peripheral venous cannula in children. These collection methods have previously been shown to be interchangeable [8]. Samples were collected into S-Monovette® tubes (Sarstedt, Australia), containing 1 volume of 3.2% citrate per 9 volumes of blood. Within 20 min of collection, blood samples were centrifuged at 180*g* for 15 min at room temperature (Megafuge 1.0R, Heraeus) to acquire platelet-rich plasma (PRP).

PRP was incubated at 37 °C for 15 min with aspirin (Mayne Pharma International Pty. Ltd.) or phosphate buffered saline as a control. Aspirin was prepared by creating a 2000 μg/mL stock solution in PBS, and made up to a final concentration of 500 μM, 250 μM or 50 μM. Samples were incubated again at 37 °C for 15 min with arachidonic acid (AA), (Chronolog, PA, USA), at the concentrations of either 150 μM or 500 μM. This approach was based on established methods [9]. All incubation was carried out in static conditions. PRP was centrifuged further at 2500*g* for 15 min to acquire PPP, which was then stored at −80 °C for ELISA testing.

Prior to testing, frozen PPP/aspirin samples were thawed at 37 °C for 10 min. Samples were analysed using a commercially available TXB_2 Thromboxane ELISA (Cayman Chemical Company, Ann Arbor MI, USA) as per manufacturer's instructions with undiluted PPP. ELISA plates were read for absorbance at 405 nm using a Fluostar Optima Microplate Reader (BMGLabTech, Mornington, Victoria, Australia). This method was selected as TXB2 is an established marker of platelet activation, and can be correlated to platelet inhibition by aspirin.

PRP samples were spiked with tirofiban (Juno Pharmaceuticals) or saline, as control. Final tirofiban concentrations were 5 ng/ml, 10 ng/ml and 50 ng/ml. Samples were incubated at 37 °C whilst stirred, prior to being activated with ADP (Chronolog, PA, USA) at either 1.25 μMor 5 μM.

Aggregation in the activated PRP sample was analysed using Light Transmission Aggregometry (LTA), with the Stago Diagnostica Medical TA-8V Platelet Aggregometer (SD Innovation, Marseilles, France), calibrated with PPP as per manufacturer's instructions. As LTA is considered the gold-standard for investigating platelet aggregation it was selected to measure the effect of Tirofiban.

The results are expressed as as a boxplot with median (50th percentile) and interquartile range (25th and 75th percentile) for the different experimental conditions performed. Differences between groups were compared using a non-parametric Wilcoxon-Mann-Whitney test, with $p < 0.05$ considered statistically significant. Statistical software package Graphpad Prism 7 (Graphpad Software Inc., California) was used for data processing and analysis.

3. Results

Participant demographics are reported in Table 1, and were stratified by age group – young children, older children and adults. There was a minimum of 6 participants in each group.

The antiplatelet effect of aspirin and tirofiban across different age groups is shown in Fig. 1. There was a significant increase in the concentration of TXB_2 in the presence of 125 μM AA in the younger children compared to adults ($p = 0.0027$). There were age-specific differences in the concentration of TXB_2 across samples spiked with three different concentrations of aspirin and across both the low and high concentration of AA (p-values 0.0007 to 0.0426) (Fig. 1A). These

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Table 1
Demographics for the study participants.

	Aspirin			Tirofiban		
	2 to 7 years	8 to 15 years	Adults 20 to 41 years	2 to 7 years	8 to 15 years	Adults 20 to 41 years
Total Number of Subjects	8	6	6	10	6	7
Median Age (range) in years	5.7 (3.0 to 7.8)	10.4 (8.5 to 14.5)	28.9 (20.2 to 41.8)	3.9 (2.2 to 4.5)	12.8 (9.5 to 14.1)	25.2 (20.2 to 41.8)
Sex (M[a]/F[b])	7/1	3/3	3/3	5/5	4/2	3/4
Mean Platelet Count in PRP × 10^9/L (range)	404 (310 to 505)	419 (306 to 530)	416 (366 to 453)	516 (344 to 623)	445 (339 to 621)	492 (353 to 638)

[a] M – Male.
[b] F – Female.

changes were especially evident when comparing the younger children and adults.

The only age-specific difference in the activity of tirofiban was between younger children and adults ($p = 0.0401$), at the highest concentration of ADP and tirofiban (Fig. 1B).

4. Discussion

The age-specific differences in the haemostatic system of children and adults have been well established and are explained by the concept of developmental haemostasis. We have previously investigated the impact of developmental haemostasis on the age-specific effects of anticoagulants [10,11]. Whilst the antiplatelet effects of aspirin and tirofiban have been extensively studied in adults, this is the first study to determine the effect of these drugs on paediatric platelets compared to platelets from adults.

