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Characterisation of platelet function and pro-inflammatory phenotype in type 1 diabetes mellitus and double diabetes

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Chapters 1 and 4 refer to the following publications:

Sagar, R.C, Naseem K.M and Ajjan, R.A. Antiplatelet therapies in diabetes. Diabet. Med. 2020; 37, 726– 734.

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For both, I was involved in the conceptualisation with RAA and completed the literature review and manuscript draft. RAA and KMN were involved in the content revision.

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Acknowledgements

Abstract

Patients with diabetes mellitus (DM) have an increased and premature risk of cardiovascular disease. Platelet-driven pathology has been implicated in the multifactorial and complex vascular abnormalities, yet this appears to be only partly studied, particularly in patients with type 1 diabetes mellitus (T1D). T1D has long been considered a more homogenous cohort than T2D but with an ever-growing obesity epidemic, there is an emerging group of T1D individuals displaying insulin resistance, often described as having double diabetes. Early evidence supports even greater risk of poor cardiovascular outcomes in this subgroup.

This thesis has thoroughly investigated the functional and bioenergetic phenotype of platelets in young adults with T1D. Crucially, this little studied cohort was chosen to eliminate confounders such as pre-existing co-morbidities and perhaps more crucially interfering concomitant medications. In addition to the role of glycaemia, the effects of insulin resistance on platelet function was assessed to explore how patients with double diabetes may be impacted. Results indicate that even in young adults with T1D there is evidence of hyperreactive platelets with diminished sensitivity to inhibition by prostacyclin (PGI$_2$). Moreover, this appears exacerbated by sub-optimal short- and medium-term glycaemic control with insulin resistance further adding to the adverse platelet profile.

Contributing mechanisms were also explored through the assessment of platelet bioenergetic and metabolic profiles. My data strongly suggest a metabolic rewiring process in platelets from patients with T1D, characterised

by reduced glycolysis and increased mitochondrial respiration which may be driven, at least in part, by a shift from glucose usage towards fatty acids.

Together these data suggest that the disease environment leads to metabolic reprogramming of platelets that could be a causal factor in platelet hyperreactivity in individuals with diabetes. Further studies are required to determine the precise molecular mechanisms and whether targeting of platelet energy metabolism could be a novel therapy that mitigates thrombotic risk in these patients.

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Relevant publications and presentations

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R.C. Sagar, S.M. Pearson, N. Kietsiriroje, M. Hindle, K. Naseem, R. Ajjan. Sub-optimal glycaemic control and insulin resistance in young adults with type

1 diabetes increases platelet expression of P-selectin and phosphatidylserine. OP17. 58th EASD Annual Meeting of the European Association for the Study of Diabetes. Stockholm, Sweden.

Chapter 1: Introduction

Approximately 5 million people in the United Kingdom live with diabetes, 90% of whom have type 2 diabetes mellitus (T2D) with 8% having a diagnosis of type 1 diabetes mellitus (T1D) [1]. 10% of the annual NHS budget is spent on treating people living with diabetes (DM), with the vast majority of this expenditure relating to the associated complications. T1D is associated with excess all-cause and in particular, cardiovascular, mortality along with premature cardiovascular morbidity [2-4]. A better understanding of those at greatest risk and the determinants of such complications is crucial to not only reduce the financial burden within an ever-increasingly stretched NHS but also improve quality of life for those living with T1D.

1.1 Vascular disease, endothelial dysfunction and diabetes

The vascular endothelium is composed of a single layer of cells which line the vascular lumen [5]. For many years the endothelium was considered to be an inactive barrier, separating circulating blood from the cells of tissues. It is now widely accepted that it actually possesses a variety of important functions and facilitates many key cell-cell interactions that are vital in maintaining normal vascular physiology [5, 6]. Its role extends extensively and includes functions in thrombosis, platelet activation, fibrinolysis and inflammation as well as maintaining vascular tone and facilitating cell adhesion. Endothelial dysfunction is therefore an important consideration in the case of vascular disease and is an abnormality commonly seen in patients with diabetes. In disease states, including diabetes, endothelial dysfunction drives a proinflammatory and prothrombotic environment that promotes vascular thombo-occlusive complications [7-9]. Furthermore, there is now clear

evidence of a strong relationship between endothelial dysfunction and platelet function driving the development of cardiovascular disease [10, 11].

Crucially, the endothelium maintains a delicate equilibrium between various factors, sustaining a healthy vasculature that can respond rapidly and appropriately to injury and stimuli [12]. When this equilibrium shifts, endothelial dysfunction develops. Some of these balances include those between vasoconstrictive and vasodilatory factors to maintain vascular tone and between prothrombotic and anti-thrombotic molecules [5, 12]. Particularly relevant to diabetes are the elevated levels of prothrombotic factors including plasminogen activator inhibitor-1 (PAI-1) and von Willebrand factor (vWF) and vasoconstrictors such as endothelin, along with the converse reduction in some key mediators of vasodilation that are especially important in platelet-driven pathways including nitric oxide (NO) and prostacyclin (PGI_2) as shown in Figure 1-1 [8, 11, 13]. Platelet quiescence requires this balance to be maintained and thereby prevents platelet-derived immune cell infiltration of the subendothelial space. A key characteristic of endothelial dysfunction is an accelerated reduction in the bioavailability of NO. Many of the characteristics of the metabolic milieu associated with diabetes contribute to endothelial dysfunction and will be explored in specific detail later [8, 9].

Initially, most of the changes associated with endothelial dysfunction are related to functional impairment and are present in almost all forms of vascular disease as well as being consistently demonstrated in patients with diabetes, particularly in the presence of obesity [12]. Once endothelial dysfunction is more established, further changes develop including evidence of reduced vasodilation, increased reactive oxygen species (ROS) production, excess

secretion of important growth factors e.g., platelet-derived growth factors (PDGFs), increased adhesion molecules and elevated cell layer permeability [12, 14]. This increase in the permeability is a key aspect of primary plaque formation; the permeability initially leads to an accumulation of low-density lipoproteins (LDL) within the vessel wall. Elevated levels of ROS, occurring due to endothelial dysfunction, promote oxidative stress which drives LDL to become oxidised-LDL (OxLDL) [15, 16]. OxLDL is associated with increased platelet activation and a pro-inflammatory state [17, 18]. This activates recruitment of immune cells to drive the immune response which involves several cells including monocytes and neutrophils [15, 18]. As monocytes differentiate into macrophages, there is an unregulated uptake by macrophages of OxLDL leading to foam cell formation [15, 18], which in turn secrete a myriad of inflammatory cytokines including interleukin-6 (IL-6) and tumour necrosis factor (TNF)-α [19, 20], further driving the pro-inflammatory environment [21]. As this process continues, fatty streaks start to form, soon followed by the development of primary atherosclerotic lesions which then continue to grow and eventually rupture, causing enhanced activation of platelets amongst other critical mechanisms driving clot formation [22, 23]. Again, many of the metabolic changes seen in diabetes are associated with driving these pathological processes contributing to the development of premature cardiovascular disease patients with diabetes.

Plaque rupture exposes blood platelets to extracellular matrix proteins such as collagen, fibronectin and vWF that promote uncontrolled platelet activation and thrombosis [23]. This activation response is exacerbated by the release of a plethora of pathological oxidised lipids, proteins and necrotic cell debris, which promote activation and blunt endogenous inhibitory pathways [23-25].

Increased activation of platelets accelerates the localised triggering of the coagulation cascade and in turn drives the generation of a fibrin network that works to stabilise the haemostatic plug following plaque rupture [22]. Of note, in patients with diabetes, this network has also been shown to be denser than those without diabetes and there is also hypofibrinolysis [26-28]. This combination means the formed clots are harder to lyse and thereby further contributes to development of atherosclerosis and vascular disease [29, 30]. Hyperglycaemia is a key driver of this with even mild improvements in glycaemia in patients with T1D resulting in demonstrable enhancement in clot lysis time [31], consequently reducing vascular risk.

Under normal conditions, platelets will circulate without adhering to endothelial cells, yet once endothelial dysfunction starts to develop, inflammatory processes upregulate the interaction between the two via a process largely mediated by P-selectin, E-selectin and PSGL-1 [22, 32]. The adhesion of platelets to endothelial cell surfaces is further stabilised by a variety of factors including through ADAM-15, a transmembrane cell surface protein, that binds to integrin $\alpha_{IIb}\beta 3$ on platelets [33]. Furthermore, activated platelets can form aggregates with leukocytes, facilitated by the binding of platelet P-selectin to PSGL-1 on leukocytes [22, 34]. These aggregates circulate in a pro-aggregatory form and interact with endothelial cells to recruit monocytes and further promote the formation of atherosclerotic plaques within vessel walls [22].

As summarised in Figure 1-1, endothelial dysfunction and platelet functions have a continuous bidirectional interaction which contributes to a chronic inflammatory state that also drives thrombus formation and atherosclerosis in a multitude of disease states, including in patients with diabetes.
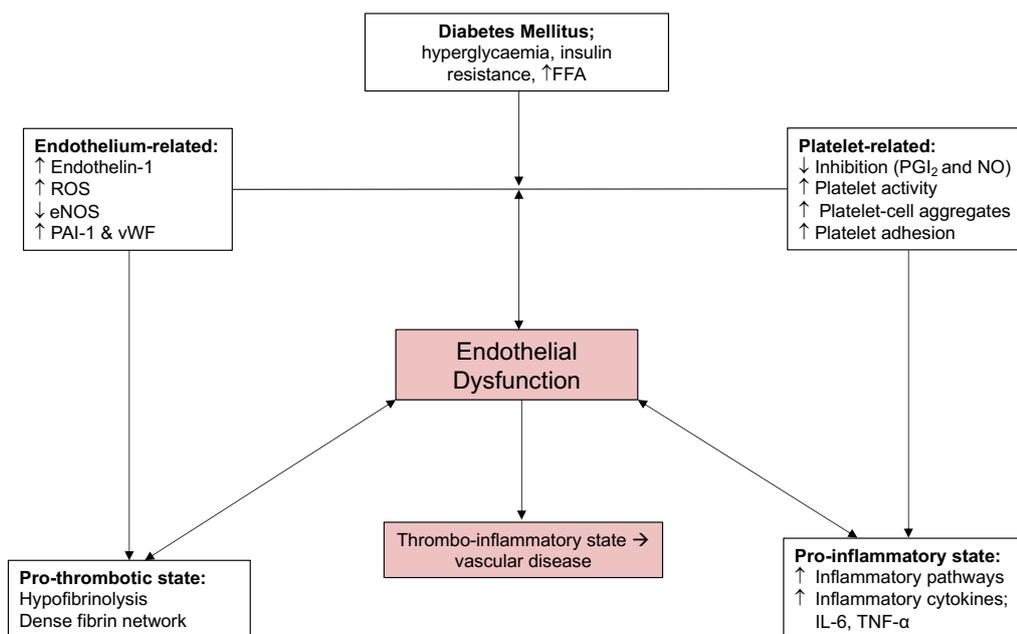
Figure 1-1: Endothelium and platelet related factors driving thrombo-inflammation and vascular disease in patients with DM.

Metabolic milieu of DM including hyperglycaemia and insulin resistance drive endothelial dysfunction via endothelium-related factors (increased endothelin-1, ROS and PAI-1 &vWF along with reduced eNOS) and platelet-related factors (reduced inhibition, increased platelet hyperreactivity, aggregation and adhesion). There is further bidirectional effects from the resulting pro-thrombotic and pro-inflammatory state further driving endothelial dysfunction and subsequent thrombo-inflammation. Abbreviations; FFA: free fatty acids, ROS: reactive oxygen species, eNOS: endothelial nitric oxide synthase, PAI-1: Plasminogen activator inhibitor-1, vWF: von Willebrand Factor, PGI2: prostacyclin, NO: nitric oxide, IL-6: interleukin-6, TNF-α: Tumour necrosis factor-α

1.2 Diabetes; classification and types

It is first important to clarify the classification and types of DM that are currently referred to within clinical practice in the UK[35].

1. Type 1 Diabetes Mellitus (T1D)- an autoimmune disease resulting in the destruction of pancreatic β cell and consequent loss of insulin production, accounting for approximately 5-10% of people diagnosed with DM [35]. There is increasing evidence to suggest patients with T1D are a less homogenous group than previously thought [36].

2. Type 2 Diabetes Mellitus (T2D)- typically presents as insulin resistance with a progressive decline in insulin secretion with loss of pancreatic β cell function, accounting for approximately 90% of people with DM [35].

3. Gestational Diabetes- glucose intolerance acquired during pregnancy in the absence of a known diagnosis if DM, which resolves following delivery[35].

3. Other types- this encompasses a wide array of more rare causes of DM, including monogenic diabetes including maturity onset diabetes of the young (MODY) or acquired causes affecting insulin production e.g., pancreatitis or post-pancreatectomy.

The term diabetes (or DM) refers to either/or T1D or T2D where no distinction has been made in the original study.

1.3 Metabolic effects of diabetes on platelet-driven vascular disease

Having now established that the pathophysiology behind the increased cardiovascular risk seen in patients with diabetes is undeniably multifactorial, with a variety of implicated pathways, exploring all of the various mechanisms in further detail is beyond the scope of this work and will hereafter focus almost exclusively on the role of platelets.

1.3.1 Platelet biology and function

Platelets are small, anucleate cells and are derived from megakaryocytes in the bone marrow with a typical lifespan of around 5-10 days [37, 38]. They have an established role in primary haemostasis responding to vascular injury [39]. Platelets adhere to the exposed subendothelial matrix through vWF and collagen through interactions with glycoprotein 1b/V/IX (GP1b/V/IX or CD42b), integrin $\alpha_2\beta_1$ and Glycoprotein VI (GPVI) respectively [38, 40]. Signalling from these receptors activates $\alpha_{IIb}\beta_3$, which is then able to bind plasma fibrinogen thereby allowing platelets to bind to each other and stabilising the thrombus [40], summarised in Figure 1-2. Activated platelets release a plethora of vasoactive mediators including adenosine diphosphate (ADP), thromboxane A_2 (TXA$_2$), vWF and fibrinogen that promote the recruitment of further platelets [38]. Several of these factors also drive further secondary platelet activation and also determine resistance of the clot to lysis [40].

Activated platelets can also be procoagulant in nature, thereby further enhancing thrombus formation following vascular injury. The procoagulant

subpopulation of platelets is largely driven by increased phosphatidylserine (PS) expression on the platelet surface [41, 42]. PS is a phospholipid, which following activation of platelets is externalised onto the platelet membrane, as shown in Figure 1-2, and acts as a binding site for factor Xa, driving the coagulation cascade and thus thrombin generation [41, 43].

Whilst this provides a brief background to platelet biology and function in a primary haemostatic capacity, it is now clear that platelets possess more far-reaching properties which will be explored in detail over the course of this chapter. In addition to the briefly described role in thrombosis at sites of vascular injury, emerging evidence also suggests platelets are key contributors to chronic low-grade inflammation as well as potential dysfunction of immune processes in patients with diabetes [44, 45]. Diabetes, irrespective of type, is associated with a variety of metabolic and functional changes that contribute to the resulting increased cardiovascular risk. It is therefore crucial to explore the effects of these various metabolic components of diabetes on platelet-driven pathophysiology.
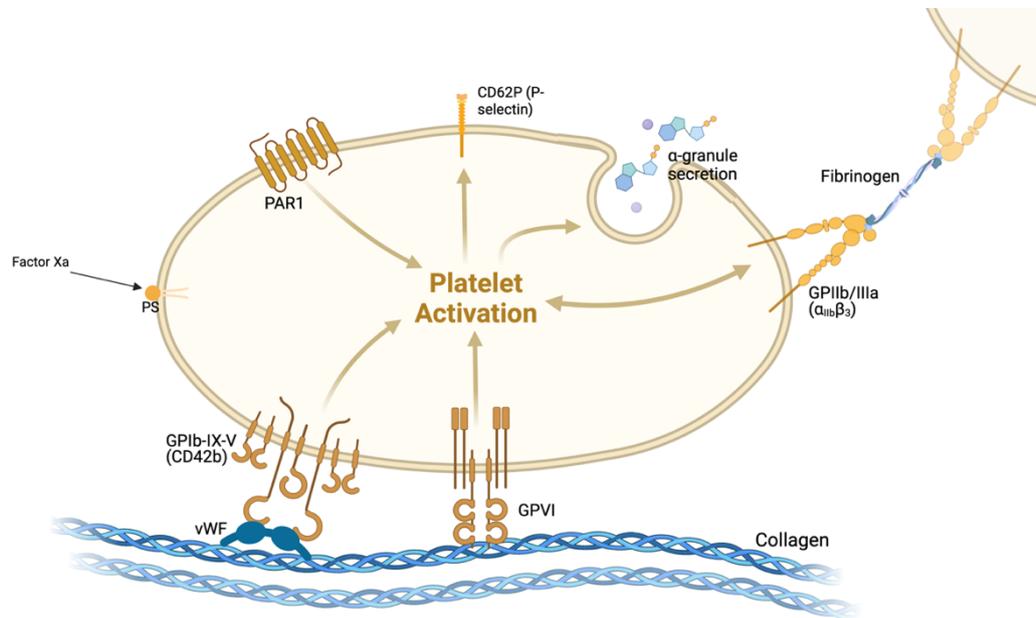
Figure 1-2: Platelet structure highlighting pathways involved in platelet activation and aggregation.

Following vascular injury, binding of platelets to the subendothelial matrix occurs via vWF binding of GPIb-IX-V. This triggers inside-out signalling which triggers the conformational change of integrin receptors on the platelet surface including $\alpha_{IIb}\beta_3$ which is the receptor for fibrinogen binding promoting platelet aggregation and stable thrombus formation. Upon activation other pathways are triggered included the release of dense and α-granules with CD62P expressed on the platelet surface. Secondary haemostasis and procoagulant activity is largely driven by the exposure of PS on the platelet surface to act as a binding site for Factor Xa. Abbreviations; PS: phosphatidylserine, GP1b/V/IX: Glycoprotein 1b/V/IX, vWF: von Willebrand Factor, GPVI: Glycoprotein VI, GPIIb/IIIa: Glycoprotein IIb/IIIa, PAR1: Protease-activated receptor-1.

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1.3.2 Insulin resistance

When the effects of insulin release on target cells are insufficient, whereby the resulting glucose disposal is impaired, there is a compensatory excess secretion of insulin by pancreatic β-cells and resulting hyperinsulinaemia

which underlies the process of insulin resistance (IR) [46]. This is typically associated with T2D but there is an increasing body of evidence to suggest the co-existence of T1D and IR, referred to as double diabetes (DD), which occurs in a significant proportion of T1D individuals and will be explored in detail later in the chapter [47]. Most studies exploring the effects of IR on platelet function are thus focussed on T2D, however this pathophysiology is likely to overlap in the cohort of patients with DD.

Platelets, much like other target cells or organs, express the insulin receptor on their surface, although the exact function of the receptor in platelets is still debated [8, 9]. In healthy people, when insulin binds to its receptor, platelet activation is inhibited. This likely occurs, at least in part, secondary to the intracellular translocation of magnesium [48]. This pathway is mediated by the activation of insulin receptor substrate (IRS-1) via tyrosine phosphorylation, which in turn increases intra-platelet cyclic adenosine monophosphate (cAMP), a key platelet inhibitor. The resulting rise in intra-platelet levels of cAMP are proposed to reduce signalling of platelet activation via the ADP receptor $P2Y_{12}$ [49, 50]. In cases of IR, as well as the absolute insulin deficiency seen in T1D, there is impaired insulin signalling and thus the described pathway is affected [51, 52]. This contributes to potential unopposed platelet activation and hyperreactivity [8, 53]. Studies on platelet reactivity in T1D and indeed DD are both limited and somewhat conflicting. Generally, evidence shows increased platelet aggregation, elevated markers of platelet activation and elevated fibrinogen binding in patients with T1D, but some data have conversely suggested there are no changes in aggregation [54-58]. In terms of mechanisms, there is evidence demonstrating lower plasma magnesium levels in a study of 45 individuals with T1D, compared

with age and sex matched healthy controls which likely contributes to this altered platelet function [59]. Furthermore, the changes in insulin receptor signalling in IR have been shown to reduce cAMP levels via $P2Y_{12}$ in patients with T2D, which results in increased intra-platelet calcium concentration contributing to increased platelet activation [60]. Other mechanisms that may influence platelet-driven cardiovascular disease in diabetes, particularly in relation to the presence of IR were first described decades ago. This includes early evidence to suggest a diminished response to inhibitors including PGI_2 and NO, particularly in people with T2D and co-existing obesity [61, 62].

As stated, one of the most crucial elements of maintaining environmental homeostasis for platelets, is ensuring a balance of endothelium secreted NO and the formation of ROS [63]. IR, along with other metabolic features in T2D, contributes to this potentially detrimental imbalance [8].

Increased accumulation of ROS, seen in IR, inactivates NO to form peroxynitrite, following generation of a superoxide anion [64]. As a result, this leads to a reduction in the overall bioavailability of NO, which is then further exacerbated by the function of peroxynitrite in driving the uncoupling of endothelial NO synthase (eNOS), with preferential production of ROS and thus inactivation of NO [7]. Another mechanism contributing to reduced endothelium-derived NO in diabetes is the decreased activity of eNOS [8, 65], as a result of both the described excess ROS production and also increased protein kinase C (PKC) activity. The increased PKC activity not only drives the reduction in eNOS but also causes increased levels of endothelin-1 which encourages both vasoconstriction and platelet aggregation [66]. The

described loss of bioavailability of NO due to reduced eNOS activity in diabetes may also directly drive platelet activation pathways.

Reduced NO levels coupled with elevated ROS levels, promote the production of transcription nuclear factor kappa β (NF-$\kappa\beta$), a transcription factor involved in several important cellular pathways within endothelial cells. The activation of the NF-$\kappa\beta$ pathway stimulates release of both chemokines and cytokines that are known to be associated with inflammatory processes and thus contributing to further atherosclerotic changes [8, 65]. There is also evidence to suggest that increased expression of NF-$\kappa\beta$ leads to elevated expression of leukocyte adhesion molecules on endothelial cells, which is considered another key aspect in driving endothelial inflammation and vascular damage[67]. Accumulation of ROS itself has significant vascular consequences; its accumulation activates additional inflammatory pathways, beyond those driven by NF-$\kappa\beta$ [7, 68]. Of particular note is the generation of advanced glycation end products (AGEs) [68, 69]. Production of AGEs affect protein function and also activate the receptor for AGEs (RAGEs). AGEs continue to drive ROS production and the RAGE activation causes an increase in the previously mentioned superoxide anion production, both of which themselves contribute to even more diminished bioavailability of NO [68].
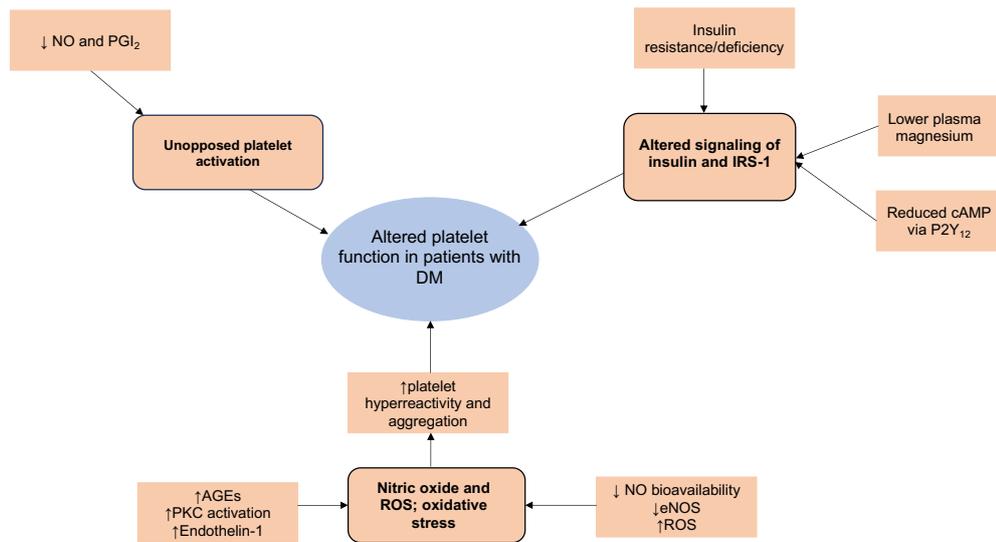
Figure 1-3: Summary of factors in DM contributing to altered platelet function driven through both unopposed platelet activation and hyperreactivity/increased aggregation.

Key proposed pathways driving altered platelet function in DM including the altered signalling of insulin and its IRS-1, both of which are implicated in T1D, T2D and DD as a result of insulin deficiency and/or insulin resistance. In patients with DM, platelets have also been shown to have diminished NO bioavailability and increased ROS driving platelet hyperreactivity and aggregation. Elevated AGEs, PKC activation and increased endothelin-1 also drive oxidative stress. Reduced NO and PGI_2 also lead to lack of platelet inhibition in DM further driving hyperreactivity. Abbreviations; IRS-1: Insulin receptor substrate-1, NO: nitric oxide, PGI_2: prostacyclin, AGE: advanced glycation end products PKC: protein kinase C, NF-$\kappa\beta$: nuclear factor kappa B, eNOS: endothelial nitric oxide synthase

1.3.3 Dysglycaemia

Dysglycaemia refers to blood glucose levels outside of the accepted normal range and typically encompasses three crucial factors; hyperglycaemia, hypoglycaemia and glycaemic variability (GV) which will be explored in this section. Elevated dysglycaemia has been repeatedly linked to poor cardiovascular outcomes [70, 71]. Each of the aforementioned contributing factors will be defined and then their effects on platelet-driven vascular disease separately discussed. Briefly they are described as follows:

1. Hyperglycaemia is defined as blood glucose levels elevated beyond acceptable normal range, in T1D typically referring to blood glucose levels above 10 mmol/mol [72]. Whilst hyperglycaemia is most commonly seen in patients with diabetes, it can occur in people without diabetes in settings of stress such as critical illness [73].

2. Hypoglycaemia refers to blood glucose levels below normal range, typically accepted as below 4 mmol/l [72].

3. Glycaemic variability refers to the daily fluctuations in blood glucose levels and occurs both in T1D and T2D, with a variety of tools used to measure this, including continuous glucose monitoring (CGM) or intermittently scanned continuous glucose monitoring (isCGM) [74, 75].

1.3.4 Hyperglycaemia

One of the most well recognised features of both T1D and T2D is hyperglycaemia and there is well documented evidence of its contribution to various aspects of platelet dysfunction and cardiovascular disease. In one study, mice models for diabetes were used to create pancreatic β-cell destruction and a permanent state of hyperglycaemia [64]. An eNOS inhibitor was then injected and the study demonstrated an increase in fibrinogen-platelet binding along with increased expression of important markers of platelet activation. Following restoration of NO bioavailability these observed pathological changes were reversed [64].

Elevated PKC has been demonstrated in the platelets of healthy controls under hyperglycaemic conditions, although this has been variable in patients with T2D [76]. The effects of hyperglycaemia on PKC activation are mediated by the diacylglycerol (DAG)-PKC pathway [77]. PKC, as mentioned, has multiple functions included reducing eNOS activity and thus NO bioavailability, increased endothelin-1 driven vasoconstriction and platelet aggregation as well as enhancing ROS generation via NADPH oxidase mediated superoxide production [78]. There is also evidence to suggest that in the context of hyperglycaemia, PKC plays a role in upregulation of both mRNA and protein expression of cyclooxygenase-2 (COX-2) and there is a resulting increase of TXA_2 production which causes a reduction of both NO and prostacyclin production, all of which are linked to endothelial dysfunction and increased oxidative stress [79]. These effects were reversed by a PKC inhibitor [79].

NO once again plays a vital role in the regulation of platelet adhesion and aggregation, under normal physiological conditions it helps prevent thrombus

formation by inhibiting platelet adhesion and aggregation [25]. Crucially, it has been shown that reduced bioavailability of NO is linked to poor cardiovascular outcomes and is likely one aspect of metabolic changes seen in diabetes and the associated platelet dysfunction [65, 80].

The effect of increased osmolarity due to hyperglycaemia may also contribute to platelet hyperreactivity which was demonstrated in both healthy controls and patients with T2D in response to varying concentrations of glucose. The higher glucose concentrations induced a rise in osmolarity and was associated with increased markers of platelet activation including P-selectin and $\alpha_{IIb}\beta_3$ [81]. Hyperglycaemia also causes non-enzymatic glycation of surface proteins on platelets enhancing their activation and reducing membrane fluidity which further encourages activation [82]. The changes associated with hyperglycaemia are summarised in Table 1-1.

1.3.5 Hypoglycaemia

Hypoglycaemia is an inevitable side effect of both insulin and some oral therapies used in T1D and T2D. Much evidence suggests a crucial aspect of management of diabetes involves the mitigation of hypoglycaemic risk, not only for patient quality of life but to reduce complications with particular focus on the associated negative cardiovascular outcomes. In fact, in the large scale ACCORD (Action to Control Cardiovascular Risk in Diabetes) trial, treatment for those randomised to the intensive glucose-lowering arm had to be discontinued due to the interim analysis demonstrating higher mortality [83].

Evidence that hypoglycaemia results in augmented levels of platelet aggregation has been around since the late 1970s [84]. The mechanisms through which hypoglycaemia and the associated platelet dysfunction contribute to cardiovascular disease are likely multifactorial [85]. There is evidence to support the notion that hypoglycaemia is another mechanism through which oxidative stress is increased [86]. Further to this, there is an association with a rise in markers of inflammation, including pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8 as well as TNF-α again demonstrated in healthy volunteers following induction of hypoglycaemia through rapid insulin-bolus [86]. In addition, hypoglycaemia also contributes to an increase in ROS production, driving the previously described NO imbalance [86].

Further to this, hypoglycaemia has also been shown to increase markers of platelet activation, many of which will be described in further detail later, including P-selectin, CD40-Ligand and platelet-monocyte aggregates on mononuclear cells [87].

Whilst much of these data stem from studies carried out in healthy volunteers, there are comparable data from patients with T1D in whom hypoglycaemia was induced within controlled research settings [88]. In both healthy controls and individuals with T1D, elevated circulating levels of markers of platelet activation and inflammation including P-selectin and IL-6 along with other proinflammatory/prothrombotic mediators e.g. PAI-1 and vascular adhesion molecules, were demonstrated following induction of hypoglycaemia [88]. The effects in the studies mentioned can be attributed to the hypoglycaemia itself rather than insulin, given in the control group, in whom euglycaemia was maintained through the use of glucose infusions, comparable results were not

replicated [87, 88]. In patients with T2D, induced-hypoglycaemia was linked to increase in other inflammatory markers including C-reactive protein (CRP) along with a marker of oxidative stress, PTGS2, again supporting the concept that hypoglycaemia contributes to cardiovascular disease through oxidative stress-related pathways [89, 90].

There may be effects of the type of hypoglycaemia occurring too, with severity and duration of hypoglycaemia also linked to degree of platelet dysfunction [91], although again further data are needed to draw definitive conclusions. There are additional effects of hypoglycaemia on reduction of fibrinolysis, further driving the prothrombotic state in patients with diabetes [27].

The effects of hypoglycaemia on thrombotic risk and platelet activation may be delayed after the acute event, with elevated markers of platelet activation (including P-selectin, $\alpha_{IIb}\beta_3$ and CD63) evident both 24 hours and 7 days after an acute hypoglycaemic event, demonstrated in patients with T2D on metformin treatment alone [92]. The main evidence demonstrating the effects of hypoglycaemia on platelets are summarised in Table 1-1.

Additionally, the physiological response to hypoglycaemia induces release of glucagon from α-cells. Glucagon is a cAMP stimulator and has been shown to itself increase platelet aggregation [93]. Further to this, there is an associated counter-regulatory secretion of catecholamines following hypoglycaemia [94]. Catecholamines are known to increase both platelet aggregation and activation [27, 95], with activation mediated via α2 adrenergic receptors [96]. There is evidence to support the fact that hypoglycaemia-induced platelet dysfunction is at least in part due to the indirect effect of adrenaline and its role in increasing platelet sensitivity to circulating agonists

such as ADP, with evidence to correlate increasing platelet activation with rising adrenaline levels [97]. It is clear the effects of hypoglycaemia on platelet function are both indirect and direct, but that in either case this is one mechanism contributing to hypoglycaemia-mediated cardiovascular disease in patients with diabetes.

1.3.6 Glycaemic variability

Glycaemic variability (GV) has become a more widely recognised feature of diabetes, as a result of the increased utilisation of both CGM and isCGM [74, 75]. It has been postulated that those with high GV may confer even greater risk of cardiovascular disease than those with persistent hyperglycaemia [98] and another study demonstrated increased risk of peripheral neuropathy in patients with T1D who had the highest GV [99]. However, high GV is associated with increased hypoglycaemia, and therefore it can be difficult to disentangle the adverse vascular effects of each. Taking this caveat into consideration, the DEVOTE study, involving over 7000 patients with T2D randomised to receive with insulin glargine or degludec, it was demonstrated that increased GV was associated with increased major adverse cardiovascular events as well as all-cause mortality [100]. Although there are somewhat conflicting data in this area, particularly in patients with T1D, this certainly warrants further research.

In relation to the specific mechanisms behind GV influencing platelet dysfunction, there is a paucity of evidence compared with the above-described factors. However, one study measured platelet reactivity through a verified $P2Y_{12}$ assay in patients with T2D undergoing percutaneous coronary

intervention with evidence demonstrating a correlation between higher platelet reactivity in those with both higher HbA1c and GV [101]. Fluctuations in blood glucose have been shown to increase oxidative stress, which is considered the most likely contributor of GV to the development of both micro- and macrovascular complications in diabetes [102]. One study involving participants with T2D, demonstrated that increased GV, measured in this case using CGM, correlated with increased levels of a marker of oxidative stress, urinary excretion of free 8-iso-prostaglandin F2α [103] and in diabetic rat models, fluctuating hyperglycaemia was also shown to increase oxidative stress and vascular damage [104]. Intermittent hyperglycaemic conditions subjected *in vitro* to human umbilical vein endothelial cells were associated with an increase in advanced oxidative protein products, indicative of oxidative stress and associated decrease in anti-oxidant capacity. This resulted in increased apoptosis within the endothelial cells from the human umbilical cords (HUVEC) [104]. The same group also demonstrated that both hyperglycaemia and higher GV resulted in both elevated platelet aggregation and increased expression of CD62P, a marker of platelet activation which will be explored in more detail later, in both HUVECs and diabetic rat models [105]. Of note, there was a more notable increase in those exposed to the fluctuating hyperglycaemia than the sustained hyperglycaemia [105]. Whilst there is far more limited evidence for the effects of GV directly on platelet function, the discussed data and the links with increased oxidative stress that will contribute to platelet dysfunction that have already been described, suggest that GV may have a role in addition to both IR and hyperglycaemia with some of the main evidence summarised in Table 1-1.

