BRITISH SOCIETY FOR HAEMOSTASIS AND THROMBOSIS

Annual Scientific Meeting 2023

Novel therapeutics & emerging technology in haemostasis & thrombosis

Wednesday 25 – Friday 27 January

Edgbaston Park Hotel & Conference Centre, Birmingham

Summer students abstracts

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Summer student abstracts

Investigating the haemostatic side effects of Pim kinase inhibitors and their impact on thrombopoiesis, megakaryocyte maturation and platelet production

Farieda Kassim
2nd Year Undergraduate student, Biomedical Sciences, Manchester Metropolitan University

Background: Drug induced thrombocytopenia and subsequent increased risk of bleeding are frequent unexpected side effects of a variety of pharmaceutical agents, in particular small molecule drug therapy[1]. These side effects can often be so severe they lead to a significant increase in mortality in long term users of such drugs[2]. Drug induced bleeding events and thrombocytopenia are often attributed to reduced platelet production and function[1]. Platelets are produced by megakaryocytes and are essential for haemostasis[3]. Megakaryocytes are derived from hematopoietic stem cells (HSCs) that reside mainly in the bone marrow (BM), differentiate, mature, and produce platelets via a process called thrombopoiesis driven by thrombopoietin (TPO)[4]. Accelerated, reduced and abnormal platelet production have implications for normal physiology, with thrombocytopenia and platelet dysfunction associated with bleeding, and thrombocytosis and platelet hyperreactivity with thrombosis.

Our long-term goal is to identify novel therapeutic targets that can either stimulate or repress platelet production and function. This ability to regulate megakaryocyte maturation, platelet number and function would be a valuable clinical tool in treating platelet related morbidities including bleeding and thrombosis.

We have identified Pim kinase as a positive regulator of platelet function, with pharmacological inhibitors that target Pim kinase, eliciting exciting anti-platelet and anti-thrombotic activity[5]. The family of Pim (proviral insertion in murine lymphoma) kinases, Pim-1, -2, and -3, are homologous, constitutively active, serine/threonine kinases that despite being shown to be highly expressed in haematopoetic cells, their roles remain largely unexplored[6]. Phase I clinical trials using Pim kinase inhibitors for the treatment of several malignancies, including myeloma, myelofibrosis and leukaemia have demonstrated drug efficacy and shown AZD1208, PIM447 and TP3654 to be safe and tolerable at therapeutic doses. However, a small subset of patients taking Pim kinase inhibitors during Phase I clinical trials experienced thrombocytopenia and bleeding events, supporting the need to investigate the haemostatic effects of Pim kinase inhibitors further, yet the reason for these observations remains unknown[7-9].

This proposal will lead to mechanistic insight underlying the role for Pim kinase in the regulation of haematopoietic stem cell differentiation and thrombopoesis, which will ascertain the translational potential of developing Pim kinase inhibitors as both haematological and anti-thrombotic therapeutic compounds.

Aims: To elucidate the contribution of Pim kinase to the regulation of megakaryocyte maturation and platelet production.

Hypothesis: Pim kinase inhibitors will delay megakaryocyte maturation and platelet production

To investigate this we will use bone marrow derived megakaryocytes from WT mice to meet the following objectives:

1. Investigate the effect of pharmacological pan-Pim kinase inhibitors on megakaryocyte development.
2. Investigate the effect of pharmacological pan-Pim kinase inhibitors in the regulation of platelet formation.

Experimental plan: We will investigate the effect of Pim kinase inhibition on bone marrow derived megakaryocyte maturation and proplatelet formation in culture, using 3 Pim kinase inhibitors that have

entered into clinical trials. We will perform dose response curve analysis of physiologically relevant concentrations of pan Pim kinase inhibitors; AZD1208, PIM447 and TP3654 (0, 0.1-10 μM) on megakaryocytes derived from the bone marrow of murine (C57/BL6) femurs.