Aspirin and tirofiban may be used either alone or in conjunction as a prophylactic treatment for patients with ventricular assist devices (VAD), Blalock-Taussig (BT) Shunts and Fontan; as well as for patient management in the context of Extracorporeal Membranous Oxygenation (ECMO) and cardio-pulmonary bypass for cardiac surgery. These treatments have previously been monitored using thromboelastography, LTA and VerifyNow assays. However a lack in standardisation between clinical diagnostic laboratories and operator-dependant variability makes these means of measuring platelet function inappropriate [12].

This is particularly important in the context of the fact that platelets from children have recently been shown to be different to those from adults in terms of their phenotype and function [1,13]. The increased responsiveness of paediatric platelets to AA is consistent with previously published research that determined the hyper-responsiveness of paediatric platelets to agonists such as ADP using whole blood flow cytometry [13]. Other studies using platelet aggregometry have identified limitations to this methodological approach and recommend it should only be used for disease screening and not as a diagnostic test [12]. The high inter-individual variation in platelet response at baseline, as well as to agonists and antiplatelet agents observed in our study is consistent with other similar studies [13,14]. It has been hypothesized that this inter-individual variation may be attributed to genetic variations in the COX enzyme, environmental factors and aspirin resistance [14]. However, the exact mechanism contributing to the inter-individual variability related to platelets in the paediatric population is yet to be determined.

The results of our study demonstrate that the antiplatelet effect of aspirin may be affected by developmental haemostasis *in vitro*, with age-specific differences in platelet inhibition. In our study, the concentration of TXB2 was directly proportional to the level of platelet activity in samples. We observed the response to AA to be significantly different in children compared to adults. This is consistent with previous evidence of hyper-reactivity of paediatric platelets to AA compared to adults. We further demonstrated that a reduced inhibition of platelet activation is translated as a reduced effectiveness of aspirin in children. This result may be attributed to the initial hyper-reactivity of the platelets in children, which can potentially be caused by inter-individual variability in the COX enzyme in the paediatric population.

Our results using aggregometry suggest age-specific effects of tirofiban at high doses. However, considering the limitations of platelet aggregometry, it is unclear if this result reflects physiology or is a result of the sensitivity of the assay.

5. Limitations

This study used only TXB2 ELISA to analyse platelet function after aspirin treatment which, alone, may be insufficient to quantify platelet inhibition. It has been shown that functional assays, such as light transmission aggregometry, are highly variable for investigating aspirin compared to TXB2 quantification [15]. In light of this, and due to limited available sample volume from paediatric participants, we chose to use only a TXB2 ELISA as a direct measure of cyclooxygenase inhibition.

Future studies should extend the results of this study by using flow cytometry to comprehensively characterise age-specific changes in platelet inhibition after aspirin treatment. Due to the small sample size, it is unclear if these results may be due to other confounding variables such as gender, platelet count, plasma components or other individual variables.

An important consideration in the tirofiban aspect of this study is the utilisation of platelet aggregometry which has previously been shown to be insensitive to certain differences in platelet response [16]. Using a sensitive method such as whole blood flow cytometry, we have shown that responsiveness to ADP is increased in children compared to adults [13]. However, these age-specific differences were not seen using platelet aggregometry. Hence, it is likely the small increases in responsiveness are masked by the high dose of agonist required for aggregometry. As such, platelet aggregometry is not a sensitive method when it comes to investigating age specific differences. Furthermore, the large volumes of blood required for platelet aggregometry for young children makes this method unfavourable. Future studies of tirofiban should use more sensitive techniques that require smaller volumes such as flow cytometry to measure the effects of tirofiban on ADP induced platelet aggregation to confirm these observations.

The next step in improving our understanding of the effect of antiplatelet agents in children is to use *ex vivo* samples from children receiving antiplatelet therapy, to get us one step closer to appropriate *in vivo* clinical studies required to determine evidence-based dosage recommendations.