Dysglycaemic state	Effects on platelets
Hyperglycaemia [64, 77, 78, 80-82]	↑ PKC mediated by DAG-PKC pathway ↓ eNOS activity and NO bioavailability ↑ ROS and oxidative stress ↑ COX-2 mRNA and protein expression ↑ Glycation of platelet surface proteins ↑ Platelet activation (P-selectin & $\alpha_{IIb}\beta_3$)
Hypoglycaemia [84-90, 92]	↑ ROS and oxidative stress ↓ NO bioavailability ↑ Pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) ↑ Platelet activation (P-selectin, CD40L, GPIIb/IIIa) and platelet-cell aggregates ↑ Inflammation (CRP, PTGS2)
High GV [101-105]	↑ Platelet activation (P-selectin) ↑ Oxidative stress (↑ prostaglandins)

Table 1-1: Impact of dysglycaemic states on platelet activation and implicated pathways.

Multiple pathways impact platelet function as a result of hyperglycaemia, hypoglycaemia and also GV. Key pathways include the reduction in NO bioavailability and eNOS activity particularly seen in hyper- and hypoglycaemia. This combination causes increased ROS and results in oxidative stress. All dysglycaemic states have been linked to platelet hyperreactivity with evidence of elevated levels of associated markers particularly P-selectin. Abbreviations; GV: glycaemic variability, PKC: protein kinase C, DAG: diacylglycerol, eNOS: endothelial nitric oxide synthase, NO: nitric oxide, ROS: reactive oxygen species, COX-2: cyclooxygenase-2, mRNA: messenger ribonucleic acid, GPIIb/IIIa: Glycoprotein IIb/IIIa, IL: interleukin, TNF-α: tumour necrosis factor-α, CD40L: CD40-ligand, CRP: c-reactive protein, PTGS2: prostaglandin-endoperoxide synthase 2

1.3.7 Dyslipidaemia

It is widely accepted that patients with T2D commonly have lipid abnormalities and part of the management frequently includes statin therapy. Yet more recently it has been acknowledged that patients with T1D, particularly in the context of IR and sub-optimal glycaemic control also frequently have altered lipid metabolism, particularly elevated LDL cholesterol and elevated triglycerides [106].

One large scale study conducted in Germany included over 27,000 children, adolescents and young adults up to the age of 26 with T1D. The study demonstrated that even in the youngest patients (up to the age of 11) 22% of the patients had dyslipidaemia, rising to over one third in patients aged 17-26 [107]. Total cholesterol levels were deemed elevated in 26% of the full cohort. Just 0.4% of the full cohort were on lipid lowering medication, however this may in part reflect the lack of therapies considered safe in children [107]. The landmark UK Prospective Diabetes Study (UKPDS) demonstrated the degree of dyslipidaemia in over 3,500 patients with T2D compared with healthy controls. Those with T2D had lower HDL cholesterol, 9% lower in men and 23% lower in female patients. Additionally, triglyceride levels were 50% higher in the T2D patient cohort [108]. The potential impact of dyslipidaemia in patients with T2D has also been shown. Within a cohort of almost 5,000 patients with T2D, those with an elevation in triglycerides and low levels of HDL cholesterol had a significantly increased risk of developing cardiovascular disease compared to those patients with normal triglycerides and HDL levels, indicating the potential benefit for early identification and intervention within this cohort [109].

Insulin itself plays an important role in the metabolism of lipids, with multiple important actions. It inhibits hormone sensitive lipase in adipose tissue and thereby reduces free fatty acid secretion in these tissues [110]. In the case of the absolute deficiency of insulin seen in T1D along with IR typical of T2D, it is likely that these typical lipolytic effects of insulin are somewhat prevented [110]. Lipoprotein Lipase (LPL) plays an important role in the postprandial hydrolysis of triglycerides within chylomicrons, which facilitates the uptake of chylomicron remnants into the liver via the LDL surface receptor. Insulin is

equally crucial in the reduction of chylomicron production through the upregulation of LPL activity and also increased LDL receptor activation, thus driving the breakdown of the chylomicrons and helping to reduce circulating LDL [111]. Indeed, IR has been shown to correlate with increased circulating LDL due to impairment of LDL receptor function and overall reduction of LDL receptor expression has been demonstrated in patients with T2D [112, 113].

Very low density lipoproteins (VLDLs) are synthesised in the liver and are converted to intermediate-density lipoproteins and LDLs in circulation through hepatic lipase activity [114]. Traditionally, focus has been on the role of LDL cholesterol in cardiovascular disease, but it has been increasingly recognised that elevated VLDL levels also contribute to the development of cardiovascular disease and has been associated with an increased risk of acute coronary syndrome and all-cause mortality, independent of LDL cholesterol [115]. Through both direct and indirect effects, insulin reduces hepatic VLDL production, thus in both T1D and T2D this inhibitory effect is negatively affected. As mentioned, insulin reduces the overall production of free fatty acids through its inhibition of hormone sensitive lipase in adipose tissues. Free fatty acids act as the substrate for VLDL production and thus their reduced synthesis directly impairs VLDL secretion too [116]. As described insulin's function in activating LPL also drives catabolism of VLDL. Finally it has a direct function on hepatocyte VLDL production although the exact mechanism has not been fully characterised [117].

The effects of T1D and T2D on dyslipidaemia are therefore multifactorial and are likely another mechanism by which patients with diabetes have platelet dysfunction. Total cholesterol levels have been shown to correlate with

increased platelet aggregation in individuals without diabetes [118]. VLDL has also been shown to increased collagen-dependent platelet aggregation as well as correlated with increased thromboxane, a surrogate for platelet activation [119]. One study demonstrated a correlation between total cholesterol, LDL and triglycerides and increased levels of platelet-monocyte aggregates as well as markers of platelet activation, CD62P (P-selectin) and CD36 in young patients with T1D [120].

1.4 Diabetes-induced platelet dysfunction

Whilst the above-described pathophysiological changes associated with the metabolic effects are key in understanding the potential role of platelets in the cardiovascular complications seen in T1D and DD, we must also explore the evidence for the functional effects of these changes, in both thrombotic and inflammatory capacities.

1.4.1 P-selectin

Elevated levels of platelet activation have been reported both in patients with T1D and T2D when compared with healthy controls. Many of the early studies supporting these functional changes focussed on elevated TXA_2 biosynthesis [121, 122]. Davi *et al.*, demonstrated that patients with T2D were characterised by higher levels of platelet activation compared with healthy controls, as evidenced by elevated TXA_2 secretion [122]. Further to this, in individuals with other features of the metabolic syndrome including

hypertension and dyslipidaemia, similar excess TXA_2 secretion has been shown, suggestive of increased platelet activation [123]. Importantly, existing atherosclerotic disease was not shown to trigger this platelet activation and thus suggests heightened platelet activation is independent of pre-existing vascular damage, which had previously been suggested as the predominant driving force behind increased platelet activation in these patients [124]. Through other studies, the same group have also shown enhanced TXA_2 synthesis in the context of post-prandial hyperglycaemia in patients with newly diagnosed T2D and good glycaemic control with HbA1c <7% [125], suggesting that even with relatively short duration of diabetes, hyperglycaemia alone can result in changes in platelet function.

The α-granule protein, P-selectin, is a key marker of platelet activation. It is single chain glycoprotein and is encoded by the Selectin-P (SELP) gene [126]. In platelets, it is stored in α-granules and following activation is translocated to the platelet surface [126]. Measuring the level of surface P-selectin is therefore a useful surrogate marker for platelet activation. Further to this, binding of P-selectin to P-selectin glycoprotein ligand-1 on leukocytes results in the formation of heterotypic platelet-leukocyte aggregates (specifically monocytes and neutrophils) [127, 128]. The most widely studied are platelet-monocyte aggregates, as they are the most stable of these leukocyte aggregates and have been shown to further accelerate platelet adhesion, contributing to a pro-thrombotic environment [129]. P-selectin-mediated platelet leukocyte interaction leads to upregulation of the gene expression of cytokines and integrins linked to inflammatory processes and vascular damage [130]. Importantly, P-selectin levels have been shown to be

implicated in a number of disease states with several reported associations with development of cardiovascular disease and mortality [131, 132].

P-selectin has been one of the most widely used markers for platelet studies in patients with both T1D and T2D. Although most studies have focussed on patients with T2D or hyperglycaemia-induced environments in healthy controls, Jilma *et al.,* first reported elevated P-selectin expression in patients with "insulin-dependent diabetes" in 1996 [133]; T1D in recent nomenclature. There still exists a paucity of evidence in this area for people with T1D although one report showed higher circulating levels of both P-selectin and platelet-monocyte aggregates in T1D compared with healthy controls [120]. Of note, in the same study, poorer glycaemic control (measured through elevated HbA1c), also correlated with higher P-selectin expression and platelet-monocyte aggregate formation. In contrast, diabetes duration was not found to correlate with the elevated levels of P-selectin, suggesting that hyperglycaemia itself plays a crucial role in the functional platelet changes seen in T1D [120].

Elevated levels of markers of platelet activation, including P-selectin, have been reported in several studies assessing T2D [134-136]. Again, the role of hyperglycaemia appears to be key, given improved glycaemic control (mean reduction in HbA1c of 3±0.7%) following either oral hypoglycaemic agents or insulin over a 3 month period was associated with a significant reduction of P-selectin expression [134]. These findings are supported by another study which investigated patients with T2D who had undergone coronary angioplasty [137]. Here, even with modestly improved glycaemic control (mean reduction of 0.4±0.5% HbA1c) after 3 months had reduced P-selectin

expression compared with those patients who had an increase in HbA1c levels (mean rise of 0.6±1.4%) [137].

To date, there are no known studies investigating P-selectin expression in patients identified with DD (i.e., the potential additive effects of hyperglycaemia and IR are incompletely understood), thus we must draw information from the functional changes in platelets seen in the metabolic phenotype associated with DD. It has been shown that inducing hyperinsulinaemic and hyperglycaemic conditions in healthy controls is associated with both an increased expression of P-selectin along with platelet-monocyte aggregates, although platelet-neutrophil aggregate levels remained unchanged. This would suggests that both insulin resistance and hyperglycaemia may play a role in the increase platelet activation and driving a pro-inflammatory state [138]. The evidence for changes in P-selectin expression in T1D, T2D and obesity/IR are summarised in Table 1-2.

1.4.2 CD40-Ligand expression

CD40-Ligand (CD40L) is a cell surface receptor, also known as CD154, which belongs to a wider family of tumour-necrosis factors. It plays a crucial role in both innate and adaptive immunity [139]. The soluble form of CD40L is predominantly formed by platelets and it is stored, then rapidly expressed on the platelet surface prior to cleavage [139]. CD40L interacts with cells via the CD40 receptor, expressed on inflammatory cells such as monocytes and macrophages [140]. The binding of CD40L to its receptor drives the synthesis and release of a number of important inflammatory chemokines and cytokines, including IL-6 and IL-8 [141]. It has been linked to atherosclerotic processes

and also thrombo-inflammatory processes and therefore may be a key component of cardiovascular disease, including in patients with T1D and/or DD [140, 142, 143].

In patients with T1D, without clinical macrovascular complications, elevated platelet CD40L expression and platelet-monocyte aggregates has been demonstrated compared with healthy controls [144]. Further studies have reported comparable results with elevated levels of CD40L in patients with both T1D and T2D when compared with healthy age-matched healthy controls [145]. One study focussed on soluble CD40L levels in children with T1D up to the age of 16 years, with patients further sub-grouped according to presence or absence of microvascular complications including retinopathy, neuropathy or nephropathy [146]. The children with T1D had elevated CD40L compared with healthy controls, with even greater elevations seen in the group with microvascular complications. This would suggest inflammatory changes are seen even in young patients, with implications for long-term cardiovascular outcomes [146].

Once again, we are unaware of any reported studies investigating expression of CD40L levels in patients with DD yet there is evidence from one study whereby the authors demonstrated increased expression of CD40L in healthy participants following induction of a hyperglycaemic and hyperinsulinaemic environment [138]. Perhaps more relevant still to DD, one study conducted in obese individuals, albeit without diabetes, reported elevated levels of CD40-ligand (CD40L) when compared with people with BMI <25 kg/m^2, with the same trend seen when comparing levels between obese (BMI $\geq$30kg/m^2) and overweight individuals (considered BMI 25-29.9kg/m^2) [147]. Higher urinary

thromboxane metabolite, which has been correlated with CD40L levels in patients with T2D [148], as well as higher levels of platelet derived microparticles have also been shown in people with obesity, with improvement of these elevated markers following weight reduction [149, 150]. Again some of the main evidence for changes in CD40L in patients with diabetes is summarised in Table 1-2.

1.4.3 Integrin $\alpha_{IIb}\beta_3$

$\alpha_{IIb}\beta_3$ is the most highly expressed integrin on platelets and is a receptor for fibrinogen binding, thus a key factor involved in platelet aggregation [151]. Elevated levels of $\alpha_{IIb}\beta_3$ have been implicated in a number of cardiovascular diseases and are of great interest in preventing atherosclerosis processes in the form of existing $\alpha_{IIb}\beta_3$ antagonists including abciximab and tirofiban [152, 153].

Of relevance to patients with diabetes, with the evidence included in Table 1-2, the area is not as widely studied as the other aforementioned platelet markers, particularly in the case of T1D. However, in one study focussing on patients with T2D, there was a statistically higher expression of $\alpha_{IIb}\beta_3$ on the surface of activated platelets in those with T2D compared with the healthy control group (3.91±2.9% in T2D vs 2.79±2.5% in control group) [154]. A further study investigated the levels of $\alpha_{IIb}\beta_3$ as well as other markers of platelet adhesion in patients with T2D demonstrated increased basal expression of $\alpha_{IIb}\beta_3$ compared with healthy controls suggesting that even at rest there is a degree of pro-aggregatory state in these platelets [155]. There was a variable response to stimulation with low and high dose agonists, from

which it was difficult to draw definitive conclusions. This particular cohort of patients had poor glycaemic control with an elevated HbA1c (mean 83 ± 3mmol/mol) and fasting hyperglycaemia (9.5 ± 2.7mmol), again implicating hyperglycaemia in inducing functional platelet changes in individuals with diabetes [155].

Further to this, one study exploring various markers of platelet activation in patients with T2D including patients with co-existing cerebrovascular disease, reported increased expression of $\alpha_{IIb}\beta_3$ complex along with increased fibrinogen compared with healthy controls, this was more profound in the patients with the co-existing cerebrovascular disease [156]. Furthermore, a meta-analysis examined six important large scale trials investigating effects of $\alpha_{IIb}\beta_3$ receptor antagonists following acute coronary events in patients with diabetes [157]. Here, 6458 patients with diabetes were included across the 6 trials, with a signification reduction in the pooled 30-day mortality risk from 6.2% in placebo groups down to 4.6%, which outperformed the results seen in the patients without diabetes where mortality was identical in the two groups at 3.0% in over 23,000 patients in the pooled results from the various trials [157]. Even greater effects were found in patients with diabetes who underwent percutaneous coronary intervention with a mortality risk of 1.2% compared with 4% in those who received placebo. This suggests $\alpha_{IIb}\beta_3$ may well play a crucial role in the development of cardiovascular disease in patients with diabetes given these positive results on mortality reduction following acute coronary syndrome when using $\alpha_{IIb}\beta_3$ inhibitors.

Diabetes Type	Evidence of changes linked to platelet function in DM
T1D [120, 133, 137, 144-146]	↑ P-selectin expression ↑ Platelet-monocyte aggregates ↑ CD40L
T2D [134-136, 144, 145, 154-156]	↑TXA$_2$ synthesis ↑ P-selectin expression ↑ CD40L ↑ $\alpha_{IIb}\beta_3$ expression
DD (obesity/insulin resistance) [138, 147, 149, 150]	↑TXA$_2$ synthesis ↑ P-selectin expression ↑ Platelet-monocyte aggregates ↑ CD40L ↑ $\alpha_{IIb}\beta_3$ expression

Table 1-2: Summary of platelet changes seen in patients with DM affecting platelet activation/aggregation driving vascular sequelae.

There is evidence to support altered platelet function in T1D and T2D, with further evidence to show similar findings in patients with obesity and/or insulin resistance. Increased P-selectin expression, $\alpha_{IIb}\beta_3$ activity and CD40-L are suggestive of hyperreactive platelets with further evidence showing elevated levels platelet aggregates. Abbreviations; T1D: Type 1 Diabetes Mellitus, T2D: Type 2 Diabetes Mellitus, DD: double diabetes, CD40L: CD40 Ligand, TXA2: Thromboxane, GPIIb/IIIa: Glycoprotein IIb/IIIa

1.4.4 Inflammatory cytokines and chemokines

Chemokines and their associated receptors have the primary function of directing cells, including those important in inflammatory processes, to their targets and are known to actively contribute to inflammatory response following injury [158]. Within platelets, chemokines are stored within the α-granules and thus immediately released following activation, with CXCL4 and CXCL7 currently believed to be the most abundant [159, 160]. Additionally, platelets can themselves be activated by chemokines from other cell types [160]. Although further data are required, current evidence suggests platelet

chemokines contribute to atherosclerotic disease, including the aforementioned CXCL4 [161] along with elevation of other chemokines linked to inflammation in those with unstable angina [162].

Beyond vascular disease alone, there is evidence to support the elevation of levels of pro-inflammatory chemokines in patients with diabetes and a constant bi-directional relationship between platelet activation and inflammatory processes. One meta-analysis evaluated chemokines in over 10,000 patients with T1D compared with controls [163]. Significantly elevated circulating levels of chemokines from the CC subgroup were demonstrated in the patients with T1D when compared with 9,700 controls, although there was a degree of variation when evaluating individual chemokines. Other studies have also shown elevations in inflammatory chemokines in autoimmune disease [164]. Further to this, patients with microvascular disease in diabetes appear to demonstrate increased levels of pro-inflammatory chemokines. In patients with nephropathy secondary to diabetes, one study has shown elevated levels of several important inflammatory chemokines including platelet derived CXCL7 [165]. Similarly, in patients with diabetic retinopathy, data have also shown evidence of elevated levels of inflammatory chemokines [166]. Figure 1-4 summarises some of the inflammatory changes seen in DM.

1.4.5 Toll-like Receptors and platelet immune response

Until recently, it was not known that platelets possess immune-related functions, beyond the traditional haemostatic functions that have long been known about. Both human and mouse platelets have now been shown to possess Toll-Like Receptors (TLRs) on their surface [167] and they potentially

play a key role in the interface between platelet derived thrombo-inflammation and the immune system. In platelets, the activation of TLRs induces an array of responses including aggregation and inflammatory processes [168, 169]. TLRs respond to both pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs), which play a central role in both innate immune response and associated maintenance of inflammation in response to these triggers [169].

Although there are multiple mechanisms through which TLRs induce platelet activation, the NF-κβ pathway appears to be heavily implicated in TLR induced inflammatory response in platelets [169], which drives increases in integrin, fibrinogen-binding and aggregation in response to inflammatory or thrombotic triggers [170]. TLR-2 is thought to be the most abundantly expressed TLR in platelets with lower levels of both TLR-1 and TLR-6 [167]. Further to this, increased expression of platelet TLR-2 has been linked to platelet hyperreactivity and this plays a crucial role for enhanced thrombosis driven by dyslipidaemia, a state commonly associated with both insulin resistance and T2D [171]. Further links between TLR-2 along with TLR-4 in platelets and enhanced thrombo-inflammatory responses have also been demonstrated supporting the hypothesis of their involvement in cardiovascular disease [172].

Platelet TLRs have also been implicated in a number of disease states, initially in their response to bacterial and viral infections but more recently in both vascular disease and diabetes. Platelet TLR-2 mRNA expression has been shown to be upregulated in patients with acute coronary syndrome, with TLR-1 and TLR-6 also showing upregulation [173, 174]. In patients with atrial fibrillation, platelet TLR-2 expression is increased compared with those

without atrial fibrillation. Furthermore, TLR-4 has been shown to have increased activation in the presence of IR and is thus believed to contribute to the inflammatory processes in this context [175], also supported by the role of fatty acids in upregulation of TLR-4 and release of inflammatory cytokines [176]. Elevated TLR-4 expression has also been demonstrated in the setting of hyperglycaemia, suggesting its role in thrombo-inflammation in both T1D and T2D [177].

Whilst the exact mechanisms through which platelet-TLRs are involved in inflammation and in particular in patients with diabetes are unclear, there is a growing body of evidence to support TLR activation being linked, at least in part, to the activation of inflammasomes [178]. These are multiprotein oligomers that are responsible for activating inflammatory responses, thus integral to the innate immune response [179]. The NOD-like receptor protein 3 (NLRP3) inflammasome is the most well characterised of the inflammasomes, and has been an area of focus for its role in both the development of vascular disease and also in patients with diabetes [178]. NLRP3 generates, amongst other cytokines, interleukin 1β (IL-1β) which is a pro-inflammatory cytokine [180]. It promotes the expression of adhesion molecules on endothelial cells, driving a pro-coagulant state [181]. IL-1β appears to be elevated in multiple inflammatory diseases [182]. Of relevance to diabetes, particularly in the context of T2D or IR, metabolic DAMPs, including AGEs, palmitate and glucose, have been shown to upregulate NLRP3 activation and in turn the synthesis IL-1β [183]. Data demonstrated that in monocytes from patients with newly diagnosed T2D, there was increased NLRP3 inflammasome expression and thus increased IL-1β synthesis [184]. A subset of patients were treated with metformin monotherapy

and after 2 months there was a significant downregulation in IL-1β synthesis suggesting metformin may have a potential role in mitigation of inflammasome activation in T2D [184]. Further to this, direct anti-IL-1β therapies are being researched and are discussed in section 1.10.3. The combination of hyperglycaemia and hyperinsulinaemia, characteristic of the metabolic environment seen in patients with T2D, is considered to be a key driver in the activation of NLRP3 and may explain its upregulation within this cohort [184]. Similar results have been demonstrated within the endothelial cells of diabetic mice models, with NLRP3 knockdown correlating with a significant reduction in atherosclerotic burden as well as leading to a reduction of the inflammation within the endothelium [185], a hyperglycaemic environment further augmented these findings. Within the same publication, NLRP3 inflammasome activity was increased in a cohort of 55 patients with newly diagnosed T2D compared with healthy matched controls. 50 out of the 55 patients within this cohort were treated with lifestyle measures and standard care medical treatment, and the study showed that a reduction in HbA1c below 6.5% (48mmol/mol) was accompanied by a reduction in NLRP3 inflammasome activation [185]. Some of these data are summarised in Figure 1-4.

With ever-increasing evidence of the involvement of TLRs in platelet thrombo-inflammatory processes contributing to the development of cardiovascular disease, understandably questions have arisen about the potential for their use as targets for novel therapies which will be explored later.
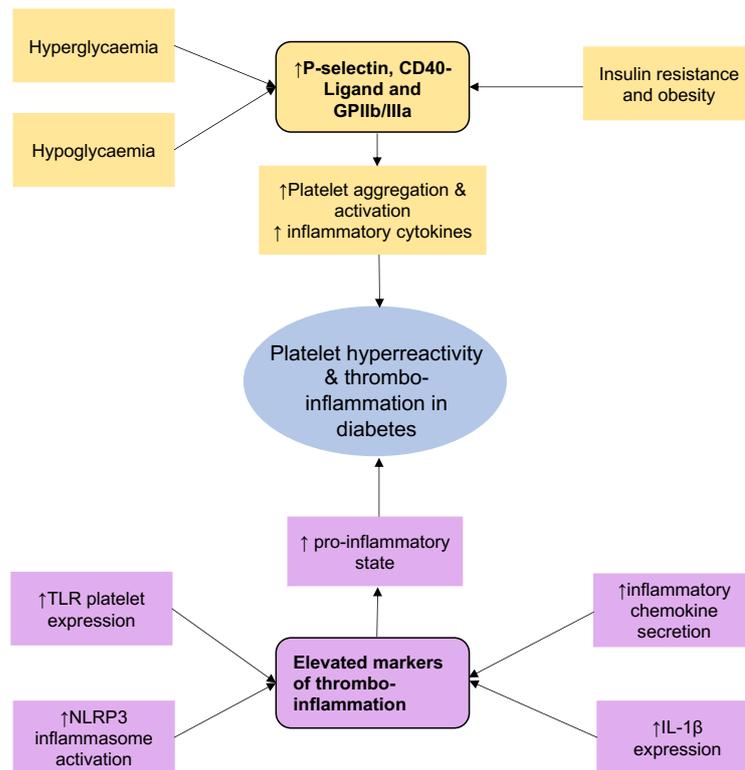
Figure 1-4: Summary of effects of DM and its metabolic milieu on platelet pathways implicated in platelet hyperreactivity and thrombo-inflammation.

The pro-inflammatory state seen in DM is driven by several factors including increased NLRP3 inflammasome activation and the associated IL-1β synthesis. Release of inflammatory cytokines and increased expression of pro-inflammatory TLRs further drive platelet associated inflammation. Metabolic milieu of DM including hyperglycaemia, hypoglycaemia and IR have been linked to increased expression of markers involved in key platelet activation pathways including P-selectin and GPIIb/IIIa. Abbreviations; GPIIb/IIIa: Glycoprotein IIb/IIIa, TLR: toll-like receptor, NLRP3: Nod-like receptor protein 3, IL-1β: interleukin 1β

1.5 Diabetes-related platelet bioenergetics

The changes associated with diabetes and platelet dysfunction described above are fundamentally energy-requiring and thus, research into metabolic shifts in cardiovascular disease has become an area of increasing interest.

1.5.1 Disease states and metabolism changes

Platelet activation is metabolically demanding, requiring rapid generation of ATP. Platelets exhibit metabolic plasticity and can switch freely between fuels and pathways such as glycolysis and oxidative phosphorylation. Alterations to these metabolic processes have been demonstrated in certain disease states, with evidence of changes in the extracellular acidification rate (ECAR), which is representative of glycolysis and also oxygen consumption rate (OCR), indicative of oxidative phosphorylation [186, 187]. Studies into these key aspects of metabolism in non-diabetes patient groups, have shown variations within these bioenergetic profiles to meet energy demands and suggests a degree of metabolic adaptability within platelets to respond to environmental changes associated with disease [188]. Whilst it is difficult to draw any conclusions from such studies about how this may affect patients with diabetes, early evidence suggests that it is an area to explore.

In reference to either T1D or T2D, studies on bioenergetics are limited and can be contradictory. One study, conducted in diabetic mouse models, evaluated the metabolic shifts in macrophages. Matsuura *et al.,* demonstrated altered glucose uptake, with associated reduction in basal glycolysis in macrophages, as measured via ECAR, supporting the notion of potential

altered bioenergetics in diabetes [189]. On the contrary, a study investigating peripheral blood mononuclear cells (PBMCs) in patients with long-standing T1D with albuminuria showed both elevated mitochondrial proton leak along with elevated glycolysis compared with healthy controls [190]. Although to date we are unaware of any studies that have been conducted specifically examining platelet metabolism in T1D, there has been one pilot study in patients with T2D, part of which examined oxygen consumption which showed a mild reduction in basal mitochondrial respiration [191]. The conflicting and lack of evidence in this area makes definitive conclusions difficult to draw and further studies are warranted, particularly in patients with T1D.

The previously outlined metabolic milieu of diabetes, including oxidative stress, insulin resistance and hyperglycaemia drive key changes to the cellular environment which in turn may affect cell metabolism including glycolysis and mitochondrial dysfunction [192-194]. Increased oxidative stress has been repeatedly demonstrated in T2D [195] and in addition to the earlier described effects, impacts oxidative phosphorylation within the platelet mitochondria, once again further driving ROS production and contributing to the imbalance of NO bioavailability [196].

One study demonstrated that elevated oxidative stress was associated with an increase in protein phosphorylation of p53 in pooled platelets from patients with T2D [193], which is associated with driving mitochondrial dysfunction and damage. This was then demonstrated through the loss of mitochondrial membrane potential when comparing the platelets from the patients with T2D with the pooled platelets from healthy controls [193]. Further studies have demonstrated that this rise in protein phosphorylation of p53 in T2D may be

contributing to accelerated platelet activation, through an aldose reductase pathway [197, 198]. Furthermore, by blocking aldose reductase, a reduction of thromboxane release has been demonstrated. This reduction can then reduce platelet activation and suggests the importance of this pathway in contributing to platelet mitochondrial dysfunction and functional platelet alterations [199].

1.5.2 Metabolic reprogramming of the platelet proteome

Proteomics and transcriptomics have been a growing area of interest across a range of diseases and blood components, including platelets. Given platelets are anucleate, the processes of transcription and translation by ribosomes are relatively limited, which creates a stable proteome primed for study through mass-spectrometry based analysis [200]. Within cardiovascular disease and diabetes, there is hope that these fields may allow individualisation of risk along with potential targets for novel therapies [201, 202].

1.5.2.1 Diabetes, cardiovascular disease and platelet proteomics

Platelet proteomics has recently gained traction as an area of focus, one study from 2009 investigated the proteome of platelets from patients with T2D compared with healthy donors. Over 100 proteins were either up or down regulated in the patients with T2D including those linked to carbohydrate metabolism, glycolysis and mitochondrial respiration [203]. A further study by Randriamboavonjy *et al*. supported this, with clear evidence of an altered

proteome in patients with T2D, associated with altered platelet adhesion [204].

Further studies into the alteration of the platelet proteome in patients with cardiovascular disease have also been published. Parguina *et al.,* demonstrated across studies that patients with either non-ST elevation Myocardial Infarction (NSTEMI) or ST elevation Myocardial infarction (STEMI) had variation in proteomes both compared with healthy controls and also those with stable coronary artery disease, with a signalling driving a propensity towards both pre-activation of platelets and more highly activated platelets [205, 206]. Further to this, the platelet proteome from patients with chronic cardiovascular disease in the form of heart failure with preserved ejection fraction, HFpEF, demonstrated differences in platelet proteome between those with HFpEF and healthy controls, including proteins associated with inflammation and those linked with poor cardiovascular outcomes [207]. In addition to cardiac pathology, one study assessed the platelet proteome in acute ischaemic stroke with the additional assessment of platelet activation [208]. There were again, clear differences in the proteome of the patients who had had a stroke compared with healthy controls, with several of the implicated proteins involved in the $\alpha_{IIb}\beta_3$ pathway which was also shown to have greater expression in the stroke cohort compared with healthy controls [208].

Whilst, at the time of writing, we are unaware of any studies analysing the platelet proteome in patients with T1D, these data suggest it is certainly an area to explore with a particular focus on cardiovascular outcomes.

1.5.2.2 MicroRNA and disease states

MicroRNA (miRNA) are non-coding RNAs transcribed from DNA and bind to mRNAs. They play an important role in the post-translation regulation of protein-coding genes and thus protein synthesis [209, 210]. The up- or down-regulation of miRNAs in disease was first and most widely described in relation to cancer but there is progressive evidence to support their importance in far more widely-reaching number of clinical conditions, including cardiovascular disease [209]. There is also emergent interest in their potential as novel biomarkers.

In addition to miRNAs in cardiovascular pathology, studies have also focussed on miRNAs and their association with endothelial dysfunction and platelet activity in patients with diabetes. One important miRNA in platelets is miR-223, implicated in the $P2Y_{12,}$ ADP-receptor pathway [211] which plays key roles in platelet aggregation and function. Other key miRNAs related to platelet activity include miR-26b and miR-140 which have been shown to target Selectin-P (SELP) mRNA, which in turn leads to enhanced P-selectin expression [212]. In one study involving 47 patients with T2D (both obese and non-obese determined by BMI), platelet mi-223, miR-26b and miR-140 were all expressed at lower levels than in the healthy controls, with the converse upregulated expression of platelet mRNA of both $P2Y_{12}$ and SELP [212]. Further to this, miR-223 knockout mice have been shown to have increased platelet aggregation and thrombus formation compared with wild-type, further supporting the notion that reduced miR-223 may contribute to cardiovascular outcomes [213, 214]. Figure 1-5 summarises these data.

Interestingly, some studies have focussed on the response to anti-platelet treatments amongst patients with diabetes. Parker *et. al*., conducted a study on patients with T2D who were on an anti-platelet therapy (including aspirin, clopidogrel or prasugrel). Diminished levels of miR-24, miR-191, miR-197 and the above mentioned miR-223 were seen in those receiving prasugrel compared with those who were on aspirin [215]. In addition, in those patients who were on either aspirin or prasugrel who had a history of previous macrovascular disease, lower levels of miR-197 were recorded when compared with patients who did not have any existing disease, suggesting that miRNAs may have potential as a biomarker of existing cardiovascular disease and even play a role in measuring response to anti-platelet therapies [215].
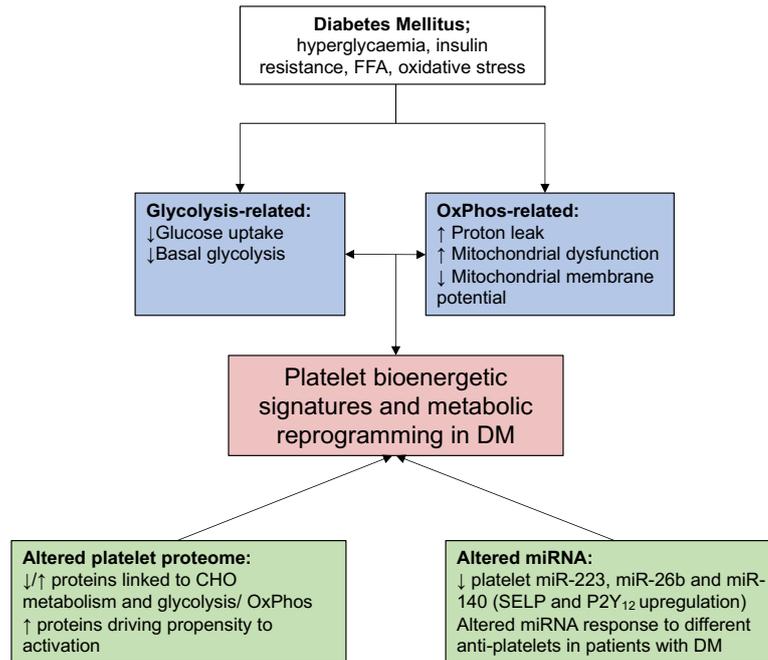
Figure 1-5: DM related-pathways and factors implicated in alterations in platelet bioenergetic signatures and metabolic reprogramming.