Objective 1: Investigate the effect of pharmacological pan-Pim kinase inhibitors on megakaryocyte development. Murine bone marrow derived megakaryocytes, isolated from bone marrow, will be cultured for 4 days with thrombopoietin supplementation, and maturation determined using flow cytometry. The proportion of CD41+ (megakaryocyte progenitors) and CD41+CD42d+ (mature megakaryocytes) event will be determined, in addition to measurement of cell size and granularity (both increase during maturation). We will also use flow cytometry and propidium iodide staining to compare DNA polyploidization (normal ploidy is 16N),

Objective 2: Investigate the effect of pharmacological pan-Pim kinase inhibitors in the regulation of platelet formation. The final stage of thrombopoiesis, is the release of platelets into the blood stream by the megakaryocyte via the formation of proplatelets. Murine bone marrow derived megakaryocytes will be cultured for 3 days with thrombopoietin and hirudin supplementation. On day 3, megakaryocytes will be isolated by BSA gradient, and left for a further 24 hours (+ thrombopoietin and hirudin) to form proplatelets on coverslips. Proplatelet formation will be visualised by staining for CD41 (megakaryocyte marker), DAPI (nuclear stain) and the cytoskeleton (Actin and tubulin) and imaged using confocal microscopy.

Techniques and training: Farieda is an excellent First class student at Man Met, demonstrating flair for modules on haematology and technical applications in biomedical sciences. Her recent Blood Science coursework essay discussing the impact of cirrhosis on thrombocytopenia was rated outstanding and highlights her interest and enthusiasm for haemostasis research.

As part of this Summer Studentship, Farieda will be supervised by Dr Unsworth, Dr Jones and members (PhD students and PDRAs) of the MMU Thrombosis group. Farieda will be given full training in handling and isolating bone marrow, including consideration of NC3R approaches; cell culture of primary cell lines; setting up and running flow cytometry experiments; performing confocal microscopy and appropriate statistical analysis and data presentation.

Farieda will also be expected to attend our weekly Cardiovascular and Thrombosis Group lab meetings, presenting her project proposal in week 1 and a project summary at the end of the studentship. This will provide Farieda with a valuable experience of a research environment and the collegiate nature in which we work and will provide a vital experience communicating scientific information and disseminating research findings. We anticipate these meetings will provide Farieda with broader background knowledge, and insight into how we troubleshoot when faced with experimental challenges.

Gantt chart: Timeline for the Studentship

	Weeks									
Project Timeline	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Induction activities, & completion of risk assessments										
Training in bone marrow preparation & flow cytometry										
Megakaryocyte maturation										
Training in proplatelet formation & microscopy										
Proplatelet formation										
Data Analysis										
Presentation to the CV and Thrombosis Group										

References: *1) Tullemans BME, J Thromb Haemost, 2018, 2) Antithrombotic Trialists C, Lancet, 2009. 3) Stone AP, 2022, Blood. 4) Machlus KR, 2013, J Cell Biol. 5) Unsworth AJ, 2021, Haematologica. 6) An N, 2013, J Hematol Oncol. 7) Coretes J, 2018, Br J Cancer. 8) Raab MS, 2019, Leukaemia. 9) Garrido-Laguna I, 2020, American Society of Clinical Haematology.*

Summer student abstracts

Analysis of platelet transmission electron microscopy in a cohort of patients with a suspected platelet-based bleeding disorder

Lily Redmond-Motteram
University of Birmingham, Yr2 BSc Biomedical Sciences

Work preceding this project:

Over the last 16 years, the Birmingham Platelet Group and the UK Genotyping and Phenotyping of Platelets (GAPP) study

(https://www.birmingham.ac.uk/research/cardiovascular-sciences/research/platelet-group/platelet-gapp/index.aspx) has investigated over 1000 cases of clinically diagnosed mild bleeding in patients with a suspected inherited platelet defect of unknown aetiology (Watson *et al.*, 2013). Patients with existing recognised platelet disorders, including Glanzmann's thrombasthenia, Bernard-Soulier syndrome (BSS), *MYH9*-related disorders, Hermansky Pudlak syndrome (HPS) and Wiskott-Aldrich syndrome have been excluded from this study. In addition, all patients referred show normal findings when performing an initial laboratory coagulation screen. In brief, we have established an extensive multi-centre phenotyping programme for patients with clinically diagnosed platelet function disorders registered at over 25 Haemophilia Comprehensive Care Centres in the UK (Darwood *et al.*, 2012). Each patient sample has been subjected to lumiaggregometry to a panel of six to nine platelet agonists alongside a healthy volunteer. In some cases, we performed additional tests including flow cytometry and measurement of cyclic AMP to gain further information. We have identified a defect in platelet function in ~60% of patients and subdivided these on the pattern of response (Figure 1). Over 70% of patients with a defective response displayed impairment in dense granule secretion, Gi receptor signalling or arachidonic acid metabolism (cyclooxygenase pathway). Subsequently, candidate gene sequencing led to the identification of three new mutations in the $P2Y_{12}$ ADP receptor (bringing the total number reported to 10), and the first reported mutations in the platelet thromboxane (two mutations) and thrombin PAR-1 receptors (Daly *et al.*, 2009; Mumford *et al.*, 2010; Nisar *et al.*, 2011; Watson *et al.*, 2010). Strikingly, three of these mutations are co-inherited with the mild bleeding disorder, type 1 von Willebrand's disease. Thus, we believe that mild bleeding that is platelet-related in origin is severely underdiagnosed and that it may often be multifactorial and should therefore be considered to be a complex trait in a majority of cases (Daly *et al.*, 2014).