Author contributions

CM and XBC performed the experiments, analysed the data and

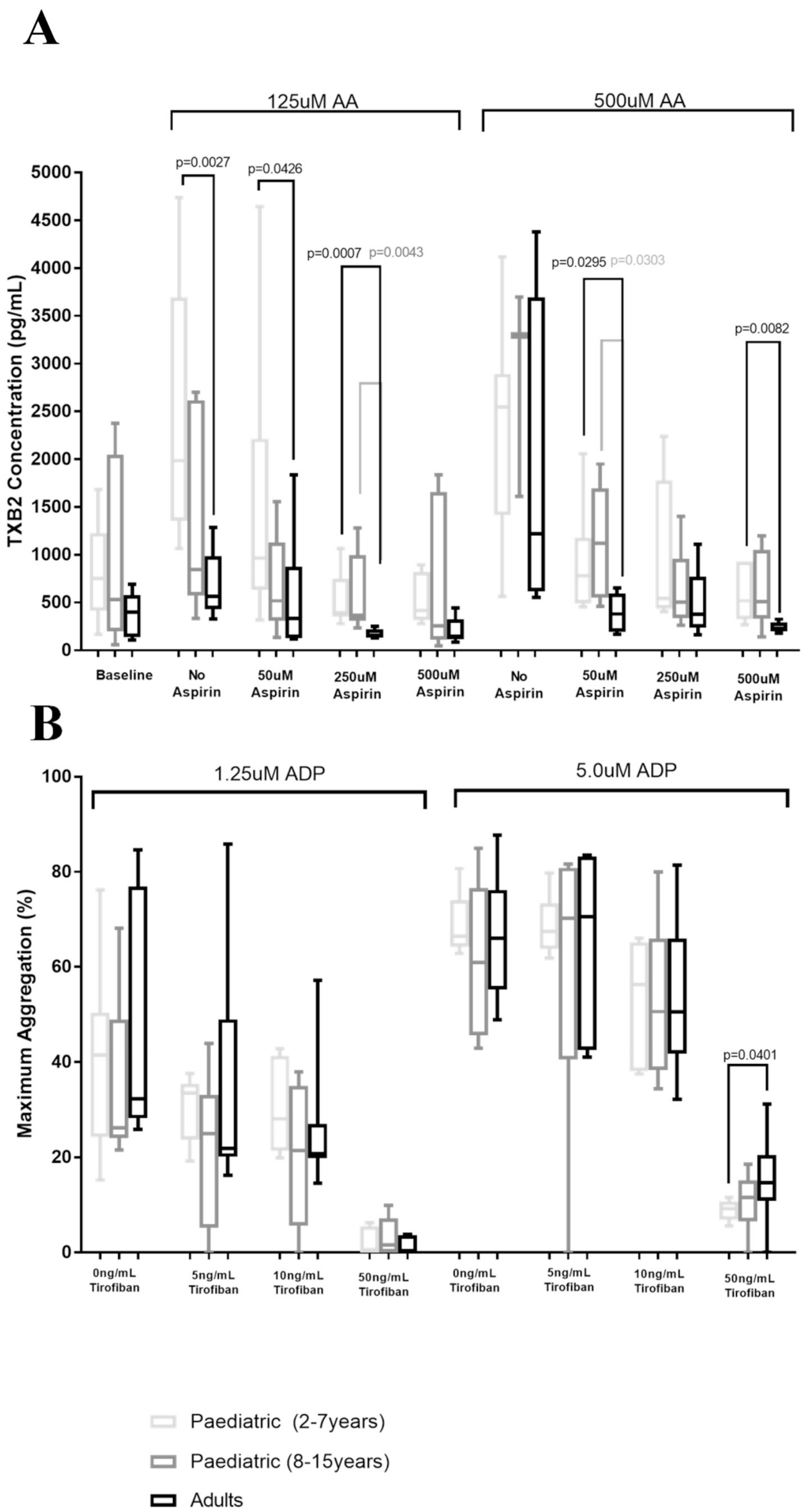

Fig. 1. A, Thromboxane B2 (TXB2) synthesis and release from platelets treated with increasing concentrations of aspirin and activated with two concentration of arachidonic Acid (AA). Measurements taken as total TXB2 concentration in Platelet Poor Plasma and expressed as a boxplot with median (50th percentile) and interquartile range (25th and 75th percentile). *p*-Values calculated using a non-parametric Wilcoxon-Mann-Whitney test and values < 0.05 were considered significant.
B, Maximum aggregation of platelets measured using platelet aggregometry of Platelet Rich Plasma treated with increasing concentrations of tirofiban and activated with two concentrations of Adenosine Diphosphate (ADP). Results are expressed as a boxplot with median (50th percentile) and interquartile range (25th and 75th percentile). p-Values calculated using a non-parametric Wilcoxon-Mann-Whitney test and values < 0.05 were considered significant.

wrote the manuscript. JC assisted with performing and planning experiments. VI oversaw the project and reviewed the manuscript. ML assisted experiment setup and reviewed the manuscript. PM reviewed the manuscript.

Declaration of Competing Interest

The authors have nothing to disclose.

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