Changes in the key metabolic processes of glycolysis and OxPhos have been shown in disease states and there are early data to show changes in animal models of DM. Further alterations including a distinct platelet proteome and changes in miRNA expression support the concept of metabolic reprogramming occurring in platelets in patients with DM, driving cardiovascular morbidity and mortality. Abbreviations; FFA: free fatty acids, miRNA: micro-ribonucleic acid, miR: miRNA, SELP: Selectin P gene, DM: Diabetes Mellitus, OxPhos: Oxidative Phosphorylation

1.6 Obesity in Type 1 Diabetes Mellitus

Cardiovascular disease, driven in part by platelet dysfunction in diabetes is a clear concern in the management of these patients to optimally mitigate this risk. Whilst diabetes alone has clear implications on the development of cardiovascular disease, further factors including obesity and associated IR must be considered. There is growing overlap between T2D and T1D, with the acknowledgement of increasing obesity and IR in patients with T1D. To

understand the need for further research into the factors contributing to platelet dysfunction, particularly in patients with T1D in the presence of IR, it is first crucial to highlight the scope of the problem.

Patients with T1D are characterised by an absolute insulin deficiency secondary to autoimmune β-cell destruction [216]. These people have traditionally been slim, with a low or low-normal body mass index (BMI), young with increased prevalence of other autoimmune conditions including coeliac disease, vitiligo and hypothyroidism [217]. Recent global trends have seen an unprecedented increase in people with obesity [218]. Patients with T1D are not exempt from this with evidence supporting a shift towards this cohort having increasing obesity and/or other features of the metabolic syndrome [219]. Many studies now quote more than one third of patients with T1D may be obese, depending on year and country, with no evidence of this figure declining [220].

The landmark Diabetes Control and Complications (DCCT) trial [221, 222], from which we continue to glean much information with regards to the long-term progression of diabetes as well crucial outcome data, showed a huge shift in prevalence of obesity in T1D over the 18 year follow up period. The baseline prevalence of 1% (largely as a result of controlled body mass index (BMI) as part of the eligibility criteria), rises to a figure of around 30% depending whether patients were in the conventional or intensive treatment arms [223]. Whilst it is impossible to distinguish whether this is a result of the insulin therapy alone or the global shift in obesity prevalence, the fact remains that an ever-increasing number of patients with T1D will have obesity and thus a better understanding is crucial to individualise care, minimise associated complications and reduce morbidity.

1.7 Double Diabetes

The term "Double Diabetes" was first coined over 30 years ago by Bergis and Teupe [47], stemming from the concept of the co-existence of both insulin deficiency, characteristic of T1D and insulin resistance, previously considered characteristic of T2D. Yet, despite the described increasing prevalence of obesity, there remains a lack of clear diagnostic criteria for this cohort of patients. Furthermore, despite several population-based studies [224] into patients with DD, it is not yet clear on how this co-existence impacts risk of cardiovascular complications and/or other factors in disease progression.

In the original Teupe and Bergis work, there was a clear identification that insulin resistance was the crucial aspect of diagnosing DD, however they reflected upon a paucity of ability to directly measure this. They suggested a family history of T2D (16% of their cohort) was critical and thus characterised their cohort of patients' DD risk according to the strength of their family history; they saw an increased insulin requirement in those they deemed "probable DD" based on family history of T2D alone, along with reported higher glycated haemoglobin (HbA1c) as a measure of medium-long term glycaemic control [47]. Although not a formal classification, this was the first attempt to produce criteria.

Since then, further efforts have been made to more comprehensively define this sub-set of patients. Pozzilli *et al.,* were the first in more recent history to do this [225]. This primarily focussed on diagnosing DD in younger patients, following the publication by Libman *et al.,* highlighting features of DD presenting in patients as young as 5 years old [226]. Key parameters presented by Pozzilli were presence of features of T2D including aspects of

the metabolic syndrome, increased BMI and a family history of T2D and T1D. They also highlighted a lack of typical features of the clinical presentation of T1D including weight loss and osmotic symptoms as well as lack of ketoacidosis as potential clues towards a diagnosis of DD and finally potential reduced number/titre of autoantibodies to islet cells compared with T1D [225]. The focus of this review was to frame DD as an overlap between T1D and T2D. Although a step toward more clear diagnostic criteria, there still remained a degree of uncertainty surrounding the clinical utility of this.

Next, Cleland *et al.,* proposed some other features to this diagnostic conundrum, in addition to the aforementioned family history of T2D and features of the metabolic syndrome (particularly focussing on the role of low HDL compared with the typically higher levels seen in T1D) and added high total insulin doses to the picture. Perhaps more crucially, they introduced the concept of using estimated glucose disposal rate (eGDR) as a numerical marker of insulin resistance, which will be explored in further detail below, as part of this diagnostic process [227].

Perhaps most important to discuss, is the choice of patients with DD as the patient cohort of focus for this study. Research studies into platelet function involving patients with T2D have always been marred by the heterogeneity and confounders such as existing macrovascular disease, anti-platelet therapies and co-morbidities. Patients with T1D are fundamentally a more homogenous group, and particularly the younger cohorts, have far fewer co-morbidities and potential interacting medications to contend with. Those with possible DD, are thus, a unique group in whom it is possible to explore

potential physiological effects of diabetes on platelet function with greater consistency.

1.7.1 Double diabetes and complications

One of the crucial questions is whether DD is really a problem and how it affects risk of both microvascular and macrovascular complications.

Referring back to the DCCT, patients with the greatest weight gain were shown to have more features of the metabolic syndrome including elevated lipids and presence of hypertension [228], associated with increased risk of cardiovascular complications [229]. Even in relatively young people, aged 5-36 years of age, evidence of reduced high-density lipoprotein levels along with higher rates of hypertension have been shown in patients with T1D in those with obesity compared with those in the normal weight category [230]. Further to this, the CACTI study demonstrated that obesity in patients with T1D, independent of other metabolic features such as dyslipidaemia, hypertension was a risk factor for developing coronary artery disease measured through coronary artery calcium scanning [231].

1.8 Estimated glucose disposal rate

Estimated glucose disposal rate (eGDR) was first validated by Williams *et al.,* designed as a tool using readily available clinical factors in a mathematical algorithm, to determine a numerical value representative of an individual's

insulin resistance [232]. Prior to this, only the invasive and impractical hyperinsulinaemic-euglycaemic clamp had been used in research settings to measure insulin resistance, not at all amenable to routine clinical use.

eGDR was validated against this gold standard, with 24 patients with T1D recruited from the original Pittsburgh EDC study, itself involving a well-described group of patients with T1D of childhood-onset (under 17 years of age). 12 male and 12 female patients, chosen to represent the full cohort underwent hyperinsulinaemic-euglycaemic clamps with subsequent insulin resistance scores calculated, based on clinical parameters including hypertension, waist-to-hip circumference, triglyceride levels, HDL cholesterol level and family history of type 2 diabetes. HbA1c was also included, due to the previously reported association between poor glycaemic control and development of insulin resistance [233]. The glucose disposal rate measured via the clamp studies was shown to have a good inverse correlation with the insulin resistance scores (r=-0.64) [232]. The insulin resistance scores were then further analysed to establish the varying contribution of individual components and later using linear regression to optimise the algorithm. Removing triglycerides and HDL cholesterol levels were found to improve the correlation [232]. Multivariate linear regression analysis was then used to allow for an estimation of glucose disposal rate directly from the insulin resistance score and the resulting algorithm was thereby deemed the estimated glucose disposal rate [232]. Waist circumference and waist-to-hip ratio were shown to have comparable predictive ability for glucose disposal rate with slight mathematical variation between these algorithms [232]. Later, eGDR using body mass index (BMI) was shown to correlate to a comparable degree as waist circumference and waist-to-hip ratio adapted from the original

linear regression but on this occasion validated in T2D patients compared once again with the gold standard hyperinsulinaemic-euglycaemic clamps [234]. Crucially, not only could this novel tool lend itself to easy integration into clinical practice given the readily available parameters but also, as a continuous variable would allow evaluation of response to treatment including insulin sensitising-therapies such as metformin.

1.8.1 Estimated glucose disposal rate, cardiovascular disease and mortality

Understanding the role of IR itself in the development of cardiovascular morbidity and mortality in patients with T1D is critical and further to this, identifying whether eGDR as a surrogate for IR can be used to predict this mortality risk. Nystrom *et al.,* used a large Swedish population registry of over 17,000 patients with T1D to determine the relationship between eGDR and mortality, along with numerical threshold values to decipher the difference in risk within the spectrum of IR itself [235]. The group stratified this large cohort according to eGDR and used groups of <4mg/kg/min, 4 to 5.99mg/kg/min, 6 to 7.99mg/kg/min and ≥8mg/kg/min, again with IR highest in those with the lowest numerical value of eGDR given the inverse correlation. All-cause mortality was highest in those with an eGDR <4mg/kg/min at 15% (versus 1% in eGDR ≥8) [235]. Similarly, cardiovascular mortality was also highest in those with the lowest eGDR (<4mg/kg/min) having a 3-fold greater risk of cardiovascular death. Furthermore, decreasing eGDR was associated with increased risk of cardiovascular events defined as any, or a combination of acute myocardial infarction (MI), stroke or ischaemic heart disease during the

follow-up period [235]. This was the case whether unadjusted or adjusted for variables including age, sex and renal-function. In fact, when adjusting for an exhaustive list of variables, the hazard ratio remained ≥ 2 for patients with an eGDR <4 compared to those with eGDR ≥ 8 [235]. Importantly, those with low IR (eGDR ≥ 8) showed comparable survival to the age and sex matched background population, with this value now often quoted as the threshold below which it can be considered that the patient has probable IR. This study demonstrated the efficacy of using eGDR in not only measurement of insulin resistance, but also its correlation with cardiovascular risk and mortality.

Whilst cardiovascular events are understandably of great concern to those with and those treating patients with T1D, microvascular complications also significantly impact quality of life and have significant financial burden on health services. In one study involving 91 patients with T1D, only patients with an eGDR <8.16mg/kg/min had evidence of any microvascular complications including retinopathy, nephropathy or neuropathy [236]. Patients with evidence of retinopathy had a lower mean eGDR (5.97$\pm$1.2) compared to those without retinopathy (9.38$\pm$2) with similar results seen when comparing those with and without evidence of neuropathy and nephropathy (determined by albuminuria values), p<0.001 in all cases [236]. Whilst a more limited number of patients compared with the larger scale Swedish cohort, this still draws attention to the role of IR in development of microvascular complications in patients with T1D and the utility of eGDR in this capacity.

Given the use of HbA1c in the eGDR algorithm, some have queried the influence of glycaemia, rather than IR itself in the development of complications associated with diabetes. Using data from the 10 year follow up

Pittsburgh EDC study, results demonstrated that glycaemia was not predictive of key end-points including coronary artery disease (CAD), with no statistical correlation whereas IR, again measured by eGDR, showed an inverse relationship i.e. those with the lowest eGDR indicative of highest IR had higher risk of CAD [237].

Data from our own local population also support the role of IR in the development of both micro- and macrovascular complication as well as the utility of eGDR as a marker for this risk. From a population of over 2000 patients with T1D across the spectrum of eGDR, patients with an eGDR <4mg/kg/min had a odds ratio of 4.84 for retinopathy, 8.35 for nephropathy and 13.22 for macrovascular complications, compared with patients with eGDR ≥8 mg/kg/min [238]. Those with eGDR ≥8mg/kg/min also had the lowest rates of any complications, irrespective of HbA1c, once again supporting the notion that IR, independent of glycaemia, is an independently important factor in determining vascular complications in patients with T1D [238].

1.9 Markers of glycaemia

It has been established earlier that hyperglycaemia, hypoglycaemia and glycaemic variability all contribute to cardiovascular disease, and one mechanism is related to modulation of platelet activation. It is thus crucial to established the various markers of glycaemia, their meaning and their associations with both macrovascular and microvascular disease.

1.9.1 Glycated Haemoglobin

Glycated haemoglobin (HbA1c), was first identified to be "abnormal" in patients with diabetes in the late 1960s [239] and was proposed as a marker of glycaemic control in 1976 [240]. It has since been relied upon as the gold-standard marker for diagnosis and monitoring of glycaemia in patients with diabetes [72] and is typically the reference used in most guidelines. HbA1c provides good estimation of average glucose over roughly a 8-12 week period and is thus a useful tool to gain insight into medium-long term glycaemia. However, in recent years there has been growing recognition for the potential limitations that exist, including anaemia, renal failure and pregnancy [241, 242]. Furthermore, whilst useful for an average over weeks to months, HbA1c does not inform patients or healthcare professionals about the daily glucose excursions and this cannot be ignored [72]. This has led to a more holistic approach to glycaemic assessment to include more daily and short-term insights alongside the traditional HbA1c.

1.9.2 Time in Range

The requirement for more in-depth, daily assessment of glucose has been vastly aided through the development and accelerated availability of sensors for continuous glucose monitoring (CGM) or intermittently scanned continuous glucose monitoring (isCGM). One such parameter that has been rapidly adopted is the time in range (TIR). TIR is defined by the time within a 24-hour period that a patient spends in a target range, which in the majority of patients is usually targeted at 3.9-10 mmol/L. Whilst it is accepted that for any given TIR there will be a degree of variability with the corresponding HbA1c value, it is largely accepted that a TIR of 70% is roughly equivalent to HbA1c of 7.0%

(53mmol/mol) [243]. TIR $\geq$70% is generally agreed by International Consensus as the target for optimal glycaemic control [72]. Certain groups have tighter target ranges, such as 3.5-7.8 mmol/L in pregnancy [244]. TIR very much lends itself to personalised targets to suit individual patients.

Recently, new guidance more commonly refers to both HbA1c and TIR targets although with regards to TIR, though these targets are largely relating to patients with T1D, given at present access to CGM/isCGM is not widely available for T2D or other forms of diabetes [245]. TIR may also play a role in predicting both microvascular and macrovascular complications. The self-monitoring of blood glucose (SMBG) from the landmark DCCT trial was re-categorised to give corresponding TIR for the participants with T1D who were included in the study. A difference of approximately 10% was found between those who developed microvascular complications and those who did not. Further to this for every decrease in TIR of 10%, there was an associated 64% increase in risk of developing retinopathy and 40% increase in risk of developing microalbuminuria [246]. In patients with T2D, a fall in TIR has also been shown to correlate with increased risk of developing retinopathy [247].

1.9.3 Hypoglycaemia

As already established, hypoglycaemia also contributes to platelet dysfunction and a pro-thrombotic environment. Especially given the risk of hypoglycaemia associated with insulin use, using CGM/isCGM to incorporate a thorough assessment of hypoglycaemia has been a welcome addition to the modern management of diabetes, in particular for people with T1D.

Hypoglycaemia or time below range (TBR) is provided in CGM reports and importantly is categorised into level 1 hypoglycaemia (blood glucose 3.0-3.9mmol/L) and level 2 hypoglycaemia (<3.0mmol/L) [72]. This removes one of the previous barriers in assessing the extent of hypoglycaemia, for patients who have impaired hypoglycaemic awareness who are perhaps at greatest risk but had previously certainly under reported this. Targets for these parameters have also been agreed, with a target of <5% for TBR, aiming for <4% for level 1 and <1% for level 2 hypoglycaemia.

1.9.4 Glycaemic Variability

Whilst the role of GV in cardiovascular disease has earlier been outlined, there are a number of calculations that can be used when referring to GV and it is thus important to outline the meaning with reference to this study and also the use of GV in clinical practice generally [248, 249]. There are a variety of reporting tools used in clinical studies to determine GV, however for the purposes of this study this has been derived from isCGM. Standard deviation (SD) and coefficient of variation (CoV, %), which is calculated as the SD divided by mean glucose are the most commonly used in clinical settings and CoV is the unit of measurement used for assessment of GV in this study and available from isCGM. This therefore equates to the deviation in glucose from the median over a 24-hour period and provides assessment of intra-day variations in glucose. Large fluctuations thus correspond with a high GV. A CoV of <36% is the typically accepted threshold value that is agreed as a clinical target [245, 250] although there is some suggestion the lower threshold of <33% may reduce hypoglycaemic risk further [250]. Further data

on GV, especially in the context of micro- and macrovascular complications are still lacking but with the ever-increasing availability of isCGM and CGM, evidence in the area will likely grow in the near feature.

1.10 Current therapies and future targets

As clearly established, cardiovascular disease in patients with diabetes is complex and multifaceted. A holistic and individualised approach towards intervention and indeed prevention is therefore crucial to minimise the development and progression of atherosclerosis, inflammation and overt vascular disease [251]. Addressing the myriad of discussed contributing factors seen in patients with diabetes including optimising glycaemic control, whilst minimising hypoglycaemia and GV, dyslipidaemia and factors contributing to insulin resistance is likely to form the mainstay of preventative interventions. Within the UK, guidelines focussed on mitigating these individual risk factors are relatively well documented [252, 253]. Yet, beyond risk factor management, guidance is much less clear about options for anti-thrombotic agents, particularly in the setting of primary prevention. There is a lack of clear evidence and existing studies, particularly those specific to patients with diabetes, are frequently contradictory and thus makes establishing clear guidance somewhat challenging. Despite this, there are many novel biomarkers and targets, some of which have been already been discussed, that may play a crucial role in future therapeutics to reduce this burden of morbidity and mortality.

1.10.1 Current evidence for primary prevention

Primary prevention for patients with diabetes within the UK has been an area of debate for some time. At one time, national and international guidelines advocated consideration of aspirin for people living with diabetes without existing cardiovascular disease, largely based on historic data from the late 1980s and early 1990s [254, 255]. This was then challenged by the Primary Prevention Project, which was unable to demonstrate comparably beneficial effect of aspirin in primary prevention in over 1,000 people with T2D, thus determining the potential harms of aspirin treatment would outweigh the intended benefits, however a clear change in management did not follow [256]. Further meta-analyses followed, failing to show clear benefit over harm for the routine use of aspirin as primary prevention [257-259], but the included studies had varying doses of aspirin and several studies lacked sufficient power, making definitive conclusions once again difficult. The landmark 'A Study of Cardiovascular Events in Diabetes' (ASCEND) trial provided far more decisive and clear data, involving over 15,000 patients of whom 94% had T2D. ASCEND randomised patients to receive either low-dose aspirin (100mg once daily) or placebo with a mean follow-up period of 7.4 years. In those patients receiving low-dose aspirin there was a 12% decrease in the primary end point of serious vascular event (including non-fatal MI and stroke/transient ischaemic attack), HR 0.88, (95% CI 0.79-0.97) [260]. Conversely, the safety data demonstrated a significantly higher proportion of bleeding events in those in the aspirin group [HR 1.29 (1.09-1.52), with gastrointestinal bleeding the most common] [260]. Whilst there was clearly a positive effect on reducing vascular events by taking the aspirin, the paper concluded that these benefits

were outweighed by the increased bleeding risk, despite there being a favourable benefit:risk ratio.

Thus, to date, the most widely accepted view is that using aspirin for the primary prevention in patients with diabetes is not routinely recommended, except to be considered in the highest-risk patients without contraindications and is a view supported by the most recently available guidelines from the European Society of Cardiology (ESC)/European Association for the Study of Diabetes (EASD) recommendations [253]. However, it may well be the case that future revisions of such guidelines may re-examine these recommendations in such a perpetually evolving area.

1.10.2 Current evidence for secondary prevention

Whilst primary prevention in patients with diabetes remains a contentious view, it is well accepted that secondary prevention guidelines are largely in line with the treatment of cardiovascular disease for the wider population. Yet it is important to examine and recognise the data that exist specifically relating to patients with diabetes who have a coronary or vascular event.

Dual antiplatelet therapy (DAPT) using aspirin and a $P2Y_{12}$ inhibitor for one year is the current mainstay of treatment following an acute coronary event in patients with or without diabetes. Long-term monotherapy, usually with aspirin, typically follows thereafter [261]. The CURE (Clopidogrel in Unstable angina to prevent Recurrent Events) study included 12,562 patients, of whom almost one quarter had diabetes. During the study patients received a combination of aspirin and the $P2Y_{12}$ inhibitor, clopidogrel, following acute coronary

syndrome (ACS). The results demonstrated that in those patients who received DAPT versus aspirin and placebo, there was a 20% reduction in cardiovascular death, MI and stroke at 9 months post-ACS event. These results were comparable in the sub-group analysis of patients with diabetes [262]. Further subsequent studies have had comparable results and thus DAPT treatment was established post ACS [263, 264].

Subsequently, newer $P2Y_{12}$ inhibitors have also been explored for increased efficacy compared with clopidogrel. The TRITON (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel-Thrombolysis in Myocardial Infarction) study explored prasugrel and aspirin compared with the gold-standard clopidogrel and aspirin combination. This included 13,608 patients, with 3,146 having diabetes. Whilst there was an overall significant reduction in vascular events in those receiving prasugrel-aspirin, there was also increased bleeding risk. However, in the sub-group analysis of patients with diabetes, there was a comparable reduction in vascular events but without the significant increase in bleeding risk [265].

The PLATO (Study of Platelet Inhibition and Patient Outcomes) Study carried out a similar study of Ticagrelor plus aspirin versus clopidogrel plus aspirin. PLATO included 18,624 patients with both ST elevation and non-ST elevation MI, with 4,662 patients included who had diabetes. In the diabetes cohort, there was a vascular risk benefit, (HR 0.88 [95% CI 0.76 to 1.03]) in the ticagrelor group, with no clear increase in bleeding risk [266].

Finally, PEGASUS-TIMI 54 (prevention of cardiovascular events in patients with prior heart attack) addressed the use of extended dual therapy; comparing ticagrelor plus aspirin with aspirin alone. Over 21,000 individuals

with a previous history of MI within the previous 1-3 years were included and all participants had a minimum of one risk factor including diabetes [267, 268]. In fact, patients with diabetes represented around one third of the total cohort. Patients were randomised to either 60/90mg of ticagrelor along with standard aspirin or placebo plus standard aspirin [268]. At either dose of ticagrelor (plus aspirin) there was evidence of reduced risk of MI, stroke or cardiovascular death compared with patients taking aspirin alone (7.85% in 90mg ticagrelor, 7.77% in 60mg group and 9.04% in placebo, *p<0.01* for both dual therapy groups vs placebo). Specifically for the diabetes sub-group, comparable results to the full cohort were demonstrated. Whilst these results were promising, it must be noted that there was a higher overall rate of bleeding events in the dual therapy groups but in fact no significant difference was shown for both major or life-threatening bleeding. Of note, there was a numerical but non-significant decrease in bleeding rate with the 60mg dose of ticagrelor [267].

1.10.3 Aspirin Resistance

An area of contention and consideration for both primary and secondary prevention in patients with diabetes is the concept of aspirin resistance [269, 270]. Evidence from studies such as the Primary Prevention Project support the notion that aspirin lacks definite efficacy in patients with diabetes. There is a paucity of clear data in this area, but one study investigating the extent of aspirin resistance in patients with T2D demonstrated that 21.5% were resistant to aspirin therapy, with a further 17% deemed semi-responders [271]. The definition of aspirin resistance varies and is likely one potential

reason this concept has not been more thoroughly explored. In the above study, the definition was based on platelet-dependent thrombus formation in the presence of chronic aspirin treatment in 172 patients [271]. Another study, using platelet aggregation as the marker, investigated response to aspirin in 92 patients with T1D and 111 with T2D. It found similar results to the previously mentioned study, with 21.5% resistance in those with T1D and 16.2% in T2D [272]. Additionally, they identified being female was associated with increased prevalence of aspirin resistance.

The exact mechanisms through which aspirin resistance manifests in diabetes have not been fully characterised, but are likely multifactorial. The established platelet hyperreactivity and hypofibrinolysis in diabetes described in depth earlier in this chapter are two such mechanisms [270]. The metabolic milieu of diabetes also contribute with hyperglycaemia, insulin resistance and dyslipidaemia all considered to play important roles [270, 273].

In the context of diminished response to aspirin, along with the well described increased cardiovascular risk, it is clear further research into alternative management strategies such as twice-daily aspirin [274, 275] or novel therapies for those at greatest risk are required.

1.10.4 Novel targets and future directions

Individualised "precision medicine" and a patient-centred approach is becoming an increasing focus within the management of chronic conditions. The contribution of various metabolic parameters including hyperglycaemia, hypoglycaemia, insulin resistance, dyslipidaemia, smoking status and

hypertension is well established. Managing these risk factors is integral to reducing long-term risk.

In terms of direct anti-platelet therapies, there are well established existing options for both primary prevention with aspirin and secondary prevention with addition of $P2Y_{12}$ inhibitors such as clopidogrel [276], which have previously been thoroughly summarised [277]. Many guidelines relating to treating cardiovascular disease in diabetes rely on extracting data from sub-categories within wider randomised controlled trials (RCTs), with a distinct lack of diabetes specific RCTs and data relating to patients with T1D are even more sparse. Some specific trials have emerged including the Aspirin and Simvastatin Combination for Cardiovascular Events in Prevention Trial in Diabetes, (ACCEPT-D) [278] investigating 100mg aspirin (with simvastatin) in patients with either T1D or T2D aged $\geq$50 years without existing cardiovascular disease. The Aspirin Twice a Day in Patients with Diabetes and Acute Coronary Syndrome, ANDAMAN, study is assessing a twice daily 100mg regime in patients with diabetes post-ACS event [279]. Prolonged release aspirin has also been developed, with the hope this would provide 24/7 antithrombotic effects as well as liquid formulation aspirin which was associated with a reducing GI bleed risk [280, 281]. The long-term outcomes of both are not yet established.

Moving away from aspirin, phosphodiesterase III inhibitor, cilostazol, has been shown to augment $P2Y_{12}$ inhibition when used in conjunction with aspirin and clopidogrel. The principle behind phosphodiesterase III inhibition is to increase phosphorylation of vasodilator-stimulated phosphoprotein (VASP) as a result of increased intraplatelet cAMP levels which in turn leads to increased platelet

inhibition [282, 283]. Whilst licensed for claudication, there remains a need for more evidence to support its use in cardiovascular disease or post-ACS and specifically in patients with diabetes. Finally, vorapaxar, a thrombin-receptor antagonist has also been explored with the potential to offer inhibition of thrombin-driven platelet aggregation and activation [284]. When used as adjunctive therapy with standard secondary prevention, it has been shown to reduce cardiovascular events in patients with pre-existing cardiovascular disease including MI and peripheral arterial disease [284-286].

In terms of novel biomarkers, some of the explored parameters of platelet dysfunction in diabetes have been proposed for future consideration. There is also evidence for the use of markers for monitoring response to anti-platelet therapies, in part to mitigate the above-described aspirin resistance. Platelet-leukocyte aggregates are one such marker, suggested as a potentially more sensitive marker of platelet activation than P-selectin. Their role as biomarkers for cardiovascular disease gained traction with the SARS-CoV-2 pandemic [287]. Platelet-leukocyte aggregates are stable, thus may prove useful in this clinical context and have been linked both to early detection of cardiovascular disease, prognostication following percutaneous coronary intervention and also for measuring efficacy of anti-platelet therapies [288].

In recent times there has been a growing global interest in genomics, transcriptomics and proteomics, including in platelets, and this is one such area that will likely have an evolving role across a multitude of diseases and therapeutics. Some of the earlier highlighted miRNAs relating to diabetes are possible targets, along with others linked more widely to cardiovascular disease. Silencing miR-155, implicated in foam cell formation and

atherosclerosis, leads to a reduction in atherosclerotic plaques. Similarly, as described, miR-223 has a role in a variety of pathways that contribute to cardiovascular disease [289]. It is expressed in a number of cells beyond platelets and thus a target for consideration in the development of new treatments.

IL-1β has also been identified as a potential target with several inhibitors of its signal transduction developed. Anakinra, an existing interleukin receptor antagonist, is currently used as a disease-modifying anti-rheumatic drug (DMARD). One trial investigated its role in acute coronary events, with patients receiving anakinra or placebo following NSTEMI. Inflammatory markers including C-reactive protein (CRP) and IL-6 had fallen 14 days after receiving the dose [290]. In addition, Canakinumab and Gevokizumab are monoclonal antibodies selectively targeting IL-1β. Gevokizumab has mostly been involved in cancer research, but Canakinumab was the focus of the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study) trial in which over 10,000 participants with elevated CRP following MI were randomised to receive the drug or placebo with some promising results [291]. These data suggest potential in targeting IL-1β, though further evidence is required, particularly in patients with diabetes in whom this pathway appears highly influential.

Whilst it is clear that reducing cardiovascular risk in patients with diabetes must include addressing indirect factors such as hypertension, dyslipidaemia and glycaemic control there must also be focus for developing new direct treatment options moving forward as well as individualising management for patients to optimise outcomes.

1.11 Hypothesis and aims

This chapter highlights the importance of platelet dysfunction in the development of cardiovascular disease in patients with both T1D and T2D. Metabolic characteristics of diabetes including insulin resistance, hyperglycaemia, hypoglycaemia, dyslipidaemia and GV contribute to platelet dysfunction. Platelets are hyperreactive and there is a resulting pro-thrombotic and pro-inflammatory environment that further drives the development of cardiovascular disease. However, the exact contribution of each of the glycaemic abnormalities and IR to platelet activation is far from clear. Individuals with DD provide unique representation of the interplay between dysglycaemia and IR. Significantly, this cohort is more homogenous than a typical group of patients with T2D making them ideal for investigating platelet dysfunction without interfering co-morbidities and various medications.

My overall hypothesis is that individuals with T1D display an altered metabolic platelet phenotype, characterised by a proinflammatory/prothrombotic state early in the disease process and that this may be exacerbated by IR. The aims of this study include:

1. Investigate the role of dysglycaemia and IR on platelet activation and function in patients with T1D

2. Analyse the role of dysglycaemia and IR on response to platelet inhibition in patients with T1D.

3. Determine the relationship between T1D and dysglycaemia and altered platelet glucose metabolism and/or metabolic bioenergetics.

Chapter 2: Materials and Methods

2.1 Introduction

Throughout this thesis, samples from patients were taken from participants enrolled in the DEVELOP study. An overview of this including the study design, inclusion/exclusion criteria and study population are detailed below. Specific laboratory techniques including relevant reagents, analyses and methods are also described and provide a reference for the rest of the thesis.

2.2 Patient study

2.2.1 Study overview

As mentioned, samples from patients with T1D were obtained from those participating in the "Double diabetes and adverse clinical outcome: identification of mechanistic pathways" (DEVELOP) study. This was designed as a cross-sectional study enrolling adult patients with T1D from Leeds Teaching Hospitals Trust. Ethical approval was granted by the Health Research Authority (REC reference: 19/NE/0349, IRAS number 259072). Initial recruitment opened in October 2020, with early recruitment in particular impacted significantly by the COVID-19 pandemic, at the time of writing recruitment remains open with a closing date of July 2023 (extension sought and granted from the initial closing date). At the time of writing, a total of 110 participants had completed the study, with a subset of this full cohort providing samples detailed in each of the relevant chapters of this thesis.

Inclusion criteria were as follows; 1) Confirmed diagnosis of T1D for a minimum duration of 3 years and on current treatment with insulin (any route of administration accepted), 2) Aged 18 years or older at the time of study visit

and under 40 years old, 3) BMI $\geq$ 18kg/m^2. The exclusion criteria were 1) end-stage renal disease, 2) current or personal history of malignancy, 3) pregnancy 4) current use of anti-coagulant or anti-platelet medications.

2.2.2 Study Visit

Participants recruited to the DEVELOP study attended for a one-off physical visit. Written consent was obtained prior to any collection of study parameters. Inclusion and exclusion criteria were confirmed. Demographic data were recorded on a standardised data entry form. Data including weight, height, waist circumference and blood pressure were collected. Questions relating to personal medical history and family history of autoimmune conditions, T2D and ischaemic heart disease were completed. Information relating to insulin administration, doses (including basal and bolus) and adjunctive therapies were also recorded. 10g monofilament testing was completed to record neuropathy status and retinopathy status was determined by last retinopathy screening result. Blood sampling was conducted as described below, with routine clinical results including HbA1c, urea & electrolytes, liver function tests, full blood count and lipid profile all recorded. Additional tests including coeliac antibody testing and urine albumin: creatinine were collected when indicated.

A blinded isCGM, Freestyle Libre Pro® sensor (Abbott) was inserted on the upper arm. In the cases of patients already wearing a routine Freestyle Libre sensor, a second sensor was inserted onto the opposite arm. Patients were instructed to wear the sensor for as many days as possible up to 14 days. Data were only included from patients who had worn the Libre Pro sensor for

a minimum of 6 consecutive days. In depth data were then exported from each sensor including key parameters described in the first chapter; the main value derived from the isCGM data was time in range (TIR). The target was defined as a glucose level of 3.9-10 mmol/L as discussed in the previous chapter, based on international consensus [245]. Additional data were extracted for time above range (TAR), referring to time spent above a glucose value of 10mmol/l. This was further divided as per standard ambulatory glucose profiles into TAR level 1 (glucose value of 10.1-13.9 mmol/L) and TAR level 2 (>13.9 mmol/L). Hypoglycaemic parameters of time below range (TBR) included level 1 hypoglycaemia, glucose 3.0-3.8 mmol/L, and also level 2, glucose <3.0 mmol/l.

2.2.3 Blood sampling

Patients recruited to the DEVELOP study underwent standardised blood sampling. Venepuncture was conducted in a non-fasted state from the antecubital fossa, with up to 50ml of venous blood drawn with steps taken to minimise potential platelet activation from venepuncture such as using a wide-bore needle and removal of tourniquet prior to sampling. In addition to routine clinical tests such as HbA1c, liver function tests, lipid profile and full blood count, research bloods were taken. Relevant to this study blood was drawn either into tubes containing sodium citrate or commercially available Acid-Citrate-Dextrose (ACD-A, BD) or both, depending on the laboratory technique planned for the specific sample. For blood samples from healthy controls, blood was drawn in the same manner. All donors were volunteers who provided written consent. It was confirmed that all donors had not used anti-

inflammatory medications, anti-coagulants nor anti-platelet therapies. Tubes were transferred to carry out platelet studies within 2 hours of sampling.

2.3 Reagents and buffers

2.3.1 Buffers

A number of common buffers or solutions were used and referred to throughout this chapter and their components are summarised in table 2-1.

Buffer/Solution	Ingredients
Acid Citrate Dextrose-A (ACD-A)	85mM sodium citrate, 136mM dextrose, 42mM citric acid, 1mM potassium sorbate
Modified Tyrode's buffer	0.5mM $MgCl_2$, 0.55mM NaH_2PO_4, 2.7mM KCl, 5mM HEPES, 5.6mM glucose, 7mM $NaHCO_3$, 150mM NaCl, pH 7.4
Phosphate-buffered Saline (PBS)	1.5M NaCl and 100mM Sodium Phosphate, pH 7.4
Dulbecco's Modified Eagle Medium (DMEM)	0.8mM MgSO4, 1.8mM $CaCl_2$, 143mM NaCl, 5.4mM KCl, 0.91mM NaH_2PO_4, 5.0mM HEPES

Table 2-1: Summary of commonly used buffers and solutions

2.3.2 Antibodies

A number of antibodies for flow cytometry were used are summarised along with the manufacturer and catalogue numbers from in table 2-2.