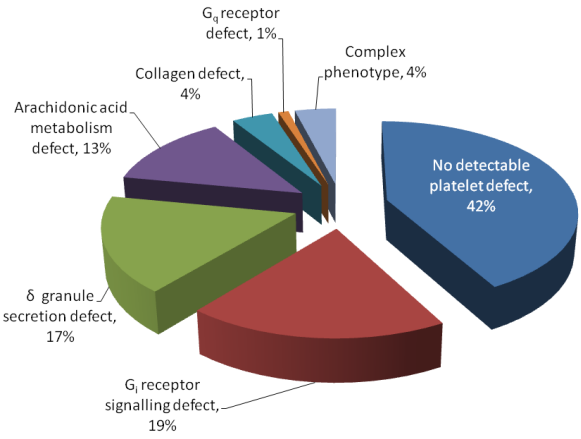

Fig 1: Pie chart showing the distribution of platelet function disorders in patients studied within the GAPP project (Watson *et al.*, 2013). Participants with a suspected PFD were referred from Comprehensive Haemophilia Care Centres throughout the UK and evaluated by lumiaggregometry as part of the GAPP study. Participants whose platelets were observed to have a functional defect were subgrouped based on the defect as shown in the pie-chart. Approximately 58% of participants in this analysis exhibited a defect on lumiaggregometry.

The failure to identify a defect in platelet function in over 40% of patients despite a clinically significant bleeding history could be due to several factors. These include the limited sensitivity of lumiaggregometry as a tool for detecting platelet defects, some overlap of response with healthy volunteers at the limits of the established normal ranges (as is the case for any laboratory test of haemostasis). Defects may only be revealed under flow conditions or in whole blood and as such may be masked by the presence of an anticoagulant or other cell-cell interactions. In addition, it is possible that bleeding is caused by defective fibrinolysis or vessel wall interactions. For these reasons we performed impedance aggregometry (Multiplate®), 96 well platelet aggregation (Lordkipanidze *et al.*, 2014), platelet aggregation on collagen at a typical arterial shear rate (1000 s^{-1}) and clot retraction assays on a subset of patients. Undertaking these assays however did not increase the number of patients identified to have a platelet function disorder over that achieved by lumiaggregometry.

Overall hypothesis:

We propose that a significant proportion of patients (>40%) with no obvious platelet defect, despite having an extensive bleeding history, have defective genes/mutations in other genes involved in haemostasis, and that some of these give rise to impaired platelet function but are not readily picked up in available tests. We will investigate this hypothesis using platelet electron microscopy to identify the possible underlying platelet defects and subsequent cause of excessive bleeding.

Specific Aim:

Identify platelet defects using transmission electron microscopy in patients with a suspected platelet-based bleeding disorders

Plan of the Research:

1. Patient selection

From a total of 1000 patients with a suspected platelet-based bleeding disorder, we have selected a cohort of patients with a possible undefined platelet defect. A total of 29 patients (and additional matched patient control samples) with a history of bleeding, consisting of 4 families and 21 individuals will be studied. These patients range between 3 and 73 years of age, at time of recruitment, and include common platelet-based bleeding phenotypes, such as epistaxis, bruising, oral cavity bleeding, menorrhagia, and haematomas. This cohort includes patients with already identified candidate gene variants including: RUNX1, TUBB1, GATA1, GP1BA, HPS2, and SLFN14, to deduce and potentially link the cause to the observed platelet disorder. Finally, we will consider isolated cases, such as patients with known granule secretion defects, prioritising the patients with the most severe bleeding histories in the first instance.