Company	Antibody (Catalogue number)
BD Pharmigen	PE Mouse Anti-Human CD62P (555524)
	FITC Mouse Anti-Human PAC-1 (340507)
	APC Mouse Anti-Human CD42b (551061)
	BB700 Mouse Anti-Human CD42b (742219)
	PE Mouse IgG1 Isotype Control (556650)
ThermoFisher Scientific	Annexin V APC (R37176)
Bio-techne	Human Glut1 Alexa Fluor 405 (FAB1418V)
	Human Glut1 Alexa Fluor 405 (FAB1415V)
	Mouse IgG2B Alexa Fluor 405-conjugated Isotype Control (IC0041V)
Abcam	2-NBDG Glucose Uptake Assay Kit ab235976

Table 2-2: Summary of flow cytometry antibodies and their manufacturers

2.4 Laboratory techniques

2.4.1 Platelet isolation

For all platelet isolation, blood was drawn into ACD-A tubes and carefully transferred from individual tubes into a clean 50ml falcon tube. Blood was centrifuged at 100g at 20°C for 20 minutes with no brake. The resulting isolated platelet-rich plasma (PRP) was then carefully removed using a wide bore cut-end Pasteur pipette and transferred to 15ml conical bottomed centrifuge tubes. Special care was taken to ensure the bottom phase with red blood cells was not disturbed to avoid contamination.

PGI_2 (200nM) was added and the PRP centrifuged at 1000g at 20°C for 10 minutes resulting in a platelet pellet and a supernatant of platelet poor plasma (PPP). The PPP is carefully removed, tube drained and platelet pellet re-suspended first in 1ml of modified Tyrode's buffer and then topped up 5.5ml. To ensure that these platelets were free of plasma, PGI_2 (200nM) was added and the tubes were further centrifuged at 1000g at 20°C for 10 minutes. The supernatant was again removed and drained, with the final platelet pellet resuspended in buffer dependent on the intended experiment. Platelets were then counted using one of the below methods before resting for a minimum of 20 minutes at 37°C.

2.4.2 Platelet counting

Following isolation from whole blood, platelets were counted as a prelude to dilution to the desired concentration. For this thesis this was conducted in one of two methods, using either a Beckman Coulter Z1 particle counter or in the absence of a particle counter using flow cytometry.

2.4.3 Light transmission aggregometry

To ensure that platelets were appropriately isolated and functional, platelet suspensions were tested using light transmission aggregometry (LTA). LTA has been used to assess platelet function for over 60 years and often provides initial data on potential functional platelet changes [292]. As shown in figure 2-1, the principle works based on light transmission through a suspension, with transmission being detected by a photocell. At rest, isolated platelets form a cloudy suspension through which light is not transmitted, that is, 0% transmission. Following addition of a platelet agonist, as shown in the example in figure 2-1, initial activation results in shape change which typically shown as a reduction in percent transmission. Following this, as platelets aggregate, more light is transmitted over time and thus the measured percentage transmission increases until a plateau at which point further aggregation and thus light transmitted is unlikely. LTA has been shown as a well-validated and useful measure of platelet function [293].
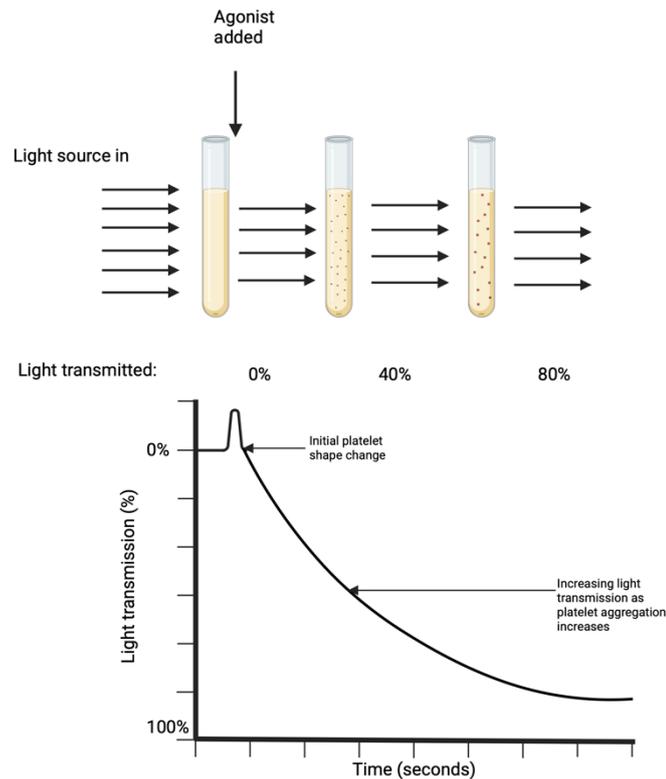
Figure 2-1: Light transmission aggregation schematic.

Shown is a representative platelet aggregation trace demonstrating light transmission measured in percentage as time progresses, following addition of a platelet agonist to induce aggregation.

Figure created using BioRender.

As mentioned, whilst LTA was not used in this thesis to test potential changes in platelet function in the patients with T1D, it was used initially to ensure my own technique for platelet isolation was adequate and ensured platelets were functioning properly. For this, platelet responses to collagen and thrombin were examined. Platelets (2.5×10^8/ml) were incubated with collagen (1μg/ml-10μg/ml); which led to a concentration dependent increase in aggregation (Figure 2-2, panel A). At low dose collagen (1μg/ml and 2μg/ml) minimal change was seen, with only 5±1% and 14±9% light transmission respectively, neither of which were statistically significant. The threshold response was at

4ug/ml with 45±26% light transmission. Maximal response was seen with both 8μg/ml 10μg/ml at 74-80% light transmission.

As shown in panel B, thrombin (0.01U/ml-0.1U/ml) also induced a clear dose response. There was a minimal change seen at the lowest concentration, 0.01U/ml, with a threshold response at 0.02U/ml (69±8% light transmission). This increased to reach a plateau around 0.04U/ml at 86±9% with no clear increase beyond this at either 0.08U/ml or 0.1U/ml, $p < 0.001$ at all 3 of these doses.
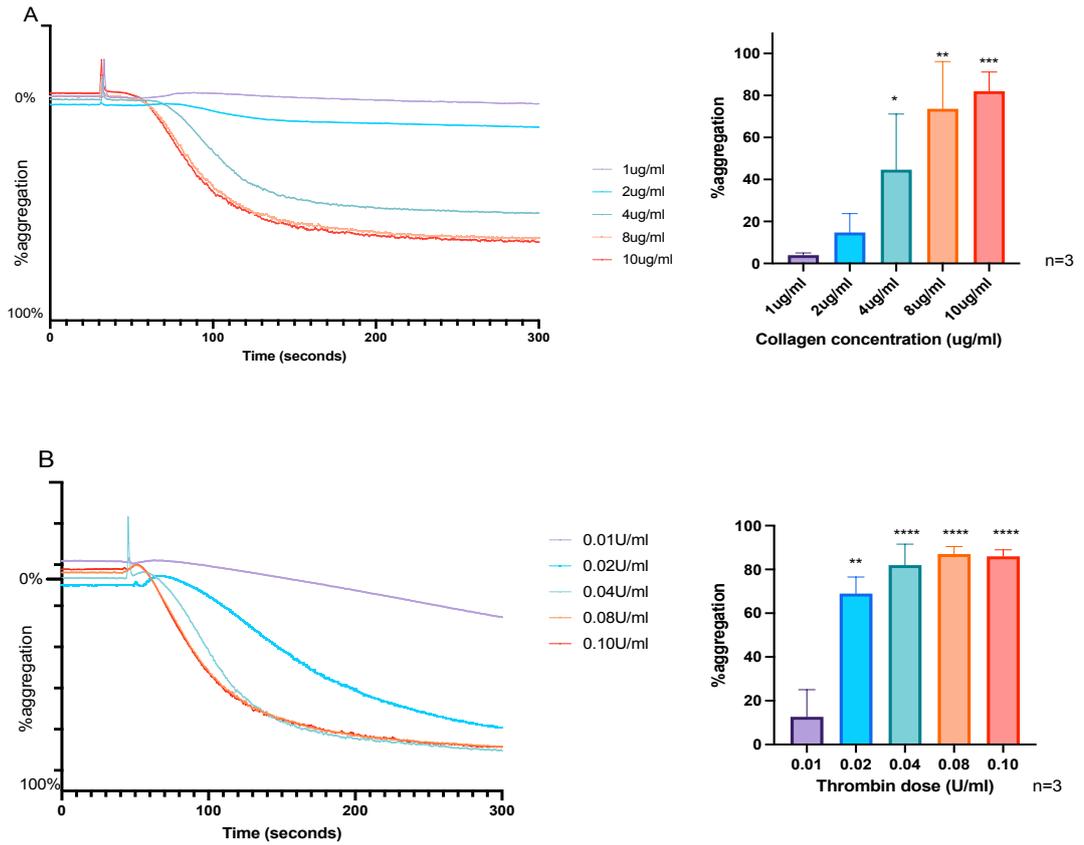
Figure 2-2: Light transmission aggregometry (LTA) using isolated platelets from healthy volunteers.

Panel A demonstrates a dose-response to collagen at incrementally increasing doses (1μg/ml to 10μg/ml). Panel B demonstrates the same for thrombin (0.01U/ml to 0.10U/ml). Graphs on the left are representative examples of the %aggregation dose-response data from one such LTA experiment. Bar charts on the right represent full data from n=3 with statistical significance of each concentration compared versus lowest dose (i.e., 1μg/ml for collagen and 0.01U/ml for thrombin). *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$. n=3.

2.4.4 Fluorescent flow cytometry

2.4.4.1 Basic Principles

Flow cytometry is a widely used technique to analyse cells and is widely applicable to a multitude of research avenues. Flow cytometry uses optics and fluidic systems to analyse a single cell or particle in suspension as they flow past laser(s) [294, 295]. This involves a sheath fluid that is pressurised to a sufficient degree to ensure cells flow in a single file, which ensures that the amount of light from the lasers that passes through reaches each cell equally. Hydrodynamic focussing is used to align the cells accurately and involves sheath fluid passing from narrow tubing into which the cells in suspension are added. The optics of flow cytometry use light from specific lasers which when directed at the cells produced a range of measurable characteristics. The light scattered is then measured in two different directions, forward scatter (FSC) and side scatter (SSC). These values are crucial as they indicate the relative size of the cell of interest and therefore can be used as an initial identification [295, 296]. Cells can also be labelled by a range of fluorophores, which are usually antibodies with tagged fluorescent labels. When excited by the lasers these are fluorescent compounds that emit light that can be detected and appropriately analysed through an attached digital interface with appropriate software to process the signal from the cytometer.

2.4.4.2 Platelets in flow cytometry

Fluorescent flow cytometry is a highly useful tool in the arsenal of options to examine platelet function. Using fluorophores, we are able to assess a variety of platelet surface receptors and can also be useful in understanding platelet interactions with other cells. This thesis primarily uses fluorescent flow

cytometry to investigate functional changes within platelets. Indeed, earlier techniques to assess platelet function such as the described LTA have been compared with evaluation of fluorescent markers of platelet activation used in fluorescent flow cytometry [297]. It has been shown that there is strong correlation between these techniques, with the advantage that flow cytometry permits a multiparameter assessment of a variety of pathways at any given time, with an ever-increasing utility in the field of platelet biology.

2.4.4.3 Multiparameter cytometry assay for assessing platelet function

As mentioned, a powerful aspect of flow cytometry is the opportunity to examine multiple different markers at a singular time point from a single sample. This is not only efficient but allows us to gain an insight into the interplay between these various aspects of platelet function. A standardised approach to investigate platelet function by flow cytometry has been used and published by the group and this was the basis for the techniques/protocols used throughout this thesis [294].

Platelets are first identified based on the FSC and SSC properties. In almost all cases, however, to minimise potential contamination, further platelet identification was conducted through the use of a platelet specific identification marker; chosen based on compatibility with other fluorophore(s) in the assay to minimise spectral overlap. Figure 2-3 shows an example of gating initial for

the physical platelet properties on the left and then using a fluorophore for platelet identification on the left.
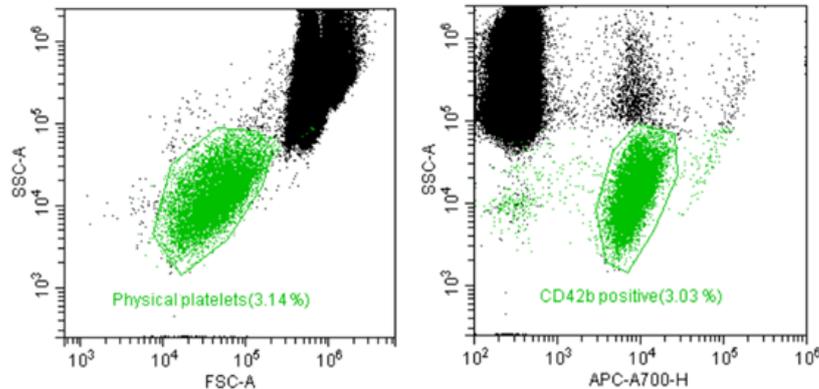
Figure 2-3: Example CytExpert data fluorescent analysis of a whole blood sample from a healthy volunteer.

Left panel shows gating for physical platelets using FSC (x-axis) and SSC (y-axis). Right panel shows gating using a fluorophore conjugated to a platelet identification marker (CD42b).

For assessment of platelet activation four parameters of focus were chosen to investigate different aspects of platelet activation. Here, the fluorophores were conjugated to antibodies raised against CD62P to measure P-selectin, PAC1 as a measure of the activation-induced confirmational change of the $\alpha_{IIb}\beta_3$ integrin complex (fibrinogen-binding) and Annexin V (AnnV) which binds to PS on the platelet surface. The specific assays are described in further detail in the relevant chapters. All of these experiments, whether two or four colour, were conducted in whole blood which necessitated the inclusion of the peptide Glycine-Proline-Arginine-Proline (GPRP), which acts as a pseudo-substrate for thrombin thereby blocking thrombin induced conversion of fibrinogen to fibrin which would prevent assessment of individual platelets.

In experiments where AnnV binding was measured, $CaCl_2$ was added (final concentration 1.8mM) since AnnV binding is calcium-dependent. The staining buffer was prepared including each of the antibodies of interest. Total staining

buffer per tube was 10μl with each antibody added at a volume previously determined and validated by the group [298]. An example of the components and volumes of staining buffer prepared for the four-colour panel is shown in Table 2-3. A negative control staining buffer for CD62P was prepared, which contains IgG-PE instead of CD62P-PE along with the other components of the standard staining buffer. EDTA was directly added to act as the negative control for both PAC1-FITC and AnnV-APC, as EDTA blocks calcium binding which is required for $\alpha_{IIb}\beta_3$ conformational change and also for AnnV binding and thus neither can occur in its presence.

Antibodies	Volume in 1 tube (ul)
PAC1-FITC	5
CD62p-PE	1.25
AnnV-APC	1
BB700-CD42b	0.25
CaCS	2.5

Table 2-3: Example staining buffer protocol for use in four-colour flow cytometry platelet activation panel.

Following venepuncture, whole blood and GPRP (50 mM, volume ratio of 10:1) were mixed gently and set aside at room temperature. Modified Tyrode's buffer, staining buffer along with agonists at the respective concentrations were added to the individual wells on a 96-well plate. In the activation panel, the whole blood containing GPRP was then added to each well followed by gentle mixing through shaking of the plate. When investigating the effects of PGI_2, whole blood was first incubated with PGI_2 at either 10nM of 100nM concentration for 2 minutes before being added to the relevant wells. After

mixing, the plate was then sealed and incubated at room temperature for 20 minutes in the dark. Following this, the seal was removed and the samples fixed in 450μl of 1% paraformaldehyde (PFA)/Modified Tyrode's buffer before being transferred into a standard 12x75mm FACS tube to be run through the Cytoflex S (Beckman Coulter). For any flow cytometry experiments conducted using isolated platelets, the same basic protocol was followed. CytExpert was used to process and export all data and when required a compensation matrix was applied (see section 2.4.4.4).

Importantly, two measures of expression were used and referenced throughout this thesis. Median or Mean Fluorescence Intensity (MFI) is a numerical measure of the full signal recorded from the population gated for and is therefore a quantitative assessment of the amount of antibody/fluorophore bound and thus indicative of the amount of antigen surface marker expressed. Percent positive is the second measure used in analysis of flow cytometry data in this thesis and is considered a more qualitative description of the proportion of the population which express the antigen or marker of interest. This is measured against a standard 2% background fluorescence using a pre-determined control; any fluorescence recorded in excess of the 2% baseline is considered a positive event. Both measures used in conjunction with one another allow a more thorough analysis to be conducted and thus provide greater insight into the markers of interest both under basal conditions and following stimulation with an agonist.

2.4.4.4 Compensation of spectral overlap for multiparameter panel

A key area of multiparameter fluorescent flow cytometry assays is compensation. This is an algorithmic calculation of the overlap between fluorophore emission across the spectrum into the adjacent laser channel bandpass filters. This calculation matrix allows the subtraction of the fluorescence from outside the target wavelength emission. Once calculated on a specific machine, this can then be applied to all experiments involving fluorophores with potential overlap of emission. Best practice indicates that this should be repeated regularly or following any interference with the lasers to ensure no changes have occurred. In this thesis, two-colour assays did not require compensation as fluorophores were chosen specifically to avoid spectral overlap. For the four colour assay experiments, compensation was always applied.

An initial compensation experiment was conducted using both antibody binding beads and cells. Tubes were made up each containing buffer along with an appropriate concentration of one of the chosen fluorescently conjugated antibodies. This was repeated until there was a tube containing each individual fluorophore to be included in the final panel. An additional 'blank' or unstained tube was also made up. For the experiment using beads, one drop of positive compensation beads was added to each tube along with one drop of negative beads to the blank. For the experiment using cells, whole blood containing GPRP at a 1/10 dilution was added. For both experiments, tubes were then mixed thoroughly, using a pipette for the cells and vortex for beads. Each tube was then run through the cytometer individually through CytExpert and an example compensation experiment is shown in Figure 2-4.

A compensation matrix is then created specific to the fluorophores used, hence in this case included PE, APC, FITC and BB700. The compensation matrices from each of the initial experiments are shown in Figure 2-5 and results show both beads and cells produced similar matrices, thus for consistency the beads compensation matrix was applied to all four-colour experiments and subsequent compensation when repeated was conducted with beads only.
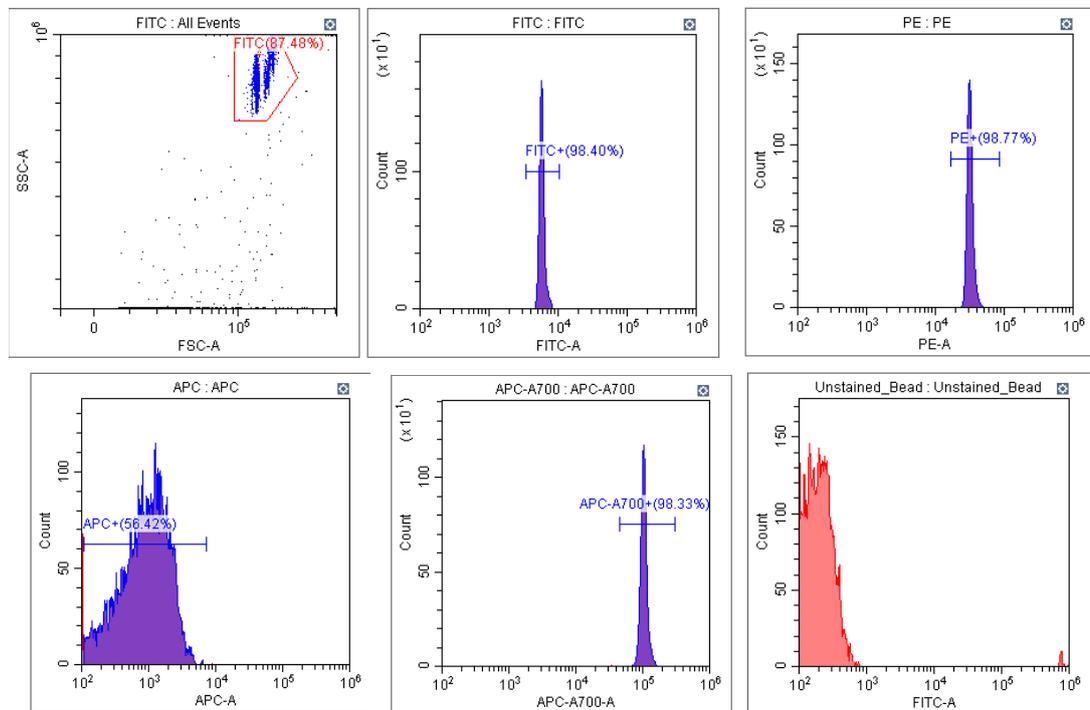
Figure 2-4: Example four-colour compensation experiment through CytExpert.

Raw data from compensation beads for each of the fluorophores included in the four-colour panel; FITC, PE, APC and APCA700 (BB700) along with unstained tube.

A- beads

Channel	-FITC%	-PE%	-APC%	-APC-A700%
FITC		1.14	0.00	2.54
PE	37.30		0.00	4.27
APC	0.00	0.00		12.50
APC-A700	0.00	0.00	28.19	

B- cells

Channel	-FITC%	-PE%	-APC%	-APC-A700%
FITC		1.09	0.00	2.06
PE	32.62		0.00	4.61
APC	0.00	0.00		11.99
APC-A700	0.00	0.00	28.17	

Figure 2-5: Example four-colour compensation matrix.

Data from compensation experiments, with panel A showing the matrix for beads and panel B for cells. The compensation matrices are for FITC (PAC1), PE (CD62p), APC (AnnexinV) and APCA700/BB700 (CD42b).

2.4.4.5 Glucose Uptake Assay

Glucose uptake by platelets was examined by flow cytometry. 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose (2-NBDG), a fluorescent analogue of glucose, was used, which can be read on the FITC channel. Platelets were isolated through the standard protocol already detailed, but re-suspended in DMEM without glucose. Where required, isolated platelets at a concentration of 5×10^8/ml were first incubated with agonists for 15 minutes followed by a 15-minute incubation in the dark with the 2-NBDG (300μM). Tubes were then run through the FITC channel with isolated platelets gated for according to FSC/SSC as previously described. Again, data were exported through the CytExpert software. With no isotype control, only MFI data are included for glucose uptake in this thesis.

2.4.5 Platelet bioenergetics - Seahorse XF96e Analyzer

The Seahorse XFe96 Analyzer (Agilent Technologies Inc.) facilitates the measurement of oxygen consumption rate (OCR) and extracellular rate (ECAR) in platelets as proxies for oxidative phosphorylation and glycolysis respectively. An array of assays can be conducted using Seahorse Analyzers, however, for the purposes of this thesis the Mito Stress Test and Glycolysis Stress Test have been conducted, with platelets tested both under basal and stimulated conditions as detailed in chapter 6. Here I used a 96-well plate approach (Figure 2-6), into which the relevant reagents for the test along with any compounds of interest can be added through injection ports. For both, the first step involves hydrating the sensor cartridge for calibration. A minimum of 12 hours prior to the planned experiment, 200μl of Seahorse XFe calibrant

(Agilent) was added to each well within the utility plate. The sensor cartridge was then carefully lowered so that each sensor was fully submerged in the calibrant, as per protocol. This was then left with a water bath and incubated at 37°C. Figure 2-6 shows the various components of Seahorse plate with further explanation of the sensor.

Sensor

Utility plate containing 200μl calibrant/well

Patented integrated injection ports (4 per well) for adding compounds, stimulators, inhibitors, substrates, and beads conjugated with antigens, let you test multiple conditions per well.

Patented transient microchamber provides superior sensitivity and signal to noise ratio

Solid state sensor probes contain polymer embedded fluorophores that are inspected through a machine vision process, allowing detection of OCR and PER simultaneously

Figure 2-6: Seahorse XFe96 Analyzer FluxPak plate.

Figure shows the sensor cartridge and utility plate into which calibrant is added and the sensors submerged prior to calibration on the day of the experiment. Sensor information also provided. Reproduced and amended from Seahorse XFe96 Analyzer materials ©Agilent Technologies Inc.

On the day of the planned experiment or study visit, prior to patient arrival, Cell Tak was diluted from stock with $NaHCO_3$ 8.4% for the relevant number of wells in the Cell culture mini plate, into which the isolated platelets ($1x10^8$/ml) were later added. Cell Tak is used to provide an adherent coating to the wells. The Cell Tak is left in the wells and incubated at 37°C for a minimum of 1 hour. Pyruvate is added to the DMEM solution in the presence or absence of

glucose depending on the intended experiment. The Mito Stress Test used glucose containing DMEM and the Glycolysis Stress Test glucose deficient DMEM.

Following initial isolation, the platelets were split to allow the final re-suspension into DMEM with glucose to be included in the Mito Stress Test, DMEM without glucose for the Glycolysis Stress Test and modified Tyrode's for any other assays as well as lysate preparation. Whilst the platelet isolation was conducted, Cell-Tak was discarded and the wells washed twice using 100μl PBS per well. 100ul of PBS solution remained in each well and the Cell culture mini plate returned to incubation at 37$°$C until ready for addition of isolated platelets.

The Flux plate was loaded with the reagents specific to the assay, described in detail below, along with any agonists or inhibitors. The Wave software, which is standard for the Seahorse Analyzer, was then used to specify the experimental parameters on the and commence calibration of the sensors. A plate map (Figure 2-7), is created to assign wells included in the assays with a required four 'blanks' in each of the four corners. Each well was repeated in triplicate to improve precision. After assigning the wells, the number of injections is inputted with a standard four injections included in both the Mito Stress Test and Glycolysis Stress Test. The experiments were therefore typically; initialisation, baseline, injection 1-4. Under standard conditions following each injection there were 3 cycles of 3 minutes for each of mixing, waiting and measuring. The total well volume was then set to 180μl and the assay commenced with initial coarse and fine calibration of the sensors. Whilst the calibration was running the Cell culture mini plate was prepared with the

isolated platelets. The PBS was discarded and 50µl of isolated platelets in the relevant buffer was added to the relevant wells. Further 50µl of non-specific buffer was added to each of the four 'blank' corners. A balance plate was also prepared with equivalent volumes of any buffer in equivalent wells. These were then centrifuged for 30 seconds at 100g, 1 acceleration and 1 break before being rotated 180° and centrifuged again for 30 seconds at 100g, this was to ensure the platelets were fully seeded in the wells and adhered to the Cell-Tak. Following this, the plate was returned to the incubator until calibration was complete. The Seahorse Analyzer ejects the utility plate (containing the calibrant) following completion of calibration and the Cell culture plate with the seeded isolated platelets is then added and the plate taken into the Analyzer and the assay then ready to run.

Following completion of the assay, data are exported through Prism or Excel for analysis.
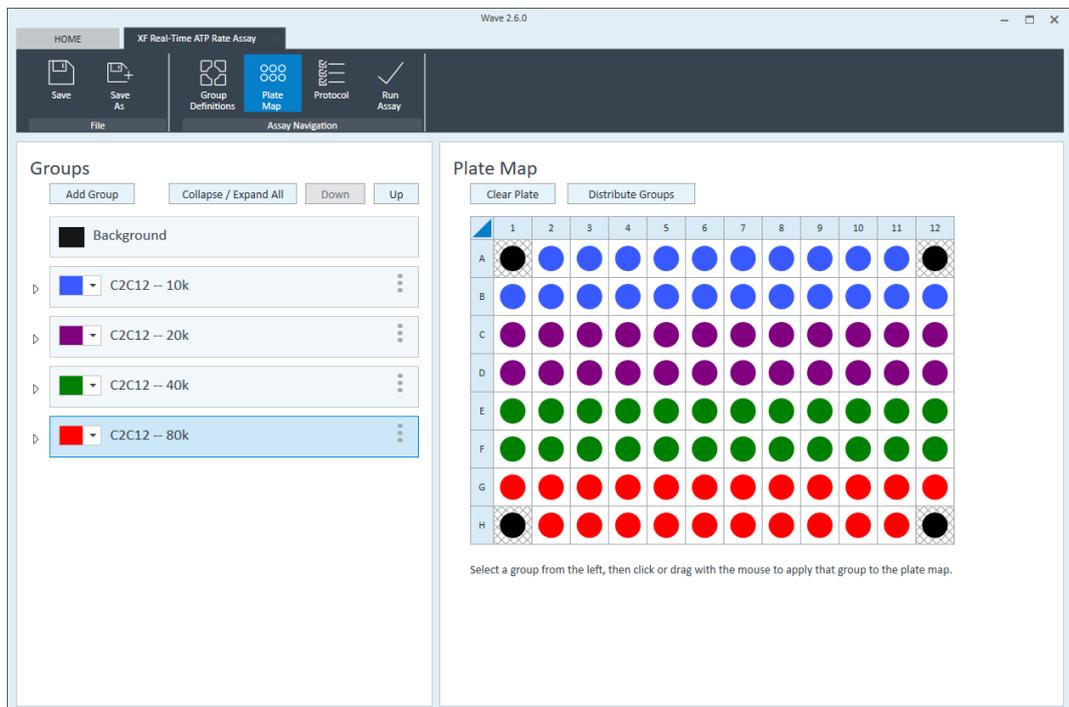
Figure 2-7: Example Wave Plate Map

Plate shows four conditions and 'blanks' shown in black in the four corners of the plate.

2.4.5.1 Seahorse Assays and reagents

Two different assays using the Seahorse XFe96 Analyzer were used in this project and are described in full within the relevant chapters. In brief, the Mito Stress Test is a validated assay for the assessment of mitochondrial function including measurements of basal mitochondrial respiration, proton leak, ATP-linked respiration and maximal respiration. The standard reagents required for the assay include Oligomycin, FCCP (carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone) and Rotenone/Antimycin A. Their sites of action within the electron transport chain as shown in Figure 2-8. Oligomycin is injected first and acts on ATP synthase to reduce electron flow which decreases mitochondrial respiration. FCCP is the next reagent injected which is an uncoupling agent which acts to disrupt mitochondrial membrane potential which results in unimpeded electronic flow across the Electron Transport Chain (ETC) and facilitates maximal mitochondrial respiration. The final injection is a combination of rotenone and antimycin A, rotenone acts on complex I of the ETC and antimycin A on complex III with a resulting complete prevention of mitochondrial respiration and thus permits the measure of non-mitochondrial respiration. The calculations of different parameters are included and discussed in the relevant chapter.
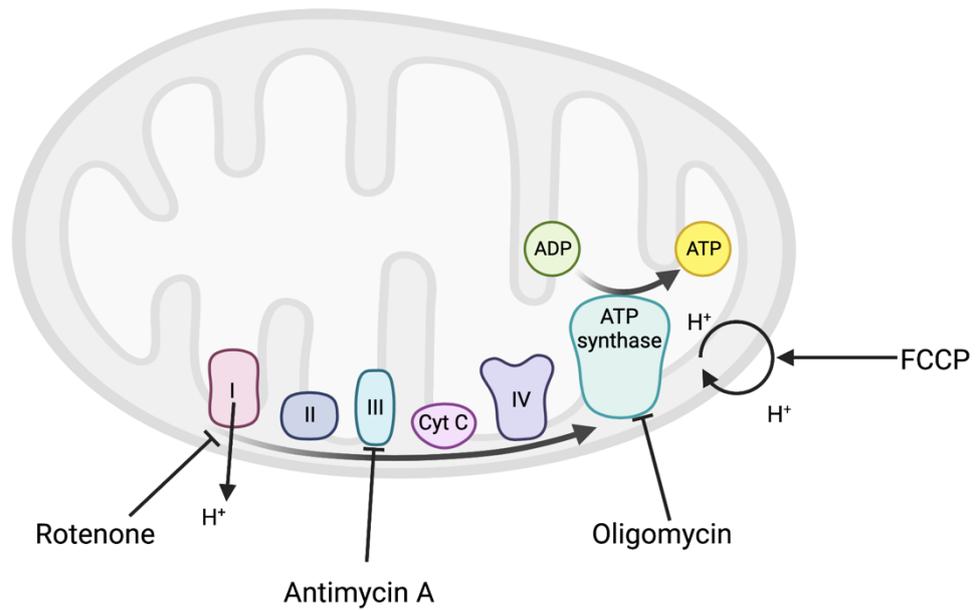
Figure 2-8: Mito Stress Test Compounds sites of action.

Schematic of mitochondrial electron transport chain demonstrating site of action of the compounds included in the Mito Stress Test.

The Glycolysis Stress Test is a validated assay to measure glycolysis by which glucose is converted to pyruvate, with the assay measuring multiple parameters including basal glycolysis, maximal glycolytic capacity and non-glycolytic acidification with the calculation of these parameters discussed later in the relevant chapter. As mentioned, for the Glycolysis Stress Test, the platelets are suspended in DMEM not containing glucose and thus the first injection is glucose. This allows the measurement of ECAR following glucose which directly equates to basal glycolysis (not including non-glycolytic acidification). Following this, oligomycin is next injected, acting as already described on ATP-synthase. The inhibition of ATP production causes a shift from mitochondrial respiration to glycolysis thus allowing measurement of maximal glycolytic capacity. Finally, 2-deoxy-glucose (2-DG) is injected. This

is a glucose analogue that competitively binds to hexokinase, as shown in figure 2-9, the key first enzyme in the glycolysis pathway thereby preventing glycolysis.
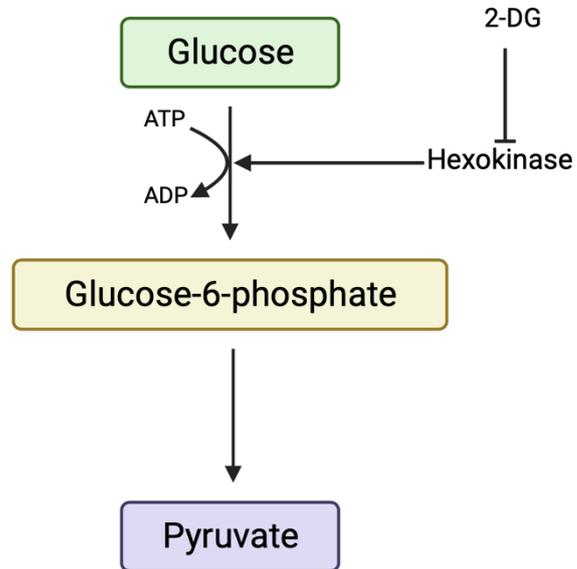
Figure 2-9: Glycolysis Stress Test Compounds sites of action.

Schematic of glycolysis demonstrating site of action of 2-DG.

Chapter 3: The optimisation of a multiparameter flow cytometry assay for the assessment of platelet function

3.1 Introduction

As outlined in chapter 2, (section 2.4.1) multiparameter flow cytometry lends itself to the investigation of platelet function both from the aspect of activation, under basal and stimulated conditions, but also in response to inhibitors. Crucially, multiparameter assays provide data on multiple markers at the same point in time and in response to the same stimuli making the data more robust and clinically applicable. Prior to conducting such assays using samples from study patients, a series of optimisation experiments were first conducted to determine the optimal conditions both for an activation panel as well as a panel examining response to inhibition, using the same markers of platelet activation.

Platelets become activated from their circulating quiescent state in response to vascular injury [40, 299]. A number of pathways are triggered as part of this response with each having a different role in facilitating platelet activation [299]. This includes conformational changes of integrins [300], which facilitates the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ and secretion of both dense and α-granules. One of the most widely used markers of platelet activation is P-selectin, already detailed in Chapter 1 and found within the α-granules at rest and later expressed on the surface following activation [301]. In addition, following robust activation a sub-population of platelets become procoagulant, defined by the externalisation of PS onto the surface, providing a haemostatic site for binding of factor Xa, supporting thrombin generation [302]. Therefore, PS has been shown to be a suitable marker to represent the procoagulant activity of platelets and is particularly valuable in assessing basal activation along with capacity for activation given its typically low-level

exposure under "normal" physiological conditions [302, 303]. Upon activation, there is a conformational change in the extracellular domain of $\alpha_{IIb}\beta_3$ which facilitates binding of fibrinogen, and this triggers an irreversible pathway of intracellular events contributing to platelet aggregation and adhesion, making $\alpha_{IIb}\beta_3$ another useful marker of platelet activation.

The described quiescent state in which platelets circulate under normal physiological conditions, relies upon a crucial balance of the pro-aggregatory factors and opposing inhibitors, perhaps most importantly prostacyclin (PGI_2) and nitric oxide (NO) [39]. Whilst increased platelet activation alone is an important mechanism to understand when exploring cardiovascular risk in patients with diabetes, investigating the potential paradoxical effects of platelet inhibition in this group is also crucial to assess the extent to which platelet dysfunction may contribute to the long-term cardiovascular outcomes.

This chapter therefore presents the data around optimisation of initially two parameter and then four parameter flow cytometry assays first to assess platelet activation alone and thereafter the sensitivity to inhibition using PGI_2.

3.2 Methods

In this chapter, all optimisation was conducted on samples from healthy volunteers under established ethics within the Leeds Institute of Cardiovascular and Metabolic Medicine (MREC 19-006). Background health status for controls was not formally collected although prior to sampling, it was established that no interfering medications such as anti-platelet medication had been taken. Samples were drawn in a non-fasted state both from patients

and volunteers and where possible sampling was from the antecubital fossa using a 21G butterfly needle and vacutainer, validated for use in platelet studies [304].

3.2.1 Flow Cytometry and platelet activation markers

For the purposes of validating a multiparameter flow cytometry panel to assess the different aspects of platelet activation, initial optimisation was carried out on samples from healthy volunteers. Whole blood was used throughout all activation panel flow cytometry experiments, drawn into sodium citrate 3.2% containing vacutainers. Initial stages involved optimising two-colour assay for each of the individual parameters of interest including P-selectin, conformation change of integrin $\alpha_{IIb}\beta_3$ and PS. Each marker was optimised along with CD42b (GPIbα), a constitutively expressed component of the GPIb-IX-V complex on platelets used for platelet identification. Antibodies raised against these targets were chosen based on previous publications along with appropriate fluorescent conjugates. The antibodies included; CD42b-BB700 or CD42b-APC (representative of GP1bα expression and therefore platelet identification) depending on the assay, CD62P-PE for P-selectin expression, PAC1-FITC for active $\alpha_{IIb}\beta_3$ and Annexin V-APC for PS exposure. Throughout this thesis, the phrase $\alpha_{IIb}\beta_3$ expression refers to the active conformational change of $\alpha_{IIb}\beta_3$. Annexin V (AnnV) is a protein that binds to the previously described externalised PS found on the platelet surface following activation, with increased surface PS implicated both in apoptosis and promotion of procoagulant environment, thus acting as a marker of platelet hypercoagulability [43, 303].

With the intention to investigate platelet activation both in a resting state *i.e.,* basal conditions, and in an activated state *i.e.,* stimulated, a dose titration was required for the agonists against each antibody. For stimulation, Protease Activated-Receptor-1 agonist, SFLLRN (0.5 μM to 20 μM), was chosen as a stable agonist that has been well validated by the group. SFLLRN is used to mimic thrombin mediated platelet activation [305] and is prepared from stock concentration of 10 mM, stored at 4°C. Alternative and additional agonists included cross-linked collagen related peptide (CRP-XL, 1 μg/ml to 40 μg/ml, prepared from stock 2mg/ml stored at -20°C) and Convulxin (CXN, 50 ng/ml to 500ng/ml, prepared from stock 50 μg/ml stored at -80°C) as GPVI agonists [306-308]. Platelet identification was initially through a gate on forward (FSC) and side scatter (SSC) followed by a platelet positive gate using a fluorophore for CD42b. Data were analysed and presented as both percent positive cells and Mean or Median Fluorescence Intensity. 'MFI' refers to mean fluorescence intensity for both CD62P and PAC1 expression and refers to median fluorescence intensity for AnnV.

3.2.2 Statistical analysis

All data referred to within this chapter were analysed using GraphPad Prism version 9.5.1 (528). Where required, normal distribution was assessed using Shapiro-Wilks. For the comparison of two independent continuous variables, either the Student t-test or Mann-Whitney test was used depending on the normative distribution of the data.

3.3 Results

3.3.1 Optimisation of two-colour flow cytometry assays

3.3.1.1 CD62P expression (P-selectin)

The first two-parameter assay was developed with antibodies against CD62P and CD42b, conjugated to PE and APC respectively. Whole blood was stimulated with SFLLRN (0.5 μM to 20 μM) [294, 309]. SFLLRN induced a concentration dependent activation of platelets. Figure 3-1 demonstrates that CD62P measured as percent positive cells increased rapidly in response to increasing doses of SFLLRN. Basal expression was 7.9$\pm$3.0% which increased to 10.1$\pm$4.0% in response to 0.5 μM SFLLRN. There was a clear shift in expression with 1 μM SFLLRN (47.1$\pm$4.0%, *p=0.002*). With 2 μM SFLLRN, there is a significant increase in CD62P expression (78.0$\pm$4.0%, *p<0.0001*), which then approaches 100% expression, with no further rise beyond 5 μM. The data, expressed as MFI, are comparable with basal CD62P expression MFI of 178$\pm$35, rising to 180$\pm$60 and 506$\pm$403 following stimulation with 0.5 μM and 1 μM of SFLLRN respectively. Again, following stimulation with 2 μM there was a large increase in expression, with an MFI of 51108$\pm$4443*, p=0.0007*. MFI allows us to see a slight increase beyond 5 μM, with MFI rising from 57096$\pm$10125 at a dose of 5 μM to 66792$\pm$7778 and

63340±5566 following doses of 10 µM and 20 µM of SFLLRN respectively, *p<0.0001* for 2-20µM SFLLRN.
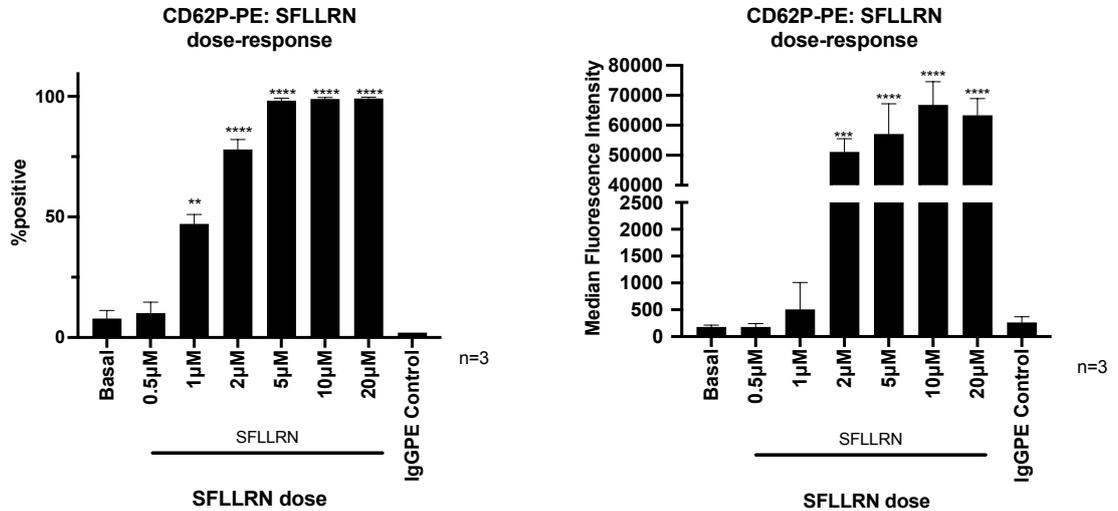
Figure 3-1: Two-parameter platelet activation measured by fluorescent flow cytometry; SFLLRN dose-response for CD62P expression in whole blood from healthy volunteers.

Left panel represents percent positive CD62P expression following stimulation with SFLLRN in a dose-dependent manner. Right panel represents median fluorescence intensity (MFI) data. **p<0.01, ***p<0.001 and ****p<0.0001. n=3.

3.3.1.2 PAC1-binding (active αIIbβ3 expression)

The next two-colour assay used antibodies for CD42b-APC as the platelet identification marker and PAC1-FITC as a marker of active $\alpha_{IIb}\beta_3$ expression. There was a dose-dependent increase in PAC1 expression in response to 0.5 µM to 20 µM SFLLRN. In Figure 3-2, these data are presented both as percent positive and MFI. PAC1 binding under basal conditions was, 0.8±0.4%, lower than the basal percent positive CD62P expression. Following stimulation with 0.5µM SFLLRN there was an increase in percent positive to 27.9±2.0%. With a dose of 2µM the percent positive platelet expression of active $\alpha_{IIb}\beta_3$ increased further to 71.1±3.0%, *p=0.009* before a further rise to 96.5±4.0%

with 5 μM SFLLRN. There were further insignificant rises to 98.9±0.9% and 98.7±0.9% with the respective 10 μM and 20 μM doses *p<0.001 for 5 to 20μM SFLLRN.

Once again, very similar results were seen with the MFI, with basal MFI of 3658±1003. This was quite variable across the 3 healthy volunteers, but rose slightly to a mean MFI of 8372±4388 following stimulation with 0.5 μM SFLLRN. At 1 μM SFLLRN, MFI was 43363±32749 which then more than doubled to 93242±27395 at 2 μM, *p=0.04*. This then slightly increased to 95549±14601 at 5 μM *(p=0.006)* and then plateaued, reaching a maximal MFI of 104025±29958 at the maximum dose of 20 μM SFLLRN, *p=0.002*.
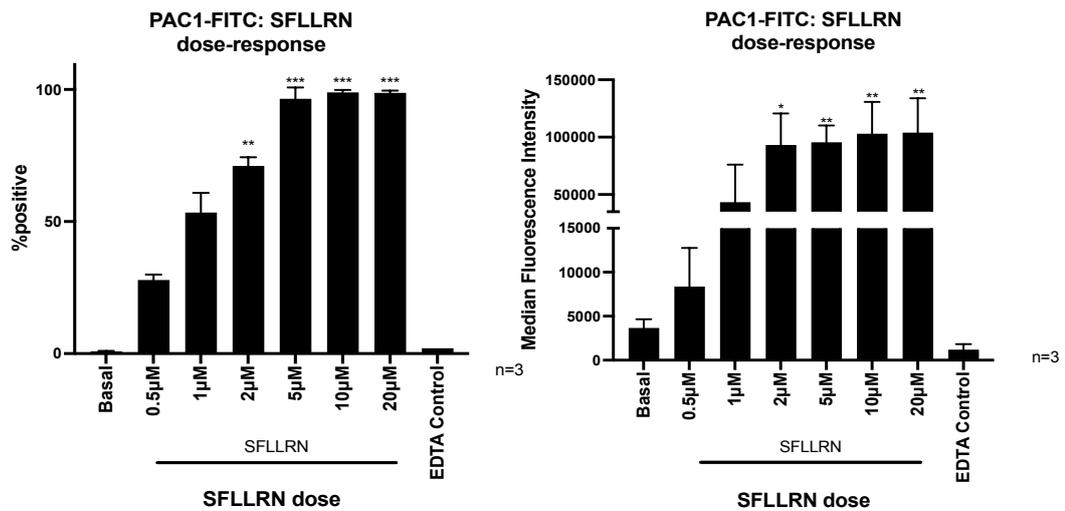
Figure 3-2: Two-parameter platelet activation measured by fluorescent flow cytometry; SFLLRN dose-response for PAC1 expression in whole blood from healthy volunteers.

Left panel represents percent positive CD62P expression following stimulation with SFLLRN in a dose-dependent manner. Right panel represents median fluorescence intensity (MFI) data. **p<0.01, ***p<0.001 and ****p<0.0001. n=3.

3.3.1.3 Annexin V-binding (PS exposure)

Following this, a final two-parameter assay was optimised for evaluation of Annexin V binding, as mentioned, this is a marker of PS exposure. The antibody available was conjugated to APC and therefore, unlike the previous two antibody/fluorophore combinations this could not be used with CD42b-APC as they would be within the same spectrum. A fluorophore with different emission spectrum was thus required for the platelet identification marker and CD42b-BB700 was chosen. As AnnV binding is calcium dependent, these experiments were carried out in Modified Tyrode's buffer supplemented with calcium (1.8 mM), as described in Chapter 2. Further to this, as has been widely demonstrated, AnnV requires a greater degree of stimulation with an agonist than either CD62P or PAC1 and typically this requires use of dual agonists [310, 311]. In this two-parameter assay, therefore a dose response was conducted with mid-range concentration (2 μM) and high concentration (20 μM) of SFLLRN along with a second agonist at two concentrations, 50 ng/mL and 500 ng/mL CXN along with dual agonists at maximal doses *i.e.,* 20 μM SFLLRN and 500 ng/mL CXN. The CXN doses were guided by established protocols [310].

Examining PS expression following stimulation appears to generate two sub-populations amongst the platelets, this presents as a bimodal peak rather than a single peak as seen in CD62P-PE and PAC1-FITC (Figure 3-3) and therefore for analysis using mean fluorescence intensity rather than median fluorescence intensity is the more representative and has been used in the MFI analysis relating to AnnV.
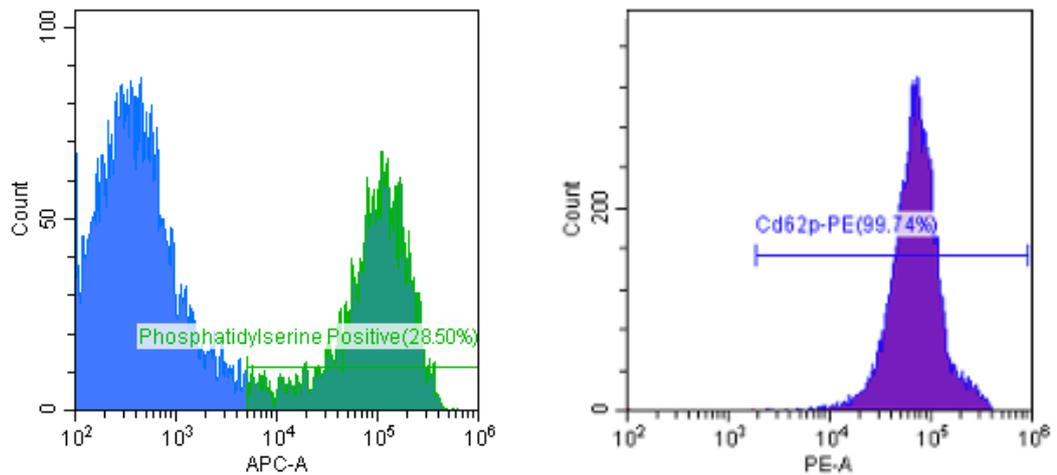
Figure 3-3: CytExpert histogram data from two-parameter fluorescent flow cytometry assays for AnnV-APC and CD62P-PE in whole blood from a healthy volunteer.

Left panel demonstrates that following maximal stimulation with dual agonists (20 μM SFLLRN/500 ng/mL CXN) there is a bimodal peak generated and thus mean fluorescence intensity is more representative of the full data, compared with single peak seen for CD62P-PE following maximal stimulation (20 μM SFLLRN) seen on the right.

In this two-parameter assay, percent positive values were much lower. As seen in Figure 3-4, under basal conditions and with single dose SFLLRN (2 & 20 μM) there was minimal increase in expression. Mean basal percent positive was just 0.1±0.0%, only rising to 0.8±0.6% at 2 μM SFLLRN and 1.3±0.5% at the higher dose of 20 μM SFLLRN. Data were comparable with single low dose CXN, 0.6±0.1% at 50 ng/mL CXN. There was a small rise at 500 ng/ml CXN to 8.2±3.2%, *p=0.008*. Only with dual agonist stimulation at high doses of both SFLLRN and CXN (20 μM and 500 ng/mL), was there a substantial increase in expression at 22.6±5.2%, *p<0.0001*.

MFI expression is shown in Figure 3-4, with the greater sensitivity in the scale this was able to show a greater rise from basal expression, 226±19, rising to

1477±955 and 1908±1186 following activation with 2 μM SFLLRN and 20 μM SFLLRN respectively. The standard deviation however demonstrates the donor-donor variability in PS expression. Single agonist stimulated expression with CXN again showed at the lower dose of 50 ng/mL there was low MFI (648±126) but a higher degree of activation with 500 ng/ml CXN, 7879±2659 (*p=0.046*), compared with the high dose 20 μM SFLLRN. This continues to a maximal MFI of 20813±9614 following dual agonist stimulation, a significant rise compared with basal conditions, *p=0.0003*.
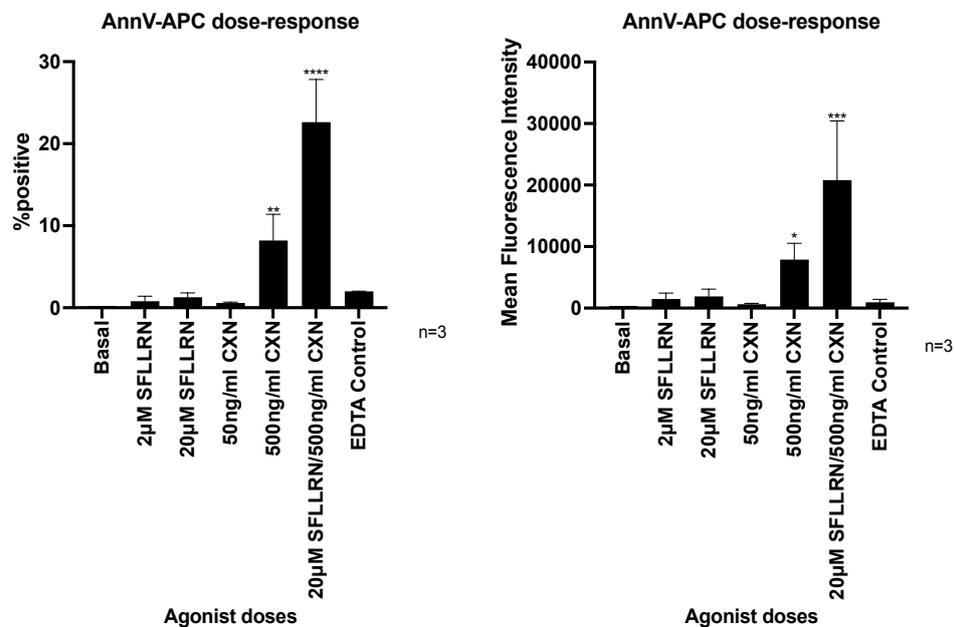
Figure 3-4: Two-parameter fluorescent flow cytometry assay demonstrating platelet AnnV-APC expression in response to low and high dose SFLLRN and CXN in whole blood from healthy volunteers.

Left panel represents percent positive AnnV expression following stimulation with SFLLRN in a dose-responsive manner. Right panel represents the mean fluorescence intensity (MFI) data. *p<0.05. **p<0.01, ***p<0.001 and ****p<0.0001. n=3.

3.3.2 Optimisation of a four-colour flow cytometry assay for the measurement of platelet activation markers

Following the adequate optimisation of each of the described two-parameter assays, the next step was to combine the antibody-fluorophore conjugates into a single four-parameter assay. Figure 3-5 is a schematic representation of the final panel with the relevant included markers highlighted.
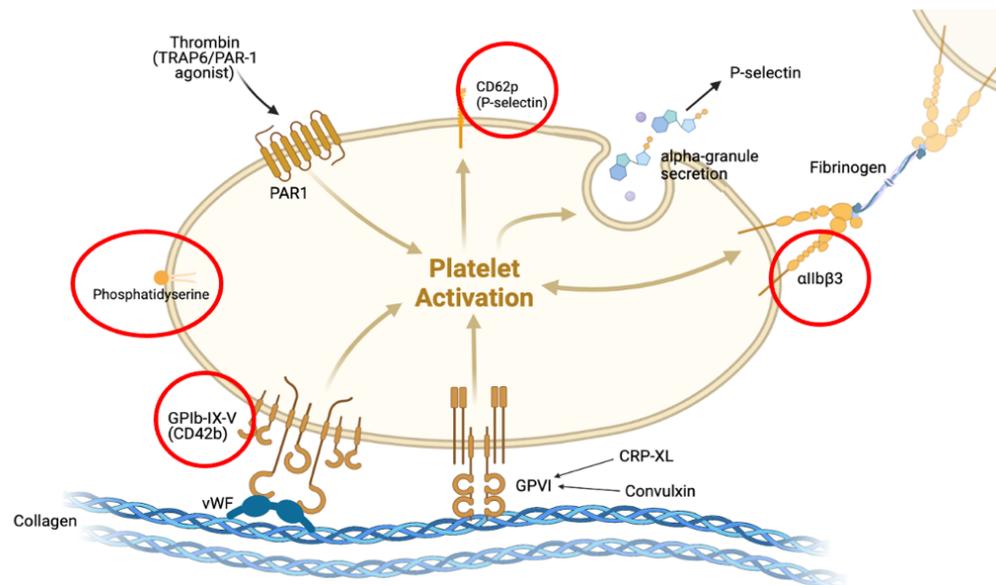
Figure 3-5: Multiparameter (four-colour) assay schematic representation of aspects of platelet activation investigated by final panel.

The four markers included in the final assay have been highlighted with red circles including; CD42b (platelet identification), CD62P (P-selectin), PAC1 (αIIbβ3) and phosphatidylserine.

Created using BioRender.com

3.3.2.1 Fluorophore selection and compensation

Combining the fluorophores into a single four-parameter assay meant spectral overlap between fluorophores. Based on the commercially-available fluorophores conjugated to the antibodies of the markers of focus, the following final fluorophore combination was selected; CD42b-BB700 as the platelet identification marker, CD62P-PE for P-selectin, PAC1-FITC for $\alpha_{IIb}\beta_3$ and AnnV-APC for PS expression. The increased number of fluorophores and associated increase in spectral overlap is visually represented by Figure 3-6.
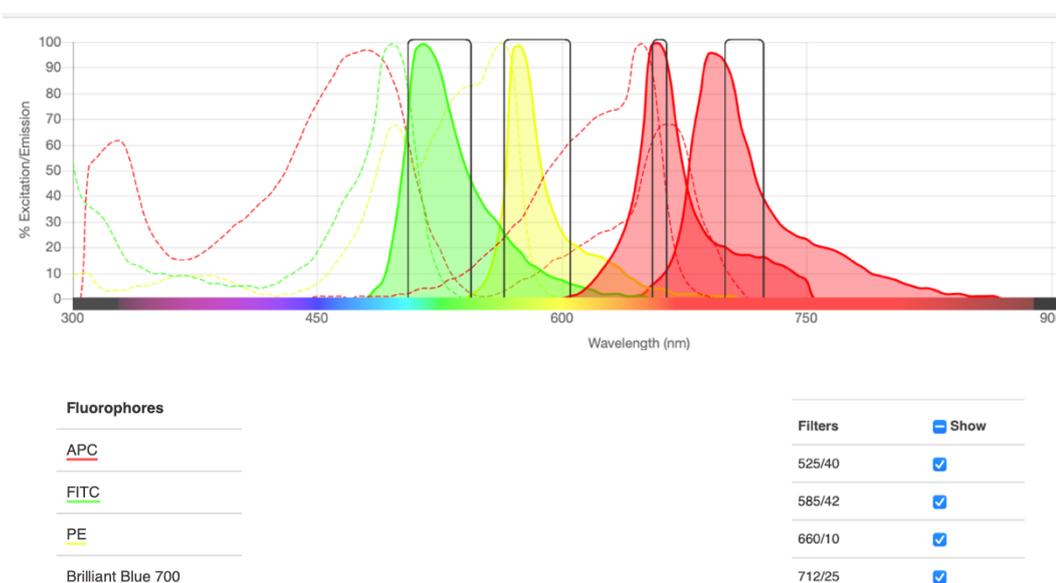
Figure 3-6: Beckman Coulter spectral viewer tool generated for four-colour assay.

This tool represents the excitation/emission wavelengths for the four chosen fluorophores and the relative spectral overlay. Vertical block lines represent the lasers, dotted lines represent the excitation patterns and the solid non-vertical lines emission spectra. As shown, there are spectra overlap on combination of the four fluorophores.

In order to mitigate the spectral overlap, a compensation experiment was performed (see section 2.4.4.4). This was repeated periodically, particularly following any recalibration or re-alignment of the lasers on the Beckman Coulter Cytoflex S. An example compensation is shown in Table 3-1.

Channel	-FITC%	-PE%	-APC%	-BB700%
525/40BP	-	1.14	0.00	2.54
585/42BP	37.30	-	0.00	4.27
660/10BP	0.00	0.00	-	12.50
712/25BP	0.00	0.00	28.19	-

Table 3-1: Representative compensation matrix.

Matrix generated using antibody-bound beads to the relevant fluorophore, in this case facilitating compensation for the four-colour assay including filters 525/40 for FITC (PAC1), 585/42 for PE (CD62P), 660/10 for APC (AnnexinV) and 712/25 for BB700 (CD42b).

3.3.2.2 The activation of platelets in response to physiological agonists

As the four-colour assay included measurement of AnnV expression a dual agonist approach was required. SFLLRN was determined as the first agonist, as previously outlined, at low and high dose (2 μM and 20 μM). CRP-XL, an alternative GPVI agonist, well established in protocols from the group was used [294]. Optimisation through a dose-response to CRP-XL (1 μg/ml to 40 μg/ml) both to ensure the panel was functional and also to determine the optimal dose of CRP-XL for inclusion in the study was carried out.

Under basal conditions, there is the highest percent positive expression of CD62P (8.2$\pm$4%) compared with 3.4$\pm$1% for PAC1 and 0.26.$\pm$0.08% for AnnV. Stimulation with low dose 1 μg/ml CRP-XL, increases expression of both CD62P (55.6$\pm$4%) and PAC1 (32.1$\pm$2.5%), but not expression of AnnV (0.3$\pm$0.1%). Using higher concentrations of CRP-XL, there is a plateau representing probable maximal expression at approximately 99$\pm$0.5% for CD62P and between 85-93% for PAC1. There is clear evidence of an increase in AnnV expression, to a peak of 14.6$\pm$3% with 40 μg/ml CRP-XL. Of note, PAC1 expression does appear to marginally, but significantly, decrease from 93.2$\pm$1.2% at 5μg/ml when AnnV expression remains low, down to 85.7$\pm$1.6% at 40μg/ml CRP-XL when AnnV is at its peak, *p=0.004*.

When analysing the data by MFI, P-selectin had a high amount of inter-donor variability with a high standard deviation. A similar pattern was seen to that described in the case of percent positive. Basally, expression of both CD62P and PAC1 were low (8297$\pm$3892 and 3503$\pm$1758) but AnnV expression was

almost undetectable (902±561). At the lowest dose of CRP-XL, 1 μg/ml, there was evidence of increased expression of both CD62P (26471±4601) and PAC1 (6400±1021) but AnnV expression remained essentially undetectable (632±202). At 2μg/ml CRP-XL there were significant increases in both CD62P (47817±22403) and PAC1 (14799±1162) expression with AnnV starting to rise at 1271±329. CD62P appeared to plateau from 5 μg/ml, with peak PAC1 expression around 10μg/ml CRP and AnnV peaking at 13121±402 with 20 μg/ml CRP-XL. Again, of note, with AnnV expression typically dual agonist stimulation is required for maximal activation.
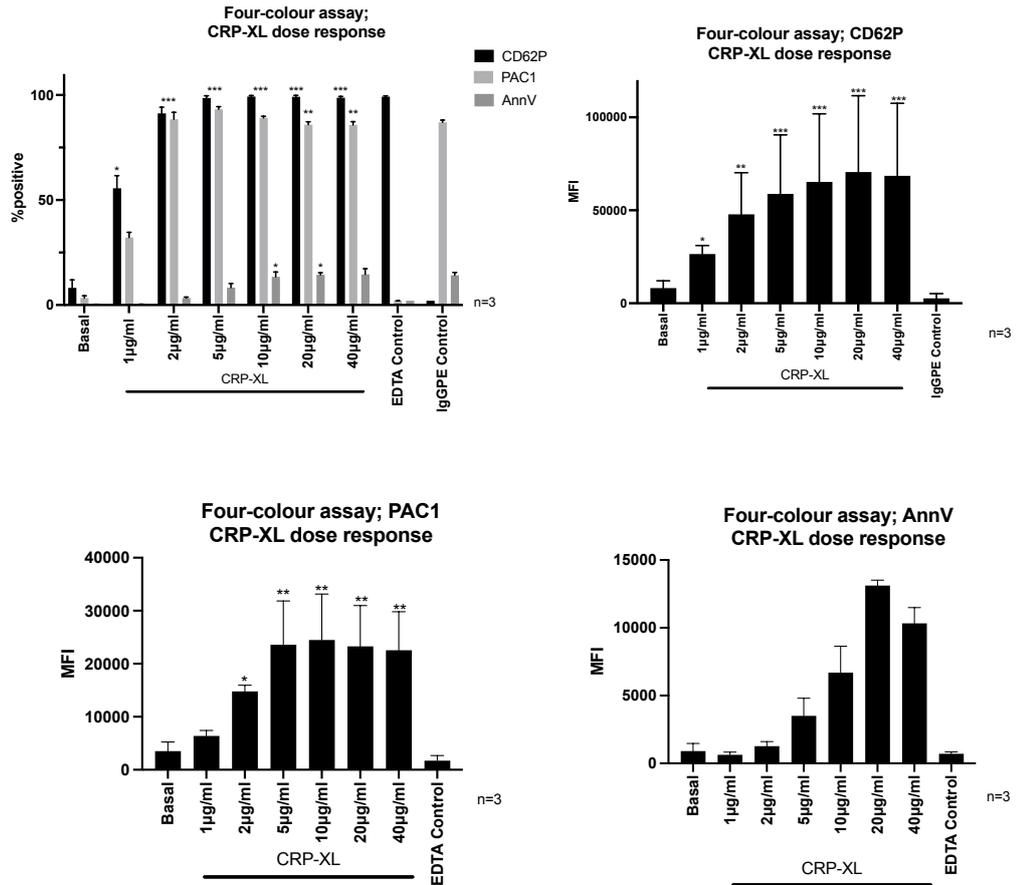
Figure 3-7: Multiparameter fluorescent flow cytometry assay demonstrating platelet expression of CD62P, PAC1 and AnnV under basal conditions and in response to increasing doses of CRP-XL in whole blood from healthy volunteers.

First panel represents percent positive expression of each marker. The other three panels represent the comparative median/mean (AnnV only) fluorescence intensity expression. Statistical analysis refers to the stimulated expression compared with basal expression for that individual marker. Control data (IgGPE and EDTA) were not presented. *p<0.05. **p<0.01 and ***p<0.001. n=3.

3.3.2.3 Optimised four-colour panel for the assessment of platelet activation under basal and stimulated conditions

Following the above optimisation, we were able to determine a combination of both single and dual agonist doses to most thoroughly evaluate all three markers of platelet activation. This included basal conditions, SFLLRN at $2\mu M$ and $20\mu M$, CRP-XL ($1\mu g/ml$ and $10\mu g/ml$) and dual agonist stimulation of SFLLRN ($20\mu M$) combined with CRP-XL ($10\mu g/ml$). These doses were chosen to ensure that low, medium and high strength agonist doses were included to assess for potentially subtle differences, rather than inducing maximal activation in both groups being examined. EDTA (10mM) was used as the control for both PAC1 and AnnV with IgG-PE as the control for CD62P. Figure 3-8 shows the final panel as carried out in an initial three healthy volunteers.
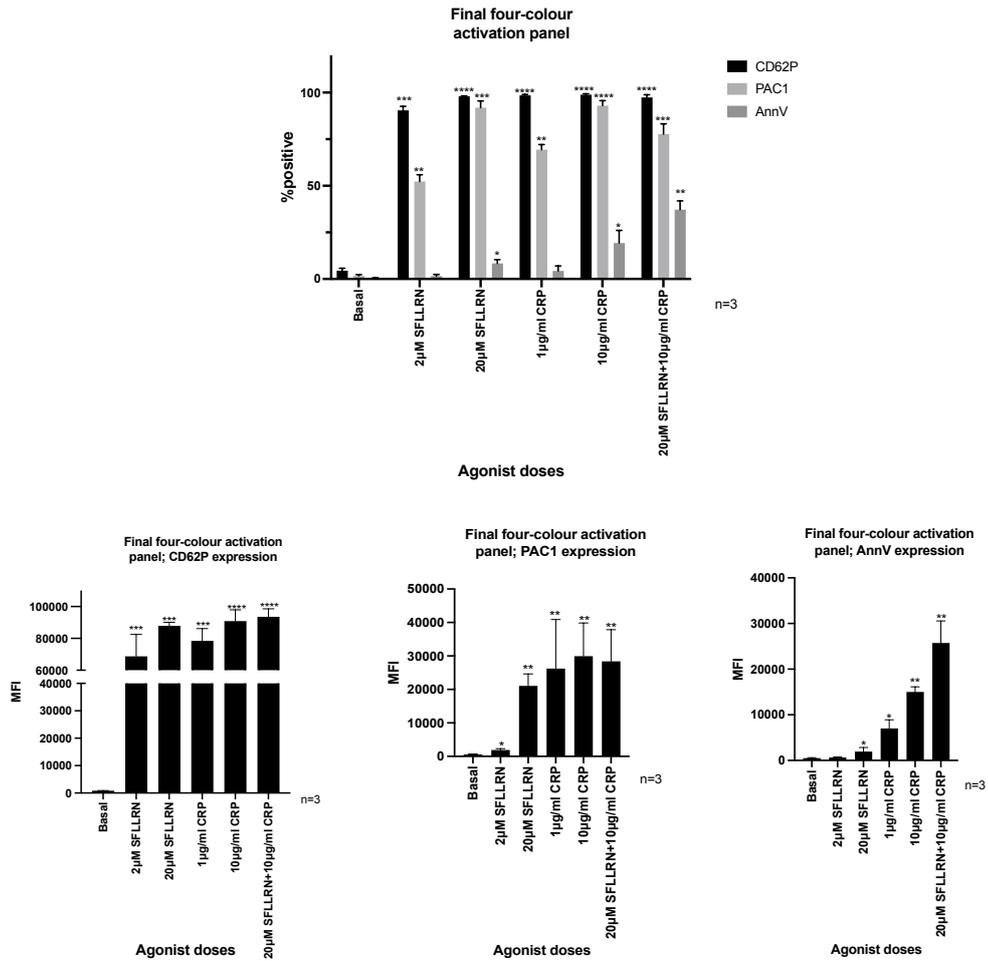
Figure 3-8: Final multiparameter fluorescent flow cytometry assay to investigate platelet expression of CD62P, PAC1 and AnnV basally and in response to low, medium and high dose (single/dual) agonists in whole blood from healthy volunteers.