2. Approaches to EM structural analysis of patient platelets

The above-mentioned patient and control samples already have resin-embedded platelet preparations to be studied. Using a JEOL 1200EX microscope, conventional transmission electron microscopy (TEM) will be carried out to examine the platelet ultrastructure in samples from these GAPP patients, following the standardised thin section TS-PEM method (Chen *et al.,* 2018). This will be performed in association with the School of Metallurgy and Materials at the University of Birmingham, whom house a highly specialised electron microscopy suite to support these studies and will provide training to the student prior to the project. As the samples have previously been embedded in Epoxy resin, the project entails performing microtomy to trim and prepare thin sections to be scoped. The ultramicrotome uses a glass or diamond knife to cut ultrathin sections of approximately 100 nm to be stained and collected onto a TEM grid ready for observation on the electron microscope. By capturing and analysing the EM image, we aim to utilise the magnification of up to 50,000,000x to analyse and examine the ultrastructure of each patients' platelets in comparison to platelets from healthy

volunteers to assess any significant differences. This shall include (but is not limited to) platelet morphology, alpha-granules and the canalicular system, the Golgi complex, and any apparent abnormal inclusions in each of the 28 patients. Therefore, we aim to contribute to the understanding of platelet structure and physiology in inherited bleeding disorders and the reported phenotypes, linking back to any possible gene variants, that is currently excluded from any standardised platelet function tests.

Summary

The proposed 10-week project will provide the named student with training in a diverse set of experimental techniques and open multiple options for further long-term research. The Birmingham Platelet Group at the University of Birmingham have an international reputation for the study of platelet defects and thrombosis research. Techniques to be used in the project will include microtomy, transmission electron microscopy, bioinformatic analysis and interpretation of gene variants which may be attributed to inherited causes of bleeding which link to the EM defect.

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Summer student abstracts

Assessing the efficacy of GPIb inhibitor Anfibatide on S100A8/A9-induced platelet activation

Caitlin Sullivan
University of Birmingham

Scientific Background and Study Design

Background S100A8/A9 is emerging as a biomarker for innate immune cell activation in a range of chronic inflammatory diseases associated with increased risk of stroke and heart attack (1). We have recently identified S100A8/A9 as a novel prothrombotic and proinflammatory damage-associated molecule pattern in severe COVID-19 patients. Unexpectedly, we discovered that S100A8/A9 induces procoagulant and proinflammatory platelets. We have strong preliminary data showing that S100A8/A9 induces platelet activation and procoagulant platelets (Figure 1). This activation occurs through a single receptor, the adhesion receptor glycoprotein Ibα (GPIbα). We identified recombinant GPIbα as the only strategy able to block platelet activation by S100A8/A9, which confirms that this activation is dependent on GPIbα (Figure 2). This interaction was validated in human and mouse platelets (Figure 2). We conducted the first proof of concept study to explore the therapeutic potential of the GPIb-S100A8/A9 axis in reducing platelet activation and procoagulant platelet formation. The formation of procoagulant platelets is not well known, and mostly, there is currently no clinically approved drugs to inhibit the formation of these procoagulant platelets. Our aim is to identify a blocking strategy for this interaction and assess the efficacy of clinically approved drug targeting GPIb-VWF interaction, called Anfibatide, in inhibiting the effect of S100 A8/A9 on platelets (2, 3).

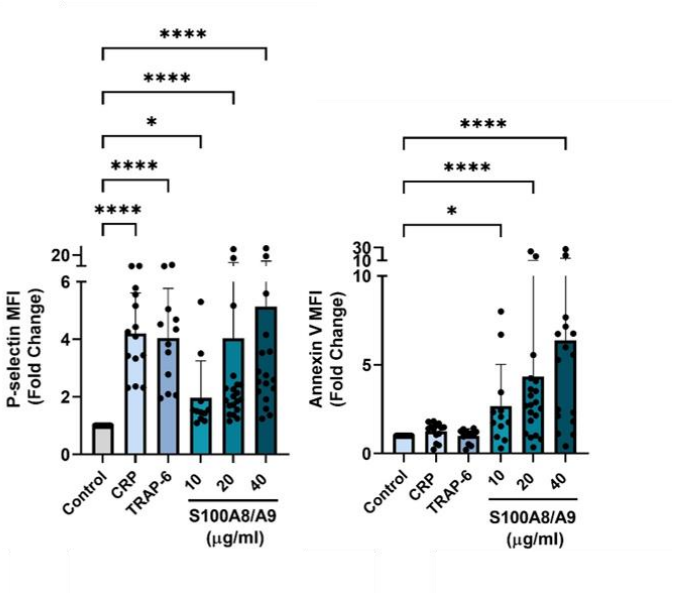

Figure 1: S100A8/A9 induces human platelet activation and procoagulant platelets as measured by increased P-selectin exposure and phosphatidylserine exposure, respectively. CRP and TRAP-6 are used as positive control.

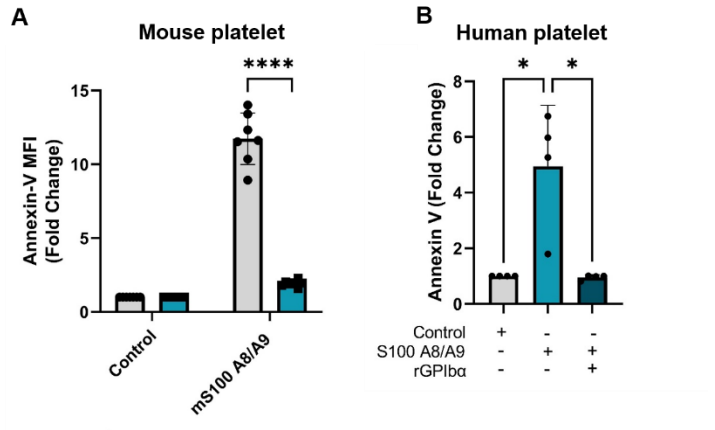

Figure 2: Mouse and human S100A8/A9 induce platelet activation and procoagulant platelets through GPIbα. A. Mouse S100A8/A9 induces procoagulant platelets in wild type (grey) but not GPIb-deficient (blue) platelet. **B**. Human S100A8/A9-mediated procoagulant platelets is inhibited by recombinant GPIbα.

In 2019, a drug targeting GPIb, called Anfibatide, was approved in immune thrombocytopenia (ITP), which is commercialised by Lee's Pharma, Hong Kong.

Aim: We aim to test for the ability of Anfibatide to block this interaction in vitro in human blood. This strategy would allow us to validate the use on Anfibatide as possible drug targeting S100A8/A9-GPIb interaction and to reduce procoagulant platelets in chronic inflammatory diseases.

Methods: Whole blood and human washed platelets will be used. Recombinant S100A8/A9 will be added to whole blood in the presence of different concentration of anfibatide. Platelet leukocyte aggregates will be assessed by flow cytometry using platelet (CD41), neutrophil (CD66b) and monocyte markers (CD14, CD16). In parallel, platelet activation will be assessed using anti-Pselectin antibody and anti-GPIIbIIIa antibody (activated form). Phosphatidylserine is a key marker to assess procoagulant platelets, the main platelet population formed by the addition of S100A8/A9. Phosphatidylserine exposure will be assessed in using Annexin V binding. These experiments will also be assessed using human washed platelets to assess the direct effect of S100A8/A9 and inhibitors on platelets. In parallel, the effect of S100A8/A9 on platelet spreading on S100 A8/A9 will be assessed using immunofluorescence imaging and measurement of PS exposure and P-selectin on spread platelets.

Expected outcome: The data generated would validate whether Anfibatide is able to block S100A8/A9 binding to GPIb.

Training: The student will be trained to isolate platelets from whole blood, assess platelet activation in vitro using flow cytometry and immunofluorescence imaging. The student will be supervised by a post doc, Dr Gina Perrella, and a PhD student Dr Martina Colicchia during her training. The duration of the training is 8 weeks, from 15th of June until 15th of August.