Top panel represents percent positive expression of each marker, bottom three panels represent the comparative median/mean (AnnV only) fluorescence intensity data. Statistical analysis refers to the stimulated expression compared with basal expression for that individual marker. Control data (IgGPE and EDTA) were not presented. *p<0.05. **p<0.01, ***p<0.001 and ****p<0.0001. n=3.

3.3.3 Determining the optimal concentration for platelet inhibition by PGI$_2$

Following the activation panel, a series of optimisation experiments using whole blood from healthy controls were conducted to determine the optimal PGI$_2$ doses to assess sensitivity to inhibition [298, 312]. PGI$_2$ (1-1000nM) was incubated with whole blood for two minutes followed by the addition of either CRP-XL (1 μg/ml), or high dose dual agonists CRP-XL (10 μg/ml) and SFLLRN (20 μM) and completion of the cytometry protocol. Figure 3-9 shows the results of the optimisation experiments. For reference, data include basal expression of each marker, following stimulation with 1 μg/ml CRP-XL and also with dual agonists (10 μg/ml CRP-XL with 20 μM SFLLRN). Expression is then shown for each marker in the presence of 1-1000 nM PGI$_2$ at each of the mentioned agonist strengths.

There was a minimal effect of PGI$_2$ on CD62P percent positive expression (Figure 3-9 panel A) with lower dose agonist, with only a small, but non-significant, change seen at 1000 nM of the prostanoid. In the presence of dual agonist stimulation, there were also no significant changes seen in CD62P expression with any dose of PGI$_2$. Shown in Figure 3-10 panel A, when examining the change by percentage reduction, there was a sigificant reduction in CD62P expression when comparing inhibition by 1000 nM PGI$_2$ with inhibition of 1 nM PGI$_2$ at 1 μg/ml CRP-XL (23$\pm$3% reduction in expression versus 0.6$\pm$0.3%, *p=0.004*) and with dual agonists (10.7$\pm$1.6% versus -0.1$\pm$0.2%, p=0.002), but ultimately the CD62P percent positive expression was not different in the presence of PGI$_2$ compared to expression with agonist alone.

In panel B of Figure 3-9, it can be seen that PGI_2 has a stronger effect on PAC1 percent positive expression. With 1 μg/ml CRP-XL, there was a mean expresson of 69.9$\pm$7.0%, this did not change with 1 nM or 10 nM PGI_2. Whereas with 100 nM PGI_2 inhibtion was much greater with 21.1$\pm$2.0% expression, *p=0.005* and with 1000 nM PGI_2 mean expression was 12.4$\pm$3.0%, *p=0.001*. With dual agonist stimulation, there was a maximal PAC1 expresssion of 84$\pm$4.0% and a significant reduction in expression was seen at 10 nM PGI_2 (56$\pm$10%, *p=0.03*), 100 nM PGI_2 (27$\pm$4.0%, *p<0.0001*) and 1000 nM PGI_2 (21$\pm$2.0%, *p<0.0001*). At 1 μg/ml CRP-XL, when assessing the data by percentage reduction compared with 1 nM PGI_2 (-2.8$\pm$3.0%), there were siginifcant differences seen with 10 nM PGI_2 (15.6$\pm$8.0%, *p=0.04*), 100 nM PGI_2 (69.6$\pm$4.0%, *p <0.0001)* and 1000 nM PGI_2 (82$\pm$5.0%, *p<0.0001*), these data are shown in Figure 3-10 panel B. Similarly, following stimulation with dual agonists, compared to 1 nM PGI_2 where there was a mean percentage reduction of 2$\pm$1.8%, there were significantly greater percentage reductions at 100 nM PGI_2 (66$\pm$6.0%, *p=0.002*) and 1000 nM PGI_2 (75$\pm$2.0%, *p<0.0001*).

Finally looking at PGI_2 effects on AnnV percent positive expression, shown in Figure 3-9 panel C, most of the discernible changes are seen in the context of dual agonist stimulation as expected with only a very marginal increase in AnnV expression following stimulation with single agonist 1 μg/ml CRP-XL. At this dose, only in the presence of 1000 nM PGI_2 was there a difference, albeit small, compared with AnnV expression at 1 μg/ml CRP-XL alone, 2.8$\pm$0.7% with 1 μg/ml CRP-XL and 1.1$\pm$0.3% in the presence of 1000 nM PGI_2, *p=0.04*. With dual agonist stimulation, AnnV percent positive expression was

29$\pm$4.0%, with significantly lower expression seen in the presence of both 100 nM PGI$_2$ (8.9$\pm$3.0%, *p=0.004*) and 1000 nM PGI$_2$ (6.8$\pm$2.0%, *p=0.007*). Looking at percentage reduction compared with 1 nM PGI$_2$ at 1 μg/ml CRP-XL (-2.5$\pm$1.0%), there were significant reductions at both 100 nM PGI$_2$ (51$\pm$13%, *p=0.02)* and 1000 nM PGI$_2$ (60$\pm$8.0%, *p=0.005*). With dual agonists, compared with percentage reduction at 1 nM PGI$_2$ (2.5$\pm$6.0%) there were significant reductions at 100 nM PGI$_2$ (69$\pm$10%, *p=0.002*) and 1000 nM PGI$_2$ (76$\pm$7.0%, *p=0.0002*) as shown in panel C of Figure 3-10.
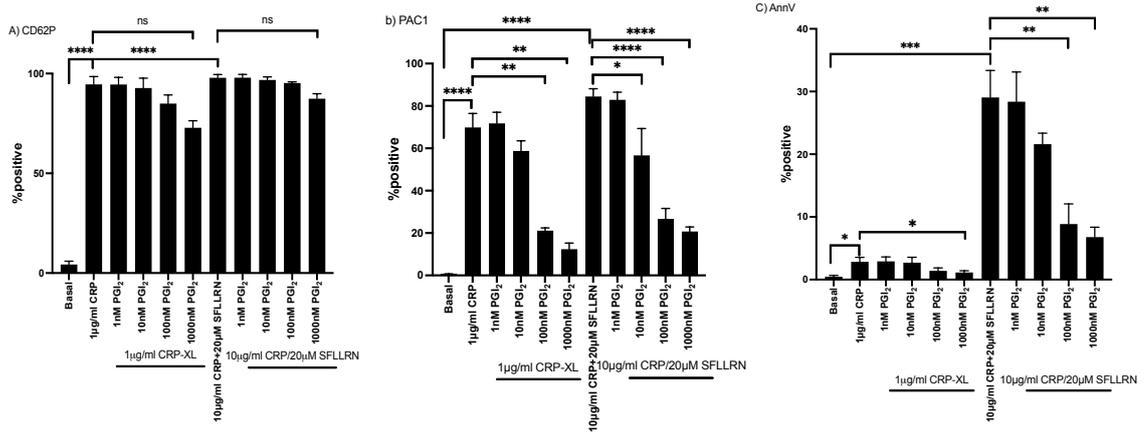
Figure 3-9: Multiparameter fluorescent flow cytometry assay measuring platelet expression of CD62P (panel A), PAC1 (panel B) and AnnV (panel C) expression under basal conditions and in the presence of agonists alone (single 1μg/ml CRP-XL and dual 10μg/ml CRP-XL and 20μM SFLLRN) and with varying doses of PGI2 (1-1000nM) for the corresponding agonists in whole blood from healthy volunteers.

Data are expressed as percent positive. Statistical analysis is conducted as basal versus stimulated and also stimulated versus expression in the presence of each of the comparative PGI2 doses. *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001. n=3.
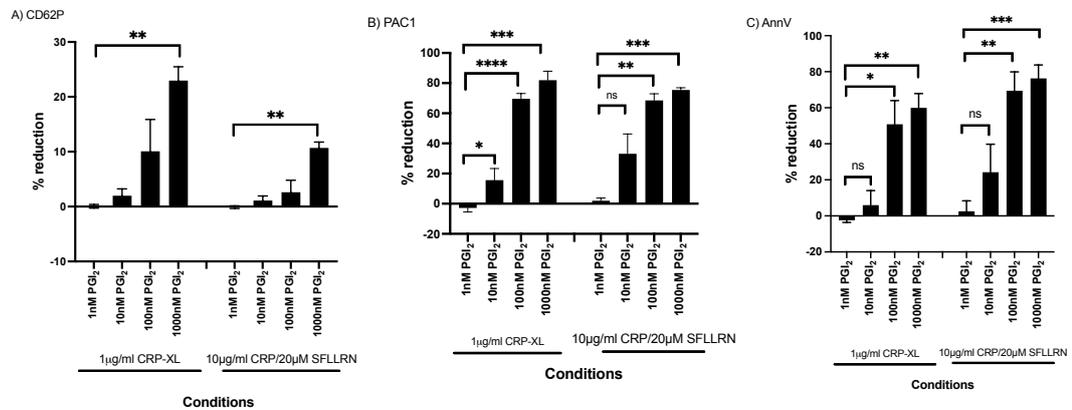
Figure 3-10: Multiparameter fluorescent flow cytometry assay measuring percentage reduction of marker expression compared to maximal expression with agonist in the presence of 1-1000 nM PGI2 for CD62P (panel A), PAC1 (panel B) and AnnV (panel C) in whole blood from healthy volunteers.

Data are expressed as percent positive. Statistical analysis conducted in comparison to %reduction in the presence of 1nM PGI2 at each comparative agonist dose. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n=3.

The comparative MFI results are represented in Figures 3-11 and 3-12, which showed comparable results. For CD62P expression, again shown in panel A at the 1 μg/ml CRP-XL dose there was some minimal reduction in CD62P expression, but this was not significant for any of the doses of PGI_2. With dual agonist stimulation, there was a minimally but significantly lower CD62P expression at 1000 nM PGI_2 (30559$\pm$2645 at maximal expression versus 23053$\pm$1501, *p=0.02*) but no clear differences at the lower doses of PGI_2. In terms of percentage reduction from maximal, with the comparison made against percentage reduction at 1 μg/ml CRP-XL in the presence of 1 nM PGI_2 (-5.8$\pm$11%), there was significantly higher percentage reduction at 1000 nM PGI_2 (24.8$\pm$11%, *p=0.02*). Similarly, with dual agonist stimulation there was a significantly higher percentage reduction in the presence of 1000 nM PGI_2 compared with 1 nM PGI_2 (24.4$\pm$4% and 1.2$\pm$2% respectively, *p=0.005*). At the lower doses of PGI_2 there were no significant differences seen.

For PAC1 expression by MFI, shown in panel B in Figures 3-11 and 3-12, there was greater response to PGI_2. At 1 μg/ml CRP-XL, mean PAC1 expression was 7199$\pm$2263, dropping to 857$\pm$143 at 100nM PGI_2 and 411$\pm$156 at 1000 nM PGI_2, *p=0.04* and *p=0.03* respectively. With dual agonist stimulation, mean PAC1 expression was 13617$\pm$3206, with significantly lower expression in the presence of 10 nM PGI_2 (4634$\pm$1460, *p=0.02*), 100 nM PGI_2 (992$\pm$102, *p=0.02*) and 1000 nM PGI_2 (509$\pm$69, *p=0.01*). When expressed by percentage reduction from agonist stimulation alone, at 1μg/ml CRP-XL, there was a mean 42$\pm$14% reduction with 1 nM PGI_2 with significantly greater reductions seen at 10 nM (75$\pm$7.0%, *p=0.04*), 100 nM PGI_2 (82$\pm$9.0%, *p=0.02*) and 1000

nM PGI_2 (92±2.0%, *p=0.02*). With dual agonist stimulation, there was a mean 30±8.0% reduction with 1 nM PGI_2. There were significantly higher reductions with 10 nM PGI_2 (66±9.0%, *p=0.008*), 100 nM PGI_2 (92±1.0%, *p=0.005*) and 1000 nM PGI_2 (96±1.0%, *p=0.005*).

Finally, for AnnV MFI expression, at 1 μg/ml CRP-XL, there was mean expression of 1249±312, which did not significantly reduce in the presence of any of the PGI_2 doses likely owing to the fact there was only a marginal increase in expression at this agonist dose. Following stimulation with dual agonists, expression rose more significantly to 23300±6846, with significantly lower AnnV expression in the presence of both 100 nM PGI_2 (4635±2038, *p=0.03*) and 1000 nM PGI_2 (888±188, *p=0.02*). As expressed by percentage reduction compared to peak agonist stimulation, at 1 μg/ml CRP-XL there was a mean 0.4±1.5% reduction with 1 nM PGI_2 with significantly greater reductions seen at 100 nM PGI_2 (29±5.0%, *p=0.007*) and 1000 nM PGI_2 (47±6.0%, *p=0.005*). With the higher dual agonist strength, there was a mean percentage reduction of 2±2.0% with 1 nM PGI_2. There were significantly greater reductions than this with all the three higher concentrations of PGI_2 with mean percentage reduction of 38±6.0% at 10 nM PGI_2, *p=0.006*, 81±6.0% at 100 nM PGI_2, *p=0.0007* and at 1000 nM PGI_2 96±1.7%, *p<0.0001.*
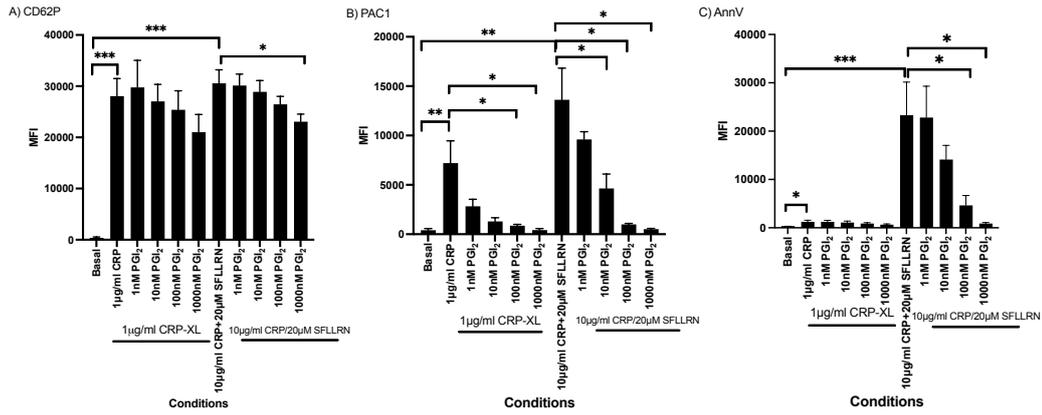
Figure 3-11: Multiparameter fluorescent flow cytometry assay measuring platelet expression of CD62P (panel A), PAC1 (panel B) and AnnV (panel C) expression under basal conditions, in the presence of agonists alone (single 1µg/ml CRP-XL and dual 10µg/ml CRP-XL and 20µM SFLLRN) and with varying doses of PGI2 (1-1000nM) for the corresponding agonists in whole blood from healthy volunteers.

Data are expressed as MFI. Statistical analysis is conducted as basal versus stimulated and also stimulated versus expression in the presence of each of the comparative PGI2. doses. *p<0.05, ** p<0.01, ***p<0.001. n=3.
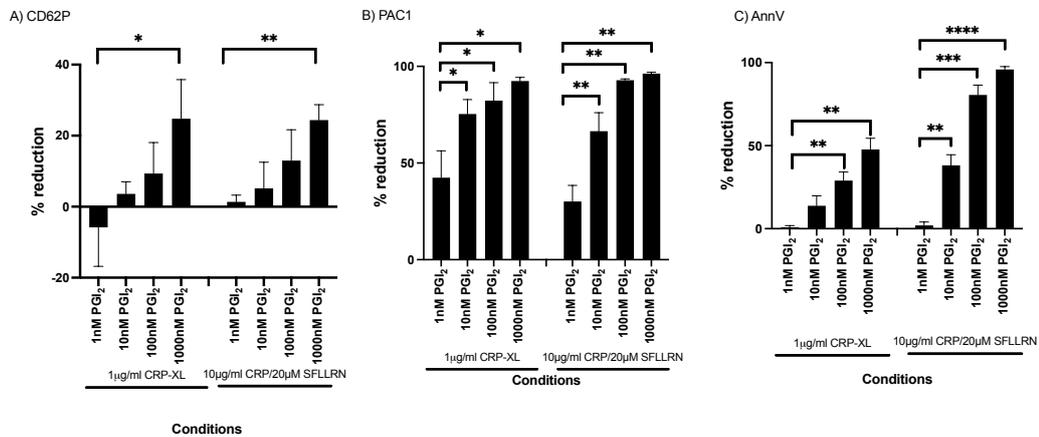
Figure 3-12: Multiparameter fluorescent flow cytometry assay measuring percentage reduction of marker expression compared to maximal expression with agonist in the presence of 1-1000 nM PGI2 for CD62P (panel A), PAC1 (panel B) and AnnV (panel C) in whole blood from healthy volunteers.

Data are expressed as percent positive. Statistical analysis conducted in comparison to %reduction in the presence of 1nM PGI$_2$ at each comparative agonist dose. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n=3.

3.4 Discussion

Establishing a robust, multiparameter flow cytometry assay to assess expression of key markers exploring different aspects of platelet activation was crucial. The optimisation experiments described allowed determination of the optimal doses of both agonists and PGI_2 to later be conducted on samples from patients enrolled in the study.

For the final multiparameter assay, the choice to use lower dose single agonists, higher dose single agonists and dual agonists was to create conditions of low, medium and high strength stimuli. The final activation panel therefore included basal conditions, 2 μM SFLLRN, 20 μM SFLLRN, 1μg/ml CRP-XL, 10 μg/ml CRP-XL and combined 10 μg/ml CRP-XL and 20 μM SFLLRN. Given the hypothesis of hyperreactivity of platelets from patients with T1D, it was important to have a spectrum of various strengths of agonists to allow identification of potential differences. Using only high strength agonist may have resulted in maximal activation in both cohorts thus making it difficult to ascertain any differences. For the inhibition panel the same conditions were used as above but with the addition of two PGI_2 doses (10 nM and 100 nM) in order to assess the potential differential response to inihibition at either end of the spectrum of activation. These doses were chosen as 1 nM PGI_2 had very minimal effect on any of the parameters and 1000nM PGI_2 resulted in almost complete inhibition and thus in both cases this would make it difficult to assess for potentially subtle differences between cohorts. Similar experiments have previously been conducted using these doses with comparable results [298].

Naturally, some limitations must be acknowledged. Despite acceptable standard deviation, it would be beneficial to have a large pool of donors for the optimisation experiments and additionally over a wider period of time to account for potential differences. Stock solutions and fluorophores will need to change over the course of the planned study, but with all experiments conducted by myself this will hopefully improve reproducibility.

The created panels will therefore allow investigation of whether patients with T1D and/or DD do indeed have a heightened response to low or medium concentrations of agonists compared with healthy controls, as well as the impact of different clinical parameters on platelet activation. This will provide evidence for potential hyperreactivity and whether these agonists produce differential expression of any of the individual markers including the possibility of increased AnnV binding and therefore PS exposure, suggestive of a more pro-coagulant state. Furthermore, the additional conditions with two doses of PGI_2 will allow investigation of the sensitivity of platelets from patients with T1D to inhibition as well as the potential impact of glycaemia and IR.

Chapter 4: Platelet activation in T1D and the impact of glycaemia and insulin resistance

4.1 Introduction

Having now established the multiple pathways involved in platelet activation under normal physiological circumstances it is next important to highlight that many disease states, including diabetes, have been shown to affect these pathways driving cardiovascular complications [313-315]. Whilst platelet hyperreactivity is most accurately described as the composite of increased activation and diminished response to inhibition, it commonly is used to describe increased capacity for platelet activation alone [316-318]. Chapter 1 outlined the existing evidence for increased activation and platelet hyperreactivity, through the measure of key markers in patients with diabetes. Most of the focus of these existing studies has been on patients with T2D, both with and without known cardiovascular disease, a population with a large number of confounding elements including concomitant medications such as anti-platelet therapies. Other experiments have either been conducted in mice models of diabetes or in healthy volunteers with experimentally-induced hyperglycaemic environments. Accurate and direct evidence for platelet hyperreactivity specifically in T1D is far less well investigated and to our knowledge there are no studies thus far directly investigating functional platelet profiles in patients with DD. A clear advantage of investigating the T1D population is the ability to dissect the role of dysglycaemia from other confounders frequently seen in T2D patients. Moreover, comparing normal and overweight T1D individuals allows the characterisation of the potential additive or synergistic role of insulin resistance on platelet reactivity in people with diabetes.

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4.2 Methods

4.2.1 Study Population

The DEVELOP study was detailed in Chapter 2 and early recruitment from the study provided samples for the patient data throughout this chapter. To reiterate, the inclusion criteria for the study included patients who had an established diagnosis of T1D >3 years, were aged 18-40 years at the time of sampling and had no known significant macrovascular complications e.g., ischaemic heart disease. All patients were on insulin therapy either delivered by continuous subcutaneous insulin infusion or multiple daily injections. Ethical approval was granted by the Health Research Authority (REC reference: 19/NE/0349, IRAS 259072).

Demographic data were taken in line with study protocols and data collected including up to date information on average insulin use over the proceeding 7 days. Samples were taken as per the study protocol, including sampling for clinical parameters and a 1.8 mL sodium citrate (3.2%) vacutainer specifically for use in the flow cytometry assays, which has previously been validated by the group [298, 319].

For control data, sampling was conducted on healthy volunteers, as described in chapter 3 methods.

4.2.2 Glycaemic and clinical markers

The DEVELOP study protocol included continuous glucose monitoring for up to 14 consecutive days, using the Freestyle Libre Pro sensor (blinded sensor). Glycaemic markers obtained from the Libre Pro sensor included TIR, average glucose value and GV (CoV). For the purposes of results, the target range was considered 3.9-10 mmol/L as per international consensus and national guidelines outlined in chapter 1. In addition, data relating to hypoglycaemia were also obtained including time below range (TBR), further categorised into level 1 hypoglycaemia (blood glucose 3.0-3.9 mmol/l) and level 2 hypoglycaemia (blood glucose <3.0 mmol/L). Conversely time above range (TAR) was also available, also further categorised into level 1 (blood glucose 10.1-13.3 mmol/L) and level 2 (>13.3 mmol/L). Median values were used for stratification by clinical parameters, where a patients fell on the threshold median value, they were included in the 'greater than' group.

4.2.3 Flow Cytometry and platelet activation markers

The detailed methods used for carrying out a standard flow cytometry assay were described in chapter 2 (section 2.4.4.3). The specific markers selected for the activation panel assay were discussed in chapter 3 (section 3.3.10) along with optimisation of the conditions. The same multiparameter assay was used throughout all experiments in this chapter with the following fluorophores included; BB700-CD42b, PE-CD62P, FITC-PAC1 and APC-AnnV.

4.2.4 Statistical analysis

All data referred to within this chapter were analysed using GraphPad Prism version 9.5.1 (528). For descriptive data, results are presented as median (interquartile range), mean±standard deviation (SD) and/or number (% of total). Where appropriate normal distribution was assessed using Shapiro-Wilks. One-way ANOVA (Kruskal Wallis) was used to test multiple continuous variables. For the comparison of two independent continuous variables, either the Student t-test or Mann-Whitney test was used depending on the normative distribution of the data.

With respect to analysis that has been stratified by the various clinical parameters including eGDR, HbA1c and TIR, categorisation has largely been done according to the median representative value. Additional analysis was conducted in the case of eGDR to mirror the large-scale Nyström *et al.,* study threshold values for presence of IR and association with cardiovascular risk (<6 mg/kg/min, 6-8 mg/kg/min, >8 mg/kg/min. A further group <4 mg/kg/min was not included due to lack of numbers in that category).

For CD62P MFI expression, and assuming an SD of 4000 for MFI (based on preliminary data), a sample size of 36 individuals would be sufficient to detect a difference of 4500 in MFI for this variable with a power of 80% at *p<0.05.* For PAC1 expression, a total for 32 individuals would be required to detect a difference 1500 in MFI, while 30 individuals are required to detect a difference of 1700 in MFI for AnnV, both with a power of 80% at *p<0.05,* based on an SD for these variables of 1450 and 1770 respectively, derived from my preliminary data.

4.3 Results

4.3.1 Patient characteristics

From the full DEVELOP patient cohort, samples from a total of 34 patients underwent the multiparameter flow cytometry assay examining platelet activation. Data from one patient were excluded from all analyses due to pregnancy and thus met exclusion criteria (unaware pregnant at time of sampling). Any further data excluded if they were clear outliers have been detailed in the relevant section. Table 4-1 summarises the characteristics of all study patients included in analysis and compares the groups according to eGDR. No patients included in this analysis were on medications known to impact platelet function *e.g.,* statin therapy or oestrogen containing contraceptives.

	Total	eGDR <8.6	eGDR ≥8.6	*p-value*
Number of participants	34	17	17	-
Male sex (%)	20 (59%)	10 (59%)	10 (59%)	>0.99
Age, years	24.0±3.8	23.4±3.3	24.4±4.2	0.44
Duration of diabetes, years	14.0±6.3	12.8±6.6	14.8±6.1	0.37
HbA1c, mmol/mol	65.3±14.0	73.5±12.7	57.6±10.6	**0.0006**
BMI, kg/m^2	27.6±5.6	30.1±6.7	25.3±3.2	**0.014**
Estimated glucose disposal rate (WC), mg/kg/min (SD)	8.1±2.1	6.4±1.5	9.7±0.8	**<0.0001**
Total daily insulin (U/24h)	66.0±25.7	80.0±29.0***	50.5±8.7	**0.0007**
Total daily insulin (U/kg)	0.79±0.3	0.91±0.3**	0.68±0.1	**0.008**
Family history of T2DM	4 (12%)	3 (18%)	1 (6%)	0.60
Presence of retinopathy	14 (41%)	6 (35%)	7 (41%)	>0.99
Adjunctive therapy (metformin)	2 (6%)	2 (12%)	0	0.48
Macrovascular complications	0	0	0	-

Table 4-1: Summary of DEVELOP study patient characteristics included in flow cytometry analysis.

Total cohort and comparison of those stratified according to median eGDR, 8.6mg/kg/min. Statistical analysis to compare patients with eGDR <8.6mg/kg/min and those with eGDR ≥ 8.6mg/kg/min.

4.3.2 Platelet activation in patients with T1D

Platelet activation was measured in samples from patients participating in the DEVELOP study and each activation marker stratified according to the various clinical parameters of interest including HbA1c, TIR and eGDR as well as further stratification by GV, TBR and plasma glucose at the time of sampling.

4.3.3 Measurement of platelet CD62P expression in patients with T1D

Data from 31 study patient samples were included in analysis of platelet CD62P expression, data were excluded from n=3 clear outliers as per PRISM statistical analysis (Grubb's Test), these results were exceptionally elevated and resolved following laser re-alignment. Figure 4-1 is representative of the results from the full data set, with the left panel reflecting percent positive and the right panel represents MFI. The MFI results have a greater variation than percent positive as shown through the wider standard deviation bars on the graph, given the much larger scale of MFI results, this was entirely expected. The results were also largely as expected when looking at the whole cohort. As this is a visual representative of the full data set, there was no meaningful comparison to be made and thus no statistical analysis was conducted.

Figure 4-1: Four-colour fluorescent flow cytometry activation panel to investigate platelet expression of CD62P under basal conditions and in response to single/dual agonists in whole blood from study patients with T1D.

Left panel represents CD62P percent positive expression basally and with the different agonist doses (SFLLRN and CRP-XL). The right panel represents the comparative median fluorescence intensity data. n=31.

4.3.3.1 The relationship between HbA1c and P-selectin expression

The first glycaemic marker by which results were stratified was HbA1c. Data were separated according to the median HbA1c measurement which was 64 mmol/mol, 15 and 16 patients above and below that cut off respectively.

As anticipated, the data analysed by percent positive show only subtle differences between the two cohorts, given they both approached maximal expression (100%) rapidly. The most significant difference was seen under basal conditions, which is shown in more detail in Figure 4-3. Across the wider activation panel, following stimulation, there were some small, but significant differences seen with 20 μM SFLLRN, 10 μg/ml CRP-XL and dual agonist stimulation (20 μM SFLLRN combined with 10 μg/ml). The lower dose agonists 2 μM SFLLRN and 1 μg/ml CRP-XL did not show any significant

differences. With 20 μM SFLLRN patients in the higher HbA1c group had mean 95.6$\pm$3.2% CD62P expression compared with 90.7$\pm$6.7% in those in the lower HbA1c group, *p=0.015*. A similar pattern was seen with single agonist 10 μg/ml CRP-XL, 95.0$\pm$4% in those with HbA1c $\geq$64 mmol/L versus 90.4$\pm$6% *(p=0.021)* in those with lower HbA1c and also with dual agonists at maximum doses, mean 95.4$\pm$4% in the higher HbA1c group versus 90.2$\pm$6% *(p=0.017)*. The comparative MFI results were similar, however given the greater sensitivity of the scale, more clear differences were seen. The basal findings are shown in greater detail in Figure 4-3. Significant differences were seen, with higher MFI in the HbA1c $\geq$64 group with all single agonist doses as well as with dual agonists. The greatest differences were seen at 20 μM SFLLRN and with dual agonists (20 μM SFLLRN combined with 10 μg/ml CRP-XL). With 20 μM SFLLRN, patients with poorer glycaemic control had an MFI of 29648$\pm$7989 versus 23629$\pm$3512 in those with lower HbA1c, *p=0.006*. Similar results were seen following dual agonist stimulation (31357$\pm$8713 in those with HbA1c $\geq$64 mmol/mol versus 23478$\pm$3826 when HbA1c mmol/mol <64, *p=0.005*).

Basally, differences between the two groups are difficult to appreciate on the wider scales and so are shown in more detail in Figure 4-3. In the cohort with HbA1c $\geq$64 mmol/mol, higher CD62P expression is shown both for percent positive, 10.3$\pm$2% versus 6.8$\pm$2% *(p=0.0003)* and MFI, 613$\pm$264 versus 428$\pm$194, *p=0.023*.

These results demonstrate those with HbA1c $\geq$64 mmol/mol had higher CD62P expression both represented by percent positive and MFI. The difference was evident both under basal conditions and after platelet

stimulation, indicative of the expression on circulating 'quiescent' platelets and also in response to various degrees of agonist stimulation.

Figure 4-2: Four-colour fluorescent flow cytometry activation panel to investigate platelet expression of CD62P basally and in response to agonists in whole blood from study patients with T1D stratified according to the median HbA1c.

Left panel represents CD62P percent positive expression and the right panel represents the comparative MFI data. *p<0.05, **p<0.01 and ***p<0.001. Median HbA1c (64mmol/mol), n=15 HbA1c ≥ 64 and n=16 HbA1c <64.
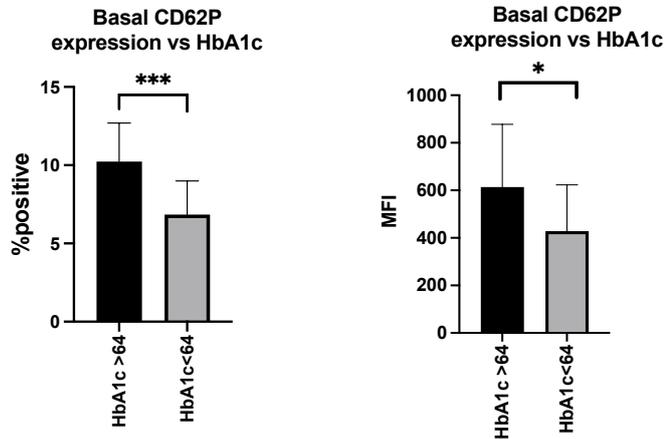
Figure 4-3: Fluorescent flow cytometry to investigate platelet expression of CD62P basally in whole blood from study patients with T1D stratified according to the median HbA1c.

Left panel represents CD62P percent positive expression and the right panel represents the comparative median fluorescence intensity data. *p<0.05 and ***p<0.001. n=15 HbA1c ≥ 64 and n=16 HbA1c <64.

4.3.3.2 The relationship between Time-in-Range and P-selectin

expression

The next clinical parameter by which results were stratified was another glycaemic marker, time in range (TIR). While HbA1c represents average glycaemia over 3 months period, TIR assesses glycaemia over the preceding 1-2 weeks. Data were again separated according to the median TIR value which was 55%. As TIR relied upon the safe return of the applied LibrePro sensor and a minimum wear duration of 6 days, not all patients could be included in this analysis, thus n=23 for this analysis (TIR<55% n= 11, TIR 55%, n=12). Figure 4-4 represents the full panel and Figure 4-5 once again provides focus on the basal results.

Starting with the full panel, for percent positive, there is a clear trend towards the group with the lower TIR (<55%) having elevated expression of CD62P compared with those with a higher TIR, albeit only the difference in expression seen following lower dose CRP-XL (1 μg/ml) was a significant difference; those with TIR <55% had mean CD62P expression of 92.7$\pm$3.0% compared with 83.9$\pm$9.0% in those with TIR $\geq$55% *(p=0.007)*. Under basal conditions, whilst there was again an elevated percent positive CD62P expression (10.9$\pm$5.0% in the TIR<55% cohort compared with 8.0$\pm$2.0% in those where TIR$\geq$55%), this did not quite meet statistical significance, *p=0.055*.

When analysing CD62P expression using MFI, similar differences shown when stratifying according to HbA1c were again seen. There was a clear trend towards an elevated MFI in those with the lower TIR and thus poorer glycaemic control. Of note, those in the lower TIR group had a far greater variation than those who had TIR $\geq$55%. As shown in Figure 4-4, with high

dose (20 μM SFFLRN) single agonist and dual agonist stimulation the greatest differences were seen between the two groups. With 20 μM SFLLRN, MFI was 30410±9491 in TIR <55% versus 23042±3290 in those with TIR ≥55%, *p=0.024.* Following dual agonist stimulation, MFI was 31036±9402 in those with TIR <55% versus 23101±4024 in those with TIR ≥55%, *p=0.022.* Basally, again shown in more detail in Figure 4-5, demonstrated an elevated CD62P expression in those with TIR <55%, 703±329 compared with patients who had TIR ≥55%, 427±195, *p=0.027.*

Figure 4-4: Fluorescent flow cytometry to investigate platelet expression of CD62P basally and in response to agonists in whole blood from study patients with T1D stratified according to the median TIR.

Left panel represents CD62P percent positive expression and the right panel represents the comparative median fluorescence intensity data. *p<0.05 and **p<0.01. n=11 TIR<55% and n=12 TIR ≥55%.

Basal CD62P expression vs TIR

Basal CD62P expression vs TIR
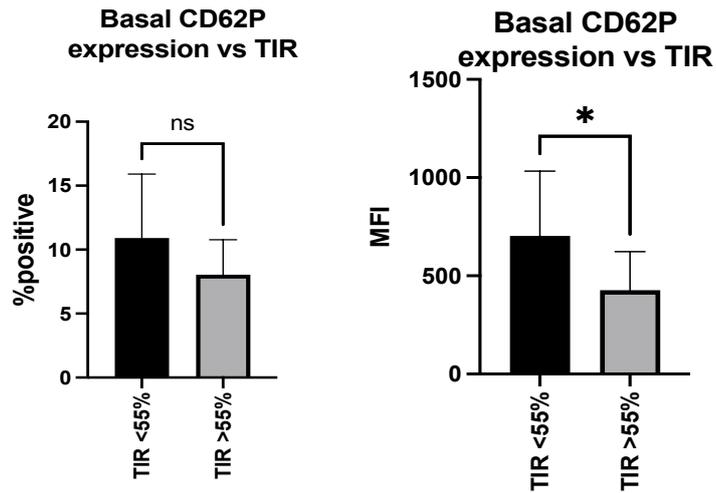
Figure 4-5: Fluorescent flow cytometry to investigate platelet expression of CD62P basally in whole blood from study patients with T1D stratified according to the median TIR.

Left panel represents CD62P percent positive expression and the right panel represents the comparative median fluorescence intensity data. $*p<0.05$ and $**p<0.01$. n=11 TIR<55% and n=12 TIR ≥55%.

4.3.3.3 The relationship between plasma glucose and P-selectin expression

Results were next stratified according to plasma glucose at the time of sampling. Unfortunately, not all plasma glucose levels were made available by the central clinical laboratory and thus a total of n= 26 were available for analysis. Groups were determined by the median plasma glucose which was 8 mmol/l and n=13 in each group. Figure 4-6 represents CD62P expression analysed by both percent positive and MFI.

First looking at percent positive, there were no significant differences between basal CD62P expression in those with the higher plasma glucose (≥8 mmol/l) than those with lower plasma glucose. With agonist stimulation (either single

or dual) there was also no significant difference seen in those with plasma glucose ≥ 8 mmol/l compared with patients with plasma glucose <8 mmol/l.

The results analysed by MFI also showed no significant difference between the two groups, with no clear trend identified. With both 2μM SFLLRN and dual agonist stimulation, there were no significant differences between the two groups.
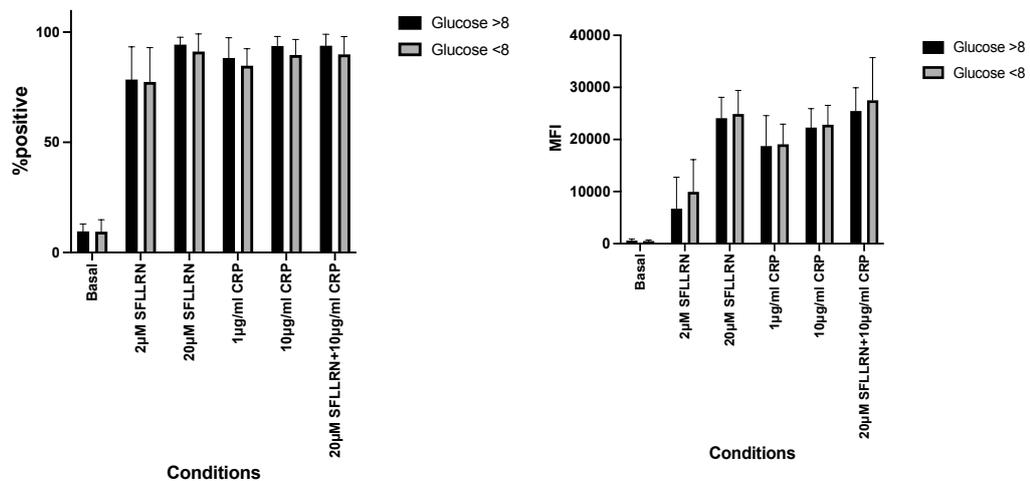
Figure 4-6: Fluorescent flow cytometry to investigate platelet expression of CD62P basally and in response to agonists in whole blood from study patients with T1D stratified according to the plasma glucose at time of sampling.

Left panel represents CD62P percent positive expression and the right panel represents the comparative median fluorescence intensity data. Glucose ≥ 8 mmol/l, n=13 and <8 mmol/l n=13

4.3.3.4 The relationship between eGDR and P-selectin expression

CD62P expression was next stratified according to IR, measured by eGDR. Again, the results were stratified according to the median eGDR value. The eGDR algorithm used included waist circumference given, as described in Chapter 1, this was validated in patients with T1D in the original eGDR algorithm [232].

All patients had an eGDR calculated thus for this analysis n=31, the median threshold value was 8.6 mg/kg/min. Based on the previously discussed large-scale Nyström et al study linking eGDR cut-off values and cardiovascular risk [235], further analysis was conducted using eGDR ≥8 mg/kg/min, 6-8 mg/kg/min and <6 mg/kg/min to assess for differences in platelet activation comparable to the cardiovascular risk demonstrated in the Swedish study. A threshold value of 8 mg/kg/min is indicative of the presence of IR. Figure 4-7 represents the data analysed by percent positive and MFI when stratified according to median eGDR value of 8.6 mg/kg/min.

First, assessing the percent positive data, there is a trend comparable to that shown when stratifying the data according to HbA1c and TIR. The only definitive difference was seen when using 20 μM SFLLRN and dual agonists for platelet activation. At 20 μM SFLLRN, patients with eGDR <8.6 (n=15) had mean CD62P percent positive of 94.4±3% compared with 91.2±6% in those without IR (eGDR≥8.6, n=16), *p=0.023.* Similar results were seen following dual agonist stimulation; 95.1±3% in those with eGDR <8.6 compared with 91.1±5% in those with eGDR ≥8.6, *p= 0.011*

Furthermore, data analysed by MFI showed comparable results. Following stimulation with 20 μM SFLLRN, those with a low eGDR had MFI 29698±8037 compared with MFI 22949±2827 in those with eGDR ≥8.6, *p=0.0089.* Similarly, with 10 μg/ml CRP-XL, MFI was significantly higher in those with a low eGDR (26097±4954 vs 20013±2901). Finally, there was also a difference seen following dual agonist stimulation, with those where eGDR<8.6 having mean of 31007±6961 compared with 22950±3373, *p=0.0027.*
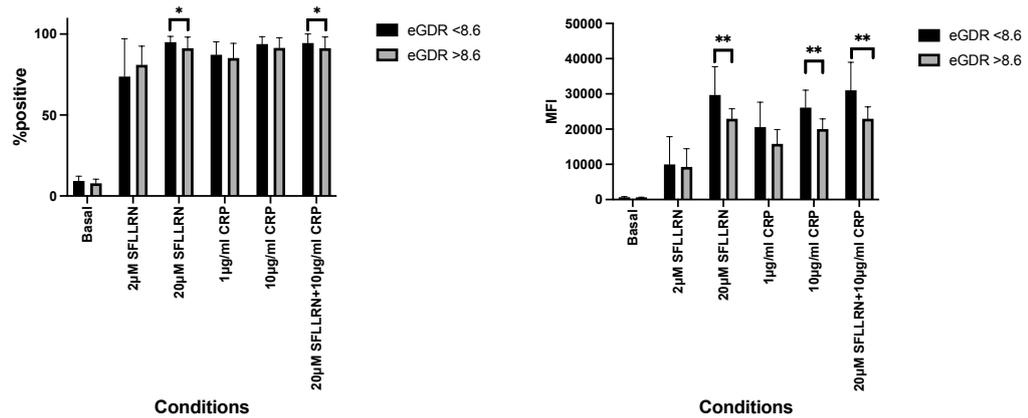
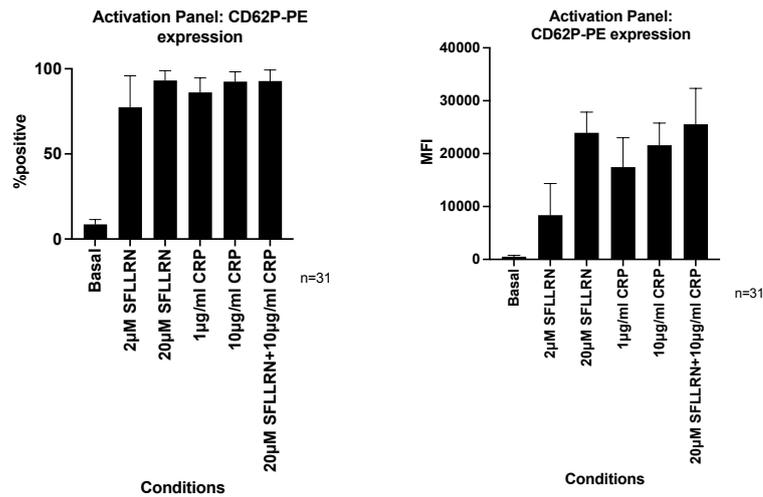**Figure 4-7: Fluorescent flow cytometry to investigate platelet expression of CD62P under basal conditions and in response to agonists in whole blood from study patients with T1D stratified according to the median eGDR.**

Left panel represents CD62P percent positive expression and the right panel represents the comparative median fluorescence intensity data. *p<0.05 and **p<0.01. n=15 eGDR<8.6 and n=16 eGDR ≥8.6.

As mentioned, the data were next analysed in three groups, eGDR <6 mg/kg/min, 6-8 mg/kg/min and ≥8 mg/kg/min. The group sizes were n=8, n=9 and n=14 respectively, given the majority of patients had an eGDR ≥8 mg/kg/min. Figure 4-8 represents this data, again including both percent positive and MFI.

Under basal conditions, there were differences seen both when comparing percent positive between patients with eGDR <6 mg/kg/min and those with eGDR ≥8 (13.1±4% versus 6.2±2%, p=*0.0027*) and a smaller but still significant difference seen between those with eGDR <6 and 6-8 mg/kg/min (13.1±4% and 7.9±2% respectively, *p=0.014*). Comparable results were seen for MFI, eGDR <6 mg/kg/min had basal MFI of 800.1±325 versus 444.2±131 and 320±107 in those with eGDR 6-8 mg/kg/min and ≥8 respectively (*p=0.0072* and *p=0.03*).

With low dose stimulation with 2 μM SFLLRN, there was only a difference between those with eGDR<6 mg/kg/min and those with eGDR $\geq$8, 84$\pm$7% versus 70$\pm$15% *(p=0.012).* When analysing the data by MFI, a more significant difference was identified between those with eGDR <6 (15017$\pm$5622) and those with eGDR $\geq$8, 5226$\pm$3565, *p=0.0059.* There was also a difference between those with eGDR <6 mg/kg/min and those with an eGDR 6-8 mg/kg/min (6404$\pm$3478, *p=0.012*).

With higher dose SFLLRN (20 μM) the difference in percent positive CD62P expression was less pronounced and only the difference between the <6 mg/kg/min group and $\geq$8 mg/kg/min was significant, 94$\pm$3% versus 90$\pm$6, *p=0.016.* Using MFI similar patterns were seen, with an MFI of 33956$\pm$7400 (eGDR <6 mg/kg/min) versus 22649$\pm$3227 (eGDR $\geq$8 mg/kg/min), *p=0.011.*

With 1 μg/ml CRP-XL no clear differences were identified between any of the groups both for percent positive or MFI. At 10 μg/ml CRP-XL, patients with eGDR <6 mg/kg/min had a significantly higher CD62P expression than those with eGDR $\geq$8 mg/kg/min, 95$\pm$5% versus 89$\pm$6%, *p=0.04*, but no difference was seen between either group and those with eGDR 6-8 mg/kg/min (89.7$\pm$8%). When using MFI, at 10 μg/ml CRP-XL, patients with eGDR <6 mg/kg/min had a significantly higher CD62P expression than those with eGDR $\geq$8 mg/kg/min (29167$\pm$2177 and 19392$\pm$2535 respectively, p=0.0003).

Following dual agonist stimulation (20 μM SFLLRN + 10 μg/ml CRP-XL), patients with eGDR <6 mg/kg/min had a significantly higher CD62P expression than those with eGDR $\geq$8 mg/kg/min analysed by MFI 34719$\pm$7860 and 22829$\pm$353, respectively, *p=0.012* and also percent positive, 95$\pm$4% and 88$\pm$8%, *p=0.02.* Although there was a trend for patients with eGDR 6-8

mg/kg/min to have a lower CD62P expression than the eGDR <6 mg/kg/min
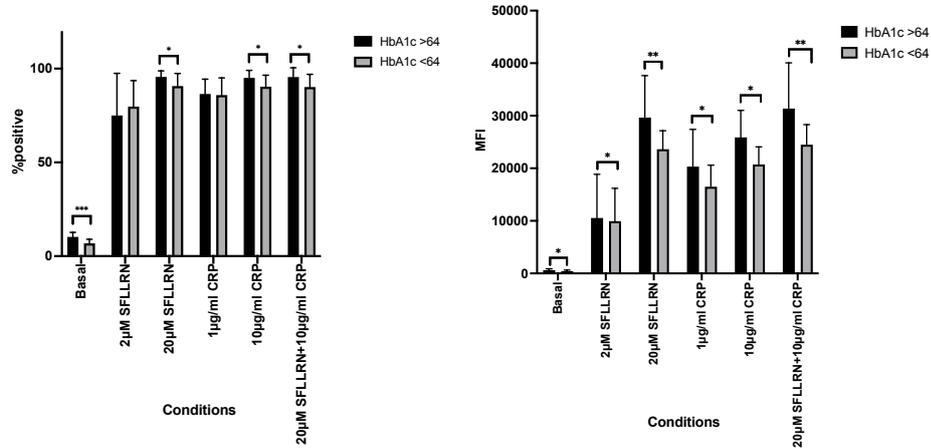
group, *p=0.09*, this did not reach statistical significance.

Figure 4-8: Fluorescent flow cytometry to investigate platelet expression of CD62P basally and in response to agonists in whole blood from study patients with T1D stratified according to the eGDR.

Left panel represents CD62P percent positive expression and the right panel represents the comparative median fluorescence intensity data. *p<0.05, **p<0.01 and ***p<0.001. <6mg/kg/min n=8, 6-8mg/kg/min n=9 and eGDR≥8mg/kg/min n=14.

4.3.3.5 The relationship between GV and P-selectin expression

Results were then stratified according to glycaemic variability, GV. Again, as these data are LibrePro sensor-derived, there were fewer patients with valid data to include so the total n=22, one patient with other available LibrePro data did not have a GV available. The data were split according to the median GV value of 44% (n=11 in both groups).

Figure 4-9 represents the data analysed by both percent positive and MFI. When the data are represented by percent positive CD62P expression, whilst there appeared to be higher expression in those with GV ≥44% both under basal conditions (11±5% versus 8±3% in GV<44%) and following agonist stimulation these differences were not statistically significant.

Similarly, when data are analysed according to MFI, there again appeared greater CD62P expression in those with the higher GV but these differences were non-significant.

Figure 4-9: Fluorescent flow cytometry to investigate platelet expression of CD62P basally and in response to agonists in whole blood from study patients with T1D stratified according to median GV.

Left panel represents CD62P percent positive expression and the right panel represents the comparative median fluorescence intensity data. GV ≥44% n=11 and GV <44% n=11.

4.3.3.6 The relationship between hypoglycaemia and P-selectin expression

I then assessed the effects of hypoglycaemia, measured by time-below-range (TBR) (<3.9mmol/l) on CD62P expression, with a total of n=23. Again, the results were stratified according to the median value of 9%; n=11 for ≥9% and n=12 for those with TBR of <9%.

Figure 4-10 represents the percent positive and the MFI values for CD62P expression. In a similar fashion to the data stratified according to GV, under basal conditions and with single agonist stimulation there were no clear differences between those with greater hypoglycaemic exposure versus those with lower hypoglycaemia. This was the case both when analysed by percent positive and also MFI. With dual agonist stimulation, the MFI expression was significantly higher in those with more hypoglycaemic exposure (28262±8923 compared with 22428±2866, *p=0.044*).
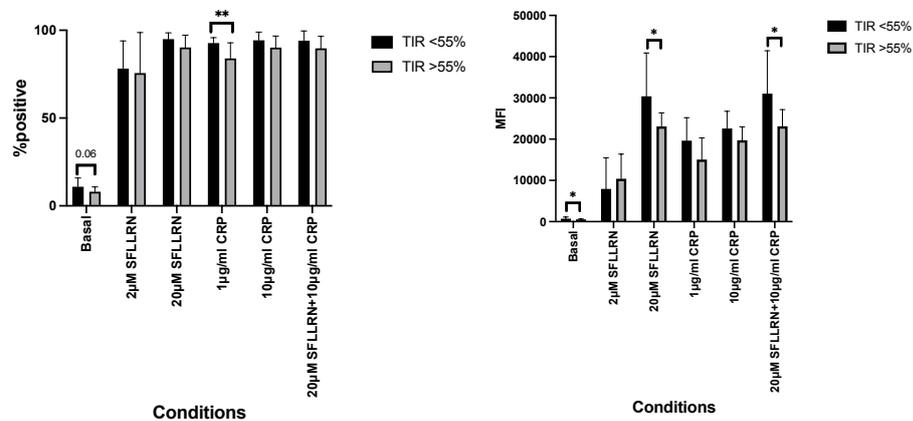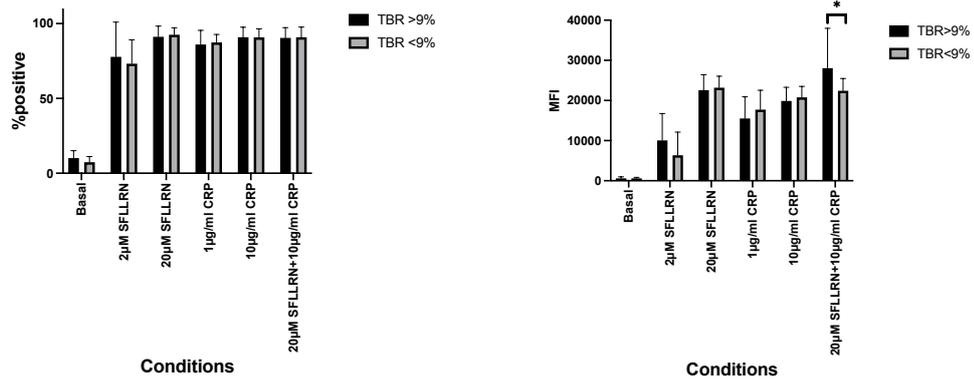
Figure 4-10: fluorescent flow cytometry to investigate platelet expression of CD62P basally and in response to agonists in whole blood from study patients with T1D stratified according to median TBR.

Left panel represents CD62P percent positive expression and the right panel represents the comparative median fluorescence intensity data. *$p < 0.05$. TBR $\geq 9\%$ n=11 and TBR <9% n=12.

4.3.4 Measurement of platelet PAC1 expression in patients with T1D

Moving onto the next marker, active $\alpha_{IIb}\beta_3$ conformational change (referred to as $\alpha_{IIb}\beta_3$ expression), represented through the measurement of PAC1. Data from 32 study patient samples were included in analysis of PAC1 expression, data were excluded from n=2 outliers as per PRISM statistical analysis. Figure 4-11 demonstrates the results from the full data set with the left panel representative of PAC1 expression measured by percent positive and the right panel representative of the same data measured by MFI. Again, there is no comparison group so statistical analysis has not been completed. Thereafter the results were again stratified and correlated with various glycaemic markers of interest, as with P-selectin.

Figure 4-11: Four-colour fluorescent flow cytometry activation panel to investigate platelet expression of PAC1 basally and in response to single/dual agonists in whole blood from study patients with T1D.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. n=32.

4.3.4.1 The relationship between HbA1c and active $\alpha_{IIb}\beta_3$ expression

Data were again stratified according to median HbA1c value of 64 mmol/mol (n=16 in both groups). Figure 4-12 represents the data across the full

activation panel, both percent positive and MFI. Figure 4-13 represents the data under basal conditions only.

First, percent positive results showed under basal conditions, there was an elevated PAC1 expression in those with higher HbA1c, though generally expression was low in both groups (1.38±1% versus 0.7±0.5%, *p=0.04*), as shown in Figure 4-13. With single agonist low dose SFLLRN and CRP-XL there were no significant differences between the two groups, nor with higher dose, 20μM SFLLRN. Patients with an elevated HbA1c appeared to have significantly increased PAC1 expression following single agonist stimulation with 10 μg/ml CRP-XL as well as with dual agonist stimulation. In the case of 10 μg/ml CRP-XL, patients with HbA1c ≥64 mmol/mol had a mean of 88±4% compared with 84±5% in the group with HbA1c <64 mmol/mol, *p=0.04*. With dual agonist stimulation, similar results were seen albeit slightly lower than the percent positive values for 10 μg/ml CRP-XL; those with an elevated HbA1c had a mean percent positive PAC1 expression of 84±4% versus 77±7% in those with HbA1c <64 mmol/mol, *p=0.007*.

Moving on to the analysis of MFI. Again, due to the wider scale in MFI, a separate Figure, 4-13, represents the basal data. Here, those with HbA1c ≥64mmol/mol had a significantly higher basal PAC1 expression than those with a lower HbA1c; 298±114 and 191±95 respectively, *p=0.01.* With single dose 20 μM SFLLRN, the data also show a significantly higher PAC1 expression in those with HbA1c ≥64 mmol/mol (13825±9627 compared with 6434±3024, *p=0.01)*. The differences were not significant with low dose single agonists (2 μM SFLLRN and 1 μg/ml CRP-XL). At the higher dose of CRP-XL, patients with an elevated HbA1c had significantly higher PAC1 expression

(13861±8735 versus 7878±2010, *p=0.03)*. Once again, the same was seen following dual agonist stimulation, the group with HbA1c ≥64 mmol/mol had mean expression of 16785±9784 compared with 7287±2711*, p=0.0023*.
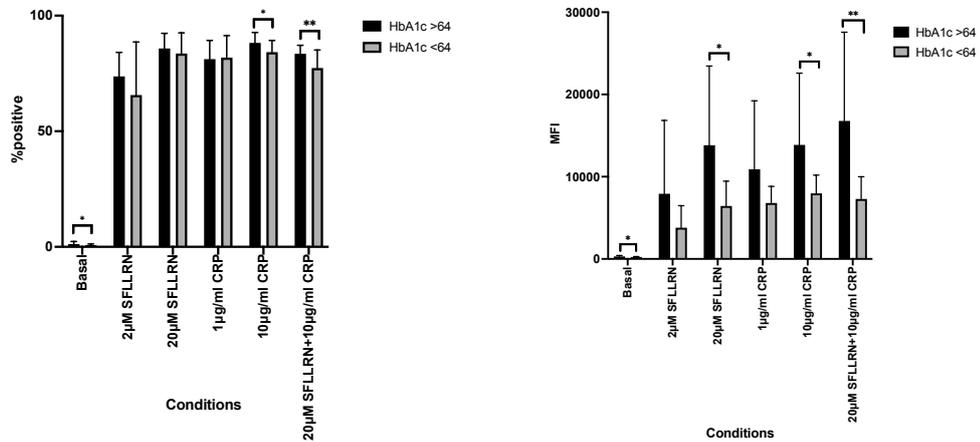
Figure 4-12: Four-colour fluorescent flow cytometry activation panel to investigate platelet expression of PAC1 basally and in response to single/dual agonists in whole blood from study patients with T1D, stratified according to median HbA1c.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. *p<0.05 and **p<0.01. HbA1c ≥64 mmol/mol n=16 and HbA1c <64 mmol/mol, n=16.
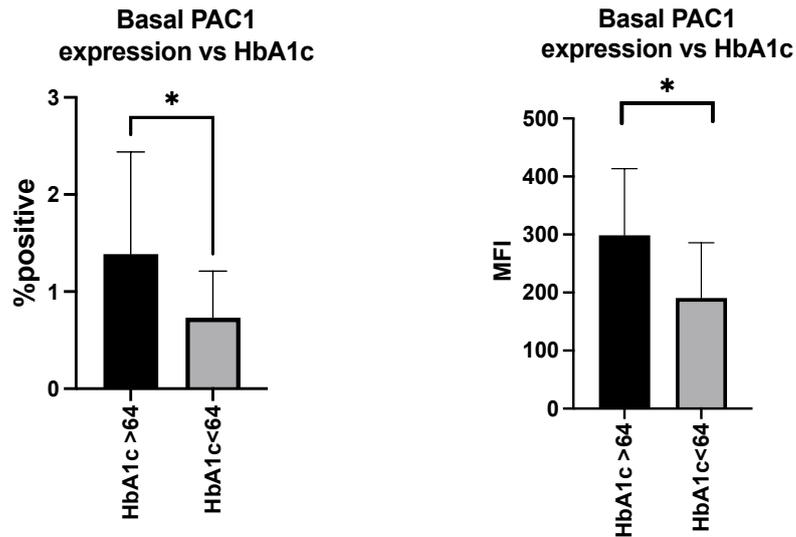
Figure 4-13: Four-colour fluorescent flow cytometry activation panel to investigate platelet expression of PAC1 basally in whole blood from study patients with T1D, stratified according to median HbA1c.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. *p<0.05 and **p<0.01. HbA1c ≥64mmol/mol n=16 and HbA1c <64mmol/mol, n=16.

4.3.4.2 The relationship between Time in Range and active $\alpha_{IIb}\beta_3$ expression

Next, PAC1 expression was examined both under basal conditions and in response to single/dual agonist stimulation stratified according to TIR. Again, the numbers included in this analysis were slightly lower due to incomplete LibrePro sensor use or non-return of the sensor, thus n=23. The mean TIR was 55%, with n=11 TIR<55% and n=12 TIR≥55%.

The full data are shown in Figure 4-14 with Figure 4-15 focussing on the basal results. In terms of the data represented by percent positive PAC1 expression, there was a small but significant difference between those with a lower TIR, 1.2±0.8% compared with 0.6±0.4% in those who had a TIR≥55%, *p=0.03*. The

trend remained in with single low and high dose SFLLRN, showing that those with a lower TIR had higher expression of PAC1 however there was a large degree of variation, shown with the wider standard deviation and thus these differences were not statistically significant. However, with low dose 1 μg/ml CRP-XL patients with lower TIR (<55%) had a significantly higher expression of PAC1, 86$\pm$4% versus 74$\pm$18% in those with TIR $\geq$55%, although again there was a larger degree of variation amongst the group, particularly in those with higher TIR, *p=0.04*. With high dose 10 μg/ml CRP-XL, similar results were seen 86$\pm$5% versus 78$\pm$18% but this difference was non-significant likely owing to the larger standard deviation in latter group. Following dual agonist stimulation, those with a lower TIR (<55%) had a higher expression of PAC1, 83$\pm$4% compared with 75$\pm$8% in those with TIR$\geq$55%, *p=0.02*.

The results represented by MFI showed a similar pattern. Basal data, as per Figure 4-15, demonstrate that those with a lower TIR had an elevated basal expression of PAC1 at 341$\pm$126 compared with 179$\pm$72 in those with a higher TIR $\geq$55%, *p= 0.0032*. With 2 μM SFLLRN, though there was again higher expression of PAC1, 11217$\pm$11901 in those with lower TIR versus 3261$\pm$2341, this was non-significant likely due to the variability in the results from those with lower TIR. At the higher dose of SFLLRN 20 μM, there was a significantly higher expression of PAC1 in those with the lower TIR (17155$\pm$11614 and 6301$\pm$3465 respectively, p=*0.02*). With low and high dose CRP-XL, again patients with the lower TIR had a higher expression of PAC1 but the difference was non-significant. With dual agonist stimulation there was significantly higher PAC1 expression in those with a lower TIR (22112$\pm$13070 versus 8064$\pm$3182, *p=0.007*).

Figure 4-14: Fluorescent flow cytometry activation panel to investigate platelet expression of PAC1 basally and in response to single/dual agonists, in whole blood from study patients with T1D, stratified according to median TIR.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. *p<0.05 and **p<0.01. TIR <55% n=11 and TIR ≥55% n=12.

Figure 4-15: Four-colour fluorescent flow cytometry activation panel to investigate platelet expression of PAC1 basally in whole blood from study patients with T1D, stratified according to median TIR.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. *p<0.05 and **p<0.01. TIR <55% n=11 and TIR ≥55%, n=12.

4.3.4.3 The relationship between plasma glucose and active $\alpha_{IIb}\beta_3$ expression

Finally, we stratified the data according to plasma glucose at the time of sampling (n=26). Median plasma glucose by which the groups were determined was 8 mmol/l and n=13 in each group. Figure 4-16 represents both percent positive and MFI PAC1 expression.

On analysing the data by percent positive PAC1 expression, there were no clear significant differences between those with higher or lower plasma glucose (≥8 mmol/l or <8 mmol/l) under basal conditions and with low or high dose SFLLRN and 1 μg/ml CRP-XL. With 10 μg/ml CRP-XL, there was a higher expression in those with elevated plasma glucose, 88±5% versus 77±14%, *p=0.03*. Similarly, following stimulation with dual agonists, those with the higher plasma glucose had a higher PAC1 expression, 84±4% compared with 71±11%, *p=0.004*.

When analysed by MFI, there were no significant differences between the two groups either under basal conditions or with single/dual agonist stimulation. Both results are shown in figure 4-16.
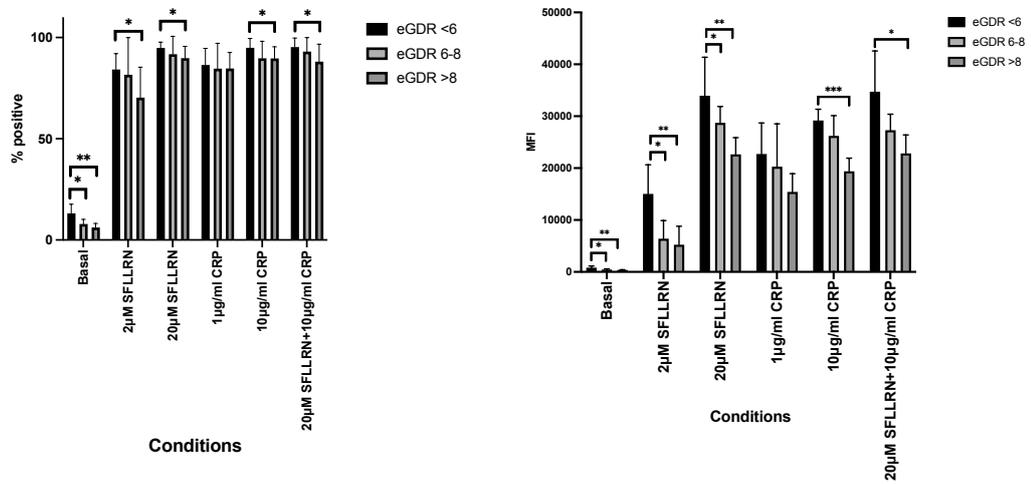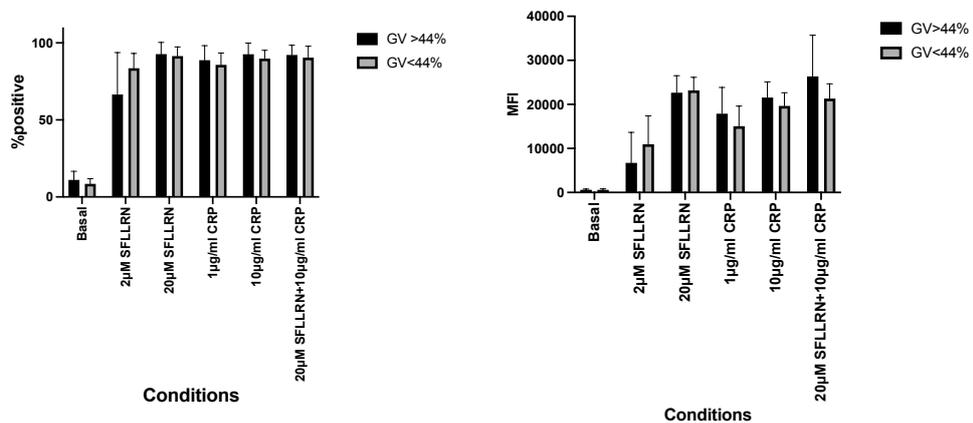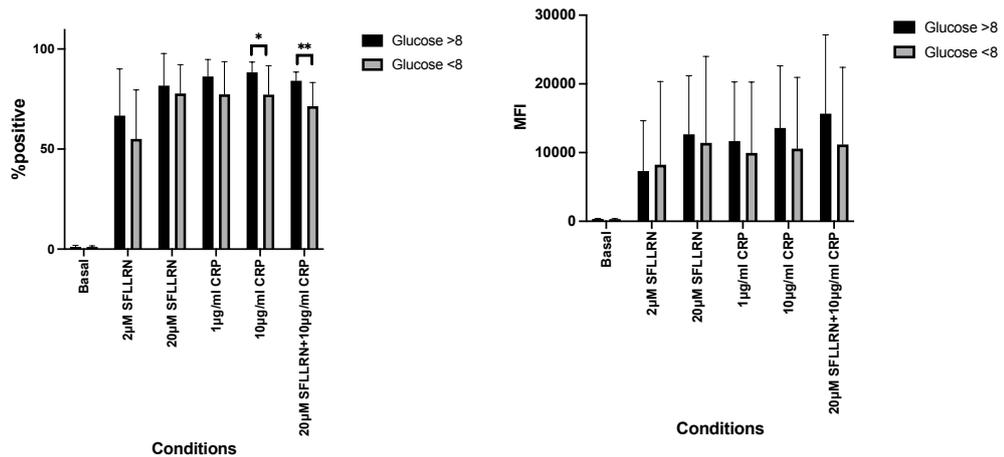
Figure 4-16: Fluorescent flow cytometry to investigate platelet expression of PAC1 basally and in response to agonists in whole blood from study patients with T1D stratified according to the plasma glucose at time of sampling.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity data. *p<0.05 and **p <0.01. Glucose $\geq$8 mmol/l, n=13 and <8 mmol/l n=13.

4.3.4.4 The relationship between eGDR and active $\alpha_{IIb}\beta_3$ expression

eGDR was the next clinical parameter by which the results for PAC1 expression were analysed. As per the P-selectin expression data, this was first done according to the median eGDR value of 8.6 mg/kg/min and then by 3 categories derived from the large scale Nyström et al paper[235]. The groups were split as n=15 with eGDR <8.6 mg/kg/min and n=16 eGDR $\geq$8.6 mg/kg/min. When analysed in the 3 categories, the group sizes were n=8 for eGDR <6 mg/kg/min, n=9 for eGDR 6-8 mg/kg/min and n=15 for those with eGDR $\geq$8 mg/kg/min.