Summer student abstracts

The effect of polyphenols on fibrin clot structure and platelet procoagulant activity

Lutale Metruth Kaselampao (Metruth)

Discovery and Translational Science Department, Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds

Background: Rapid blood clot formation (haemostasis) is the body's primary response to vascular injury that helps to control and avoid life-threatening blood loss. In this process, and in subsequent would healing, platelets and fibrin play a crucial role[1,2]. Nevertheless, excessive platelet activity and fibrin formation can lead to severe thromboembolic disorders[3]. Thrombosis is a major contributor to disease burden globally as it is a serious and potentially lethal element of common cardiovascular diseases such as stroke, ischaemic heart disease and venous thromboembolism (VTE)[4,5]. Another major global public health concern is diabetes mellitus, which affected 451 million adults worldwide in 2017 and is predicted to affect 698 million adults by 2045 without the implementation of effective preventive measures[6]. Individuals with diabetes have been shown to be twice as likely to experience a stroke[7]. Studies have also shown that individuals with diabetes have a 50% increased chance of developing VTE than non-diabetic individuals[8]. Moreover, pulmonary embolism (PE), a complication of VTE, was independently associated with type 2 diabetes mellitus (T2DM) in a multivariate analysis[9]. In the UK, heart and circulatory diseases account for 1 in 3 deaths in diabetic adults[10]. Due to the overlap between diabetes and thrombosis risk further research is critically required on ways we can prevent and reduce thrombosis in patients with diabetes.

One mechanism implicated in the development of thrombosis in obese patients, a population with increased T2DM risk, is chronic inflammation[11]. Thromboinflammation, a recent term used to describe the coordinated activation of thrombotic and inflammatory responses, has been shown to be increased in patients with diabetes and other metabolic diseases[12]. Almost two third of adults in the UK are obese[13], highlighting the critical need for effective prevention of thrombosis in individuals with a heightened inflammatory response. The interaction of platelets with neutrophils, the most abundant leukocyte, has been implicated in the development of thromboinflammation[14], but further research is required to determine potential mechanisms to attenuate this interaction.

It is well-established that dietary habits have an impact on cardiovascular and diabetes risk[15]. Polyphenols, or compounds derived from plants, have been shown to modulate post-prandial glycaemic response[16]. Platelet aggregation and platelet-neutrophil aggregates were decreased following consumption of green tea (EGCG) and cocoa-derived compounds, respectively[17,18]. Patients with diabetes have been shown to have platelets with procoagulant features[19], suggestive of platelets with increased clotting ability. However, the effect of polyphenols at modulating procoagulant platelet development and platelet-neutrophil complex formation remains to be explored. Our previous data shows that inhibition of procoagulant platelet formation alters clot structure (Gauer JS, *Blood Advs*, under review), a well-established risk factor for thrombosis[20,21], but further investigations are required to determine the impact of polyphenols on clot structure.

Aims: This project aims to investigate the effect of polyphenols from different classes (phenolic acids, flavonoids, stilbenes and lignans) known to impact glycaemic response on procoagulant platelet development and platelet-neutrophil complex formation. Furthermore, the impact of polyphenols on clot structure characteristics commonly associated with a prothrombotic clot phenotype will also be explored.

Experimental plan: Whole blood or platelet-rich plasma (PRP) from healthy volunteers will be spiked with different concentrations of polyphenolic compounds from different classes with potential to impact platelet activity, based on existing literature. Number of procoagulant platelets in PRP will be determined by laser confocal microscopy, as previously described[22] (Gauer JS, *Blood Advs*, under review). Number of platelet-neutrophil aggregates in whole blood will be determined using flow-cytometry, following methods previously established[23]. Clot structure analysis will be caried out in PRP by 1. Turbidity, to determine the kinetic profile of polymerizing clots, 2. Laser confocal microscopy, to determine differences in clot density, 3. Permeation, to examine clot pore size and 4. Thrombin generation, to establish effects on production/release of thrombin (following methods described previously)[22,24,25].

Expected value: The results from this project will help identify potential mechanisms through which dietary compounds can modulate procoagulant platelet development and platelet-neutrophil complex formation, shedding light on potential correlations between these markers and thrombinflammatory risk in patients with diabetes and other metabolic diseases. Furthermore, this project will produce novel data on the effects of polyphenols known to modulate glycaemic response on clot structure. This research project will contribute to our current understanding, and support future research, on the suitability of dietary compounds at reducing cardiovascular risk by positively impacting on thromboinflammatory risk factors. The results from this project will be presented by the candidate in the forthcoming 2023 BSHT Annual Meeting.

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