The percent positive PAC1 expression showed a similar trend to the data stratified according to HbA1c and TIR. Under basal conditions, patients with a

low eGDR did have a higher PAC1 expression (1.3±0.9% versus 0.68±0.6%) but this difference only showed a trend (*p=0.054)*. With both low and high dose SFLLRN there was a large amount of variation and whilst there appeared to be higher PAC1 expression in those with lower eGDR these differences were non-significant. With low dose CRP-XL there was also no significant difference. Following stimulation with 10 µg/ml CRP-XL those with a low eGDR had higher expression of PAC1, 88±4% compared with 80±9% in those with eGDR ≥8.6 mg/kg/min, *p=0.03*. Finally with dual agonist stimulation, patients with higher IR had a higher mean percent positive, 84±4% compared to those without IR (76±10%), *p=0.02*. This is shown in Figure 4-17.

When analysing PAC1 expression by MFI, basal expression showed no significant differences but after stimulation with 20 µM SFLLRN, a clear difference was detected (13144±9310 versus 6782±4084, *p=0.04*), with a similar pattern for dual agonists (14892±9726 versus 7899±3134, *p=0.03*). Figure 4-17 also represents these data.
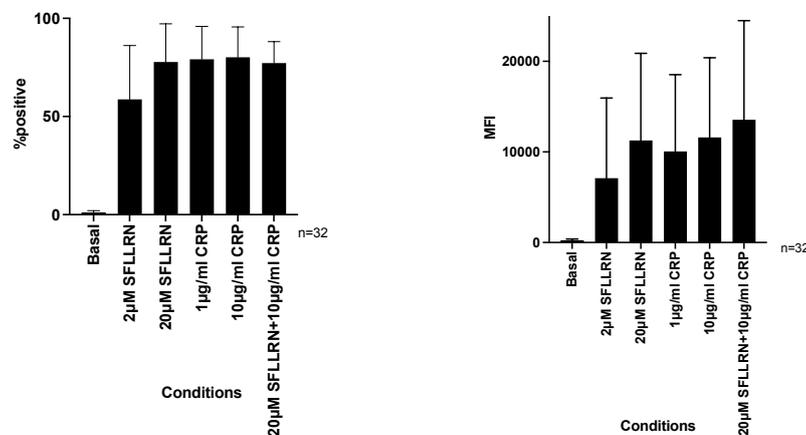
Figure 4-17: Fluorescent flow cytometry activation panel to investigate platelet expression of PAC1 basally and in response to single/dual agonists, in whole blood from study patients with T1D, stratified according to median eGDR.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. *p<0.05. eGDR <8.6 n=16 and eGDR ≥8.6 n=16.

As explained, the data were then analysed in 3 different categories informed by the Nyström et al study; these were eGDR <6 mg/kg/min, 6-8 mg/kg/min and ≥8 mg/kg/min. The group sizes were n=8, n=9 and n=15. Figure 4-18 shows this data, represented by both percent positive and MFI.

For percent positive expression, when analysing the data split into the 3 categories the trends are clearer. Basally, those in the lowest eGDR category (highest IR) had a significantly higher PAC1 expression (1.7±0.8%) than both those in the intermediate (6-8 mg/kg/min) and low (eGDR ≥8 mg/kg/min) categories (0.9±0.7%, *p=0.04* and 0.6±0.5%, *p=0.0043* respectively). There was no difference between those with eGDR 6-8 mg/kg/min and ≥8 mg/kg/min. Although there was far greater variation at 2μM SFLLRN, those with the lowest eGDR had a significantly higher PAC1 expression than those

with the highest eGDR $\geq$8 mg/kg/min, 81$\pm$1% compared with 58$\pm$2%, *p=0.008*. The differences between other categories were non-significant. Similar results were seen following stimulation with the higher dose SFLLRN, with a mean percent positive expression of 89$\pm$7% in those with eGDR <6 mg/kg/min versus 75$\pm$14% in those with eGDR $\geq$8mg/kg/min, *p=0.007*. Those with the lowest eGDR also had significantly higher expression when compared with patients with eGDR 6-8 mg/kg/min, 79$\pm$8%, *p=0.01*. With 1 μg/ml CRP-XL comparable results were seen, eGDR <6 mg/kg/min had mean expression of 88$\pm$5% compared with 83$\pm$3% in those with eGDR 6-8 mg/kg/min and 74$\pm$16%, *p=0.049* and *p=0.017* respectively. Following stimulation with 10 μg/ml CRP-XL those with the lowest eGDR had significantly higher expression at 92$\pm$2%, compared with 85$\pm$3% in those with eGDR 6-8 mg/kg/min and 77$\pm$14% in those with the highest eGDR, *p=0.0012* and *p=0.004* respectively. Finally, following dual agonist stimulation, an even greater difference was seen between those with eGDR <6 mg/kg/min at 87$\pm$3%, significantly higher than PAC1 expression in those with an eGDR $\geq$8 mg/kg/min with a mean expression of 73$\pm$9%, *p=0.0005*. There was no significant difference between those with the lowest eGDR and patients with an eGDR 6-8 mg/kg/min (83$\pm$3%), though this was significantly higher than those with eGDR $\geq$8 mg/kg/min, p=0.005.

A similar trend was seen when data were analysed by MFI. Basally, those with eGDR <6 mg/kg/min had a significantly higher MFI, 435$\pm$239, versus 206$\pm$110 in patients with eGDR $\geq$8 mg/kg/min (*p=0.04*). At low dose SFLLRN there were no significant differences between groups. Following stimulation with 20 μM

SFLLRN, those with eGDR <6 mg/kg/min had a significantly higher MFI, 19338±11749 than those with both eGDR 6-8 mg/kg/min (7287±2004, *p=0.03*) and those with the highest eGDR (5187±2872, *p=0.02*). At low and higher dose CRP-XL, no significant differences between the 3 groups were found. Following dual agonist stimulation, patients with the lowest eGDR had higher MFI PAC1 expression (21184±11312) than both patients with eGDR 6-8 mg/kg/min (8705±2755, *p=0.03*) and eGDR ≥8 mg/kg/min (7010±2456, p=*0.02*). Figure 4-18 represents both percent positive and MFI.
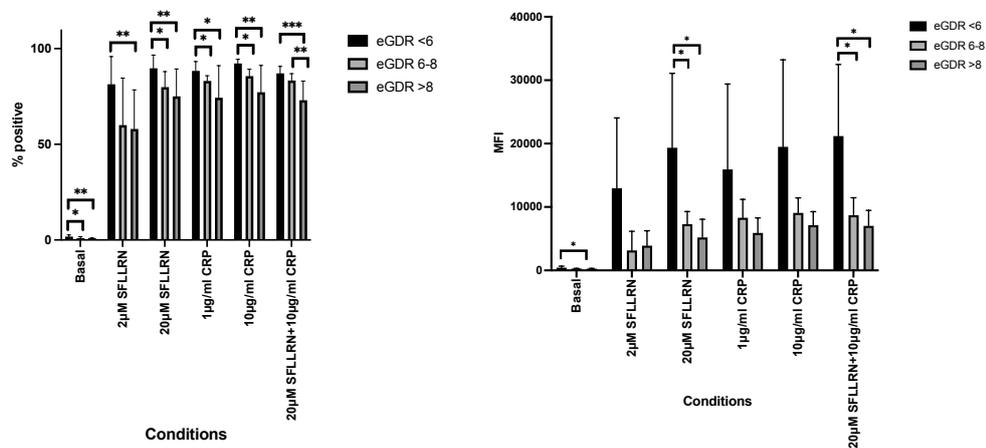
Figure 4-18: Fluorescent flow cytometry to investigate platelet expression of PAC1 basally and in response to agonists in whole blood from study patients with T1D stratified according to the eGDR.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity data. *p<0.05, **p<0.01 and ***p<0.001. <6 mg/kg/min n=8, 6-8 mg/kg/min n=9 and eGDR ≥8 mg/kg/min and n=15.

4.3.4.5 The relationship between GV and active $\alpha_{IIb}\beta_3$ expression

GV was again split according to the median value of 44%. N=11 for GV $\geq$44% and n=11 for those with GV <44%.

Figure 4-19 represents the data both by percent positive and MFI. Basally, those with the lower GV had a higher percent positive PAC1 expression though this was not significant. With both low and high SFLLRN dose stimulation, there was a large variation with no significant differences observed between the two groups. The same was seen at low dose CRP-XL. With 10 μg/ml CRP-XL, a trend for a difference was demonstrated *(p=0.08)*. With dual agonist stimulation, the same trend was seen (*p=0.054)*.

Comparable results were seen when analysing the data using MFI. There were no clear differences observed between the groups under basal conditions, nor with single agonist or dual agonist stimulation, again this is shown in Figure 4-19.
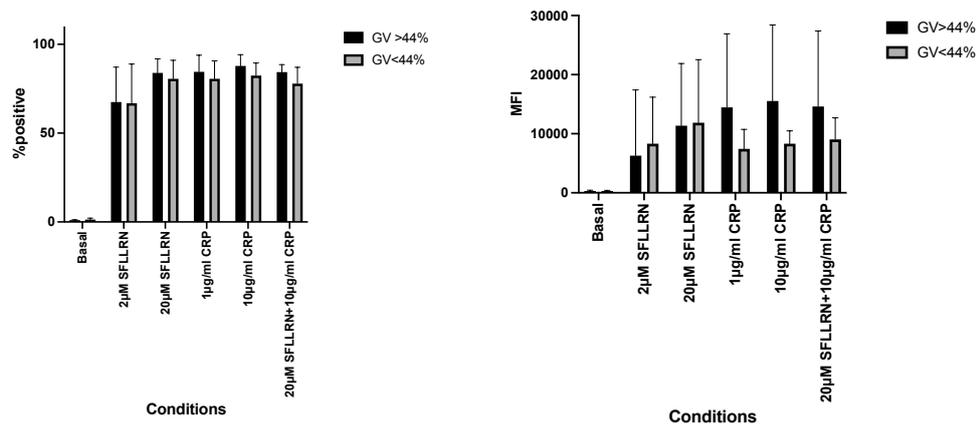
Figure 4-19: Fluorescent flow cytometry to investigate platelet expression of PAC1 basally and in response to agonists in whole blood from study patients with T1D stratified according to median GV.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity data. GV $\geq$44% n=11 and GV <44% n=11.

4.3.4.6 The relationship between hypoglycaemia and active $\alpha_{IIb}\beta_3$ expression

Results were then stratified according to time below range (TBR), referring to values below 3.9mmol/l. There was a total n=23. Again, the results were stratified according to the median value for TBR which was 9%; n=11 for $\geq$9% and n=12 for those with TBR of <9%.

Percent positive PAC1 expression demonstrated a trend towards a higher expression in those who had higher hypoglycaemic exposure but none of these differences reached statistical significance (Figure 4-20).

When analysing the data using MFI, under basal conditions those with higher hypoglycaemic exposure had a significantly higher PAC1 expression than those with lower TBR (311$\pm$117 and 176$\pm$97 respectively, *p=0.01*). A similar pattern was seen at the low and high dose single agonists for both SFLLRN and CRP-XL, however none of these reached statistical significance. Following stimulation with dual agonists, those with higher hypoglycaemic exposure had significantly greater PAC1 expression, 12865$\pm$4758 versus 7747$\pm$2893, *p=0.01*, again shown in Figure 4-20.

Figure 4-20: Fluorescent flow cytometry to investigate platelet expression of PAC1 basally and in response to agonists in whole blood from study patients with T1D stratified according to median TBR.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity data. *p<0.05. TBR ≥9% n=12 and TBR <9% n=11.

4.3.5 Measurement of platelet Annexin V expression in patients with T1D

The final marker of interest was phosphatidylserine, measured through Annexin-V (AnnV) binding, also referred to as AnnV expression, as previously explained in chapters 1 and 2. Data from 26 study patient samples were included in this analysis with data excluded from n=8 patients due to a faulty fluorophore which was subsequently replaced. Figure 4-21 is representative of the results from the full data set. As previously explained, this differs from CD62P and PAC1 as there is a bimodal peak following activation and thus the mean fluorescence intensity is more representative and thus MFI refers to mean rather than median in the following sections. As anticipated from the optimisation data, the percent positive and MFI data are considerably lower than both PAC1 and CD62P expression, particularly under basal conditions and with single agonist stimulation.
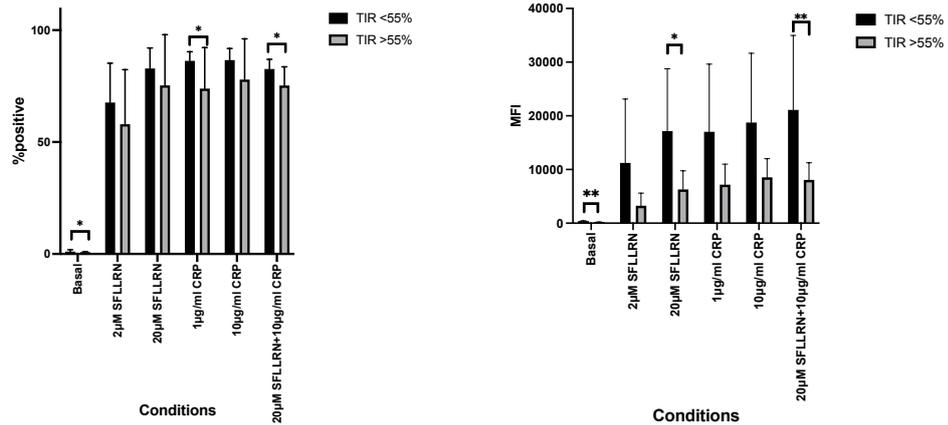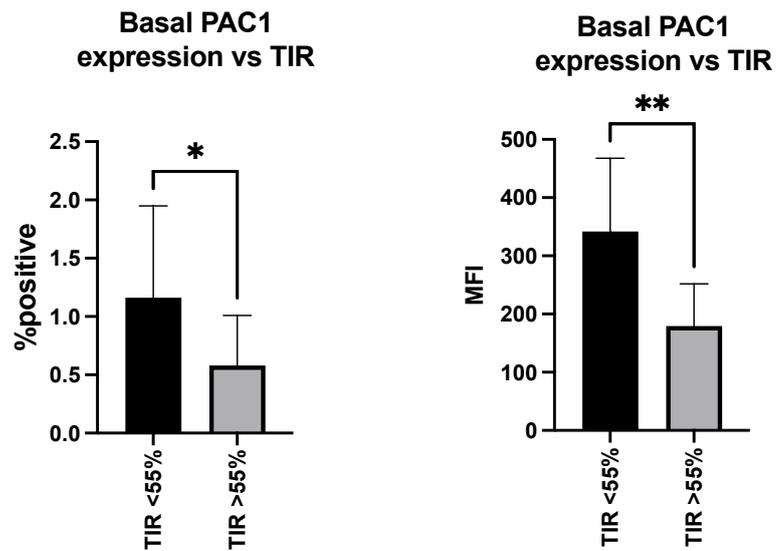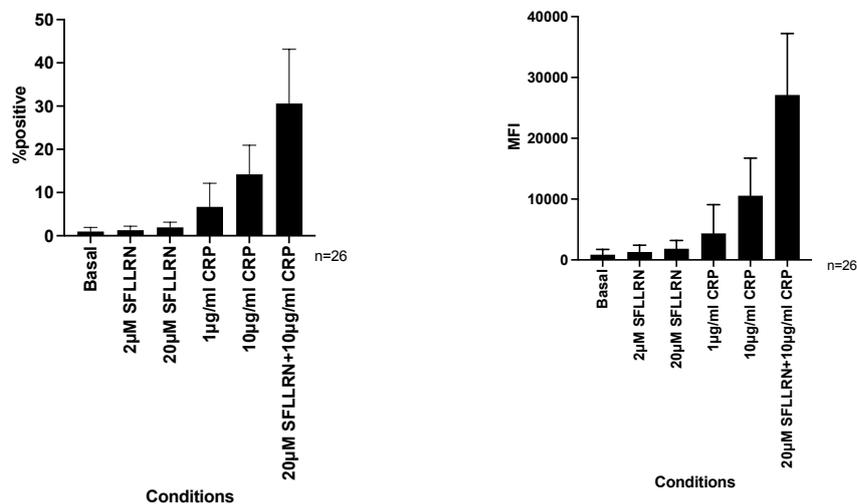

Figure 4-21: Four-colour fluorescent flow cytometry activation panel to investigate platelet expression of AnnV basally and in response to single/dual agonists in whole blood from study patients with T1D.

Left panel represents AnnV percent positive expression and the right panel represents the comparative mean fluorescence intensity (MFI) data. n=26.

4.3.5.1 The relationship between HbA1c and Phosphatidylserine expression

Data (n=26) were separated according to the median HbA1c, 64 mmol/mol. 13 patients had HbA1c $\geq$64mmol/mol and 13 patients had HbA1c <64mmol/l. Data from the whole activation panel are represented by percent positive and MFI, with results shown in Figure 4-22, with further focus on basal expression in Figure 4-23.

Whilst percent positive expression in both groups was low for AnnV, there was a higher expression in those with elevated HbA1c, 1.3$\pm$0.9% and 0.5$\pm$0.3% respectively, *p=0.02*. Following stimulation with single agonists, both low or high dose SFLLRN and CRP-XL, there were no differences between the two groups. As expected, with dual agonist stimulation, the percent positive expression of AnnV was higher and a difference more discernible. Those with HbA1c $\geq$64 mmol/mol had greater AnnV expression, 35$\pm$11% compared with patients who had HbA1c <64 mmol/mol, 24$\pm$6.0% *p=0.006*.

Similar results were seen when analysing AnnV expression by MFI. Under basal conditions, patients with HbA1c $\geq$64 mmol/mol demonstrated higher expression, 938$\pm$746 compared with 331$\pm$108 in those with HbA1c <64 mmol/mol, *p=0.007*. Again, following stimulation with single agonist, either at low and high dose there were no clear differences in AnnV expression between the two groups. With dual agonist stimulation, those with higher HbA1c had a significantly higher expression of AnnV than those with the lower HbA1c, 31025$\pm$9471 versus 22509$\pm$8530, *p=0.02*. Data are shown below in Figures 4-22 and 4-23.
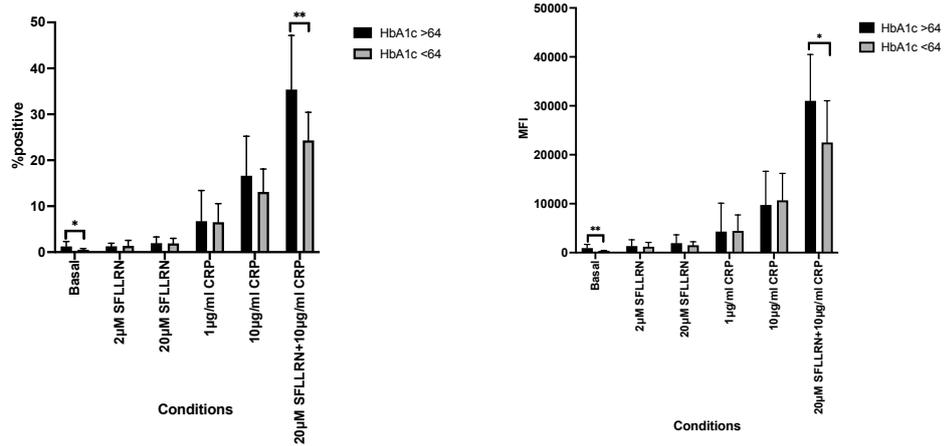
Figure 4-22: Four-colour fluorescent flow cytometry activation panel to investigate platelet expression of PS under basal conditions and in response to single/dual agonists in whole blood from study patients with T1D, stratified according to median HbA1c.

Left panel represents AnnV percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. *p<0.05 and **p<0.01. HbA1c ≥64 mmol/mol n=13 and HbA1c <64 mmol/mol n=13.
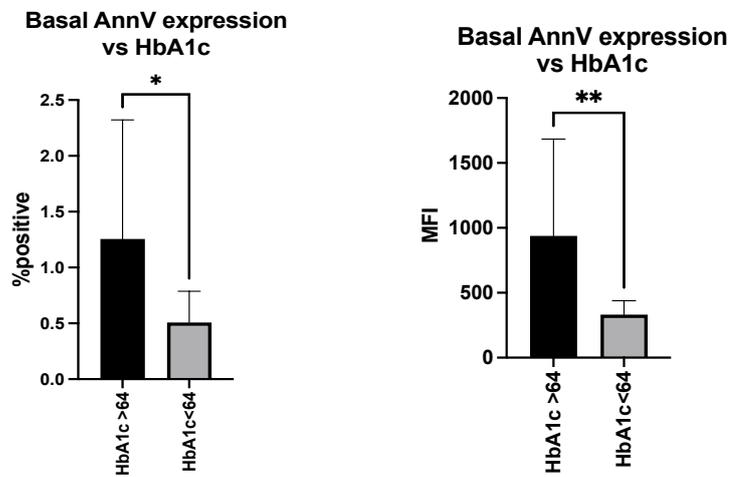
Figure 4-23: Four-colour fluorescent flow cytometry activation panel to investigate platelet expression of PS basally in whole blood from study patients with T1D, stratified according to median HbA1c.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. *p<0.05 and **p<0.01. HbA1c ≥64 mmol/mol n=13 and HbA1c <64mmol/mol n=13.

4.3.5.2 The relationship between Time-in-Range and

Phosphatidylserine expression

As per the previous markers, the results were then stratified according to time in range (TIR). The median TIR value which was 53% for those with available AnnV data. Again, the total number of samples included in the TIR analysis was lower and n=19 for this analysis (TIR<53% n= 10, TIR $\geq$53%, n=9). Figure 4-24 represents the full panel and Figure 4-25 once again provides focus on the basal results.

Basally, those with lower TIR had a significantly higher percent positive AnnV expression than patients with a higher TIR, 1.6$\pm$1% compared with 0.6$\pm$0.2%, p=0.02, although again both groups had low expression. Again, with single agonists, there was only a minimal increase in expression and no clear differences between the groups. However, following activation with dual agonists, those with the lower TIR were shown to have a higher AnnV expression, 32$\pm$10%, compared to those with a higher TIR, 22$\pm$5%, *p=0.01*.

The comparative MFI data showed a similar trend. Basally, patients with TIR <53% had statistically higher AnnV expression, 840$\pm$454 compared with those with TIR $\geq$53%, 394$\pm$232, *p=0.01*. With SFLLRN activation alone, no differences were seen between the two groups and whilst there was a trend towards higher AnnV expression in those with TIR <53% following stimulation with both low and high dose CRP-XL, the differences were non-significant. Finally with dual agonist stimulation, patients with lower TIR had an increased AnnV expression compared to those with higher TIR, 31235$\pm$7743 and 20604$\pm$7858 respectively, *p=0.009*. These data are shown below in Figures 4-24 and 4-25.
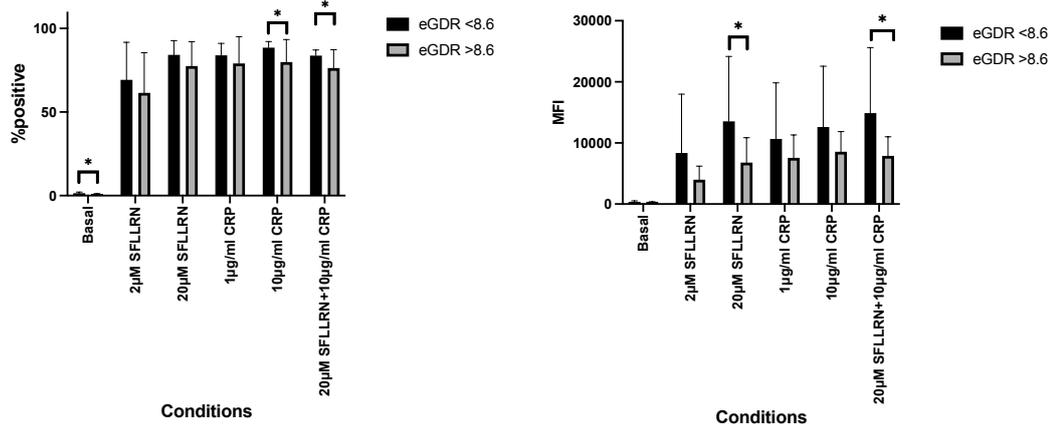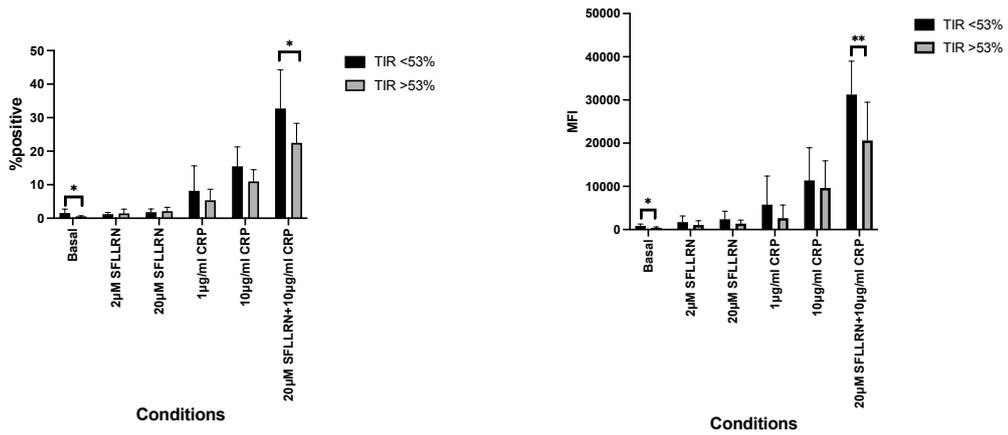
Figure 4-24: Fluorescent flow cytometry activation panel to investigate platelet expression of AnnV basally and in response to single/dual agonists, in whole blood from study patients with T1D, stratified according to median TIR.

Left panel represents AnnV percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. *p<0.05 and **p<0.01. TIR <53% n=10 and TIR ≥53% n=9.
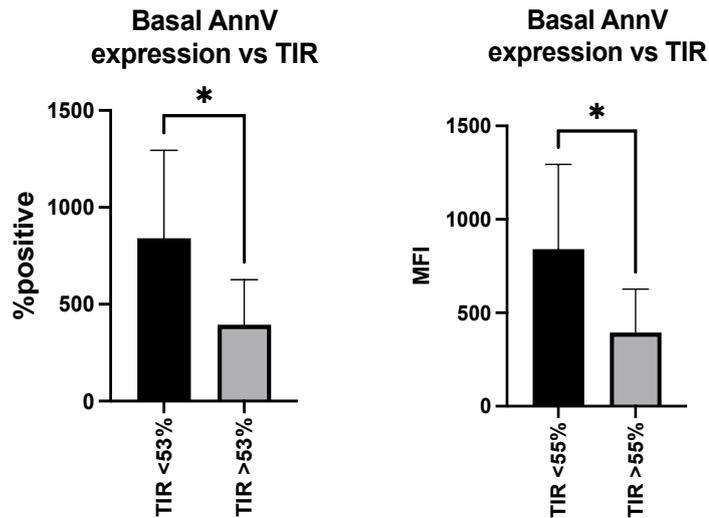
Figure 4-25: Four-colour fluorescent flow cytometry activation panel to investigate platelet expression of AnnV basally in whole blood from study patients with T1D, stratified according to median TIR.

Left panel represents PAC1 percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. *p<0.05. TIR <53% n=10 and TIR ≥53% n=9.

4.3.5.3 The relationship between plasma glucose and

Phosphatidylserine expression

The next parameter by which the data were stratified was plasma glucose at the time of sampling. Total n=24 patients had both a plasma glucose level and AnnV binding data available, with median plasma glucose of 8 mmol/l, thus n=12 in each group. Figure 4-26 represents AnnV expression analysed both by percent positive and MFI.

For percent positive expression, there were no differences between the groups seen basally and with single agonist stimulation. Following dual agonist stimulation, those with the higher plasma glucose showed a trend towards higher AnnV expression (29±11% versus 25±5%, *p=0.07*).

While a similar pattern was observed for MFI, none of the differences showed a significance or a trend (p>0.1 for all).
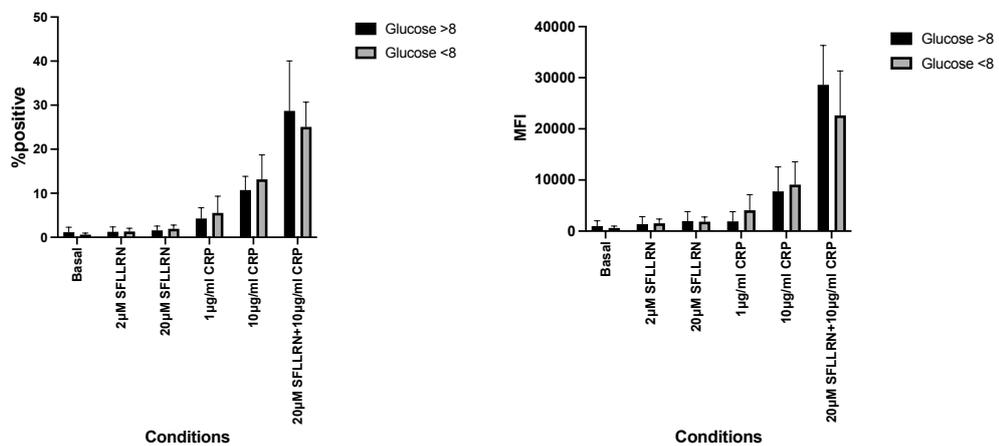
Figure 4-26: Fluorescent flow cytometry to investigate platelet expression of AnnV under basal conditions and in response to agonists in whole blood from study patients with T1D stratified according to median plasma glucose at time of sampling.

Left panel represents AnnV percent positive expression and the right panel represents the comparative median fluorescence intensity data. Glucose ≥8mmol/l n=12 and < mmol/l n=12.

4.3.5.4 The relationship between eGDR and Phosphatidylserine expression

Again, eGDR was the next parameter by which the results were stratified in the same way as the previous two markers. As all patients had an eGDR calculated, the groups were split as n=13 with eGDR <8.6 mg/kg/min and n=13 eGDR ≥8.6 mg/kg/min. When analysed in the 3 categories, the group sizes were n=7 for eGDR <6 mg/kg/min, n=8 for eGDR 6-8 mg/kg/min and n=11 for those with eGDR ≥8 mg/kg/min.

With data split according to median eGDR, patients with the lower eGDR had an increased AnnV expression under basal conditions, compared to higher eGDR ($1.4\pm1.1\%$ and $0.62\pm0.4\%$, respectively, *p=0.04*). No clear differences were seen following stimulation with single agonists (either SFLLRN or CRP-XL) at both low and high doses. With dual agonist stimulation, those with the lower eGDR had a higher AnnV expression, $35\pm12\%$ versus $23\pm5\%$, *p=0.005*.

The comparative MFI data followed a similar pattern with increased basal AnnV expression in those with low compared with high eGDR (935 ± 753 and 340 ± 183, respectively, *p=0.009*). There were no discernible differences between the groups following stimulation with either single agonist at neither low nor high doses. Following dual agonist stimulation, the numerical difference failed to reach statistical significance (27980 ± 8349 and 24384 ± 8870, *p=0.13*; Figure 4-27).
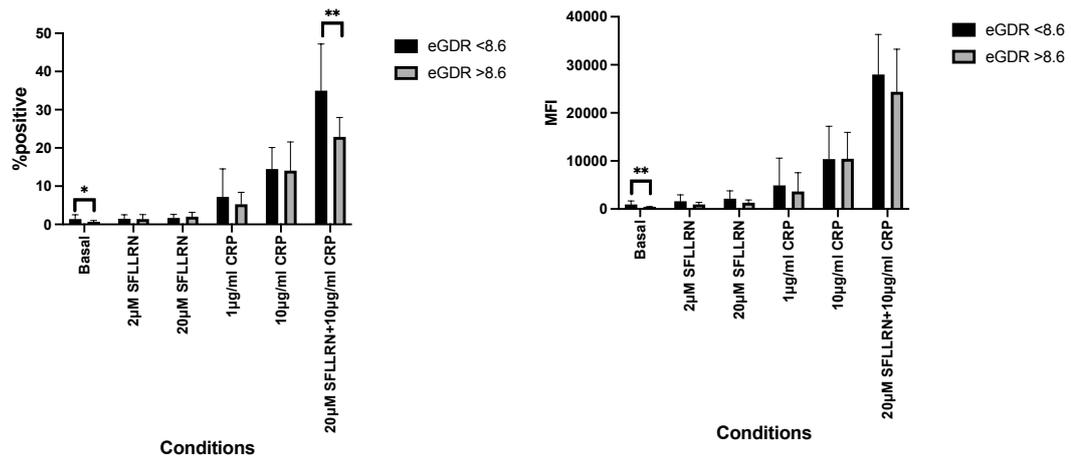
Figure 4-27: Fluorescent flow cytometry activation panel to investigate platelet expression of AnnV under basal conditions and in response to single/dual agonists, in whole blood from study patients with T1D, stratified according to median eGDR.

Left panel represents AnnV percent positive expression and the right panel represents the comparative median fluorescence intensity (MFI) data. *p<0.05. eGDR <8.6 n=13 and eGDR≥8.6 n=13.

With the groups split into 3 categories, basally, there was highest percent positive AnnV expression in those with the lowest eGDR (<6 mg/kg/min), 2.0±0.9%, which was significantly higher than those with eGDR 6-8 mg/kg/min, 0.6±0.3% and eGDR ≥8 mg/kg/min, 0.66±0.4%, *p=0.004* and *p=0.007,* respectively. There were no clear differences between any groups following stimulation with either agonist, SFLLRN/CRP-XL, alone. Stimulation with dual agonists demonstrated significantly higher AnnV expression in those with eGDR <6 mg/kg/min, 34±5%, than both patients with eGDR 6-8 mg/kg/min, 27±4% and eGDR ≥8mg/kg/min, 23±5%, *p=0.03* and *p=0.001* respectively.

As shown in Figure 4-28, these findings were mirrored by the MFI data, with mean basal AnnV expression highest in those with eGDR <6 mg/kg/min,

1139±447, (eGDR 6-8 mg/kg/min 480±195 and eGDR ≥8 mg/kg/min 341±183, *p=0.003* and *p=0.007* respectively). Again, the differences seen between all the groups following stimulation with either agonist alone were non-significant. Finally, following dual agonist stimulation, patients in the lowest eGDR group had the highest AnnV expression, 33966±7163 (eGDR 6-8 mg/kg/min 22575±3242 and eGDR ≥8 mg/kg/min 21829±6048, *p=0.005* and *p=0.004* respectively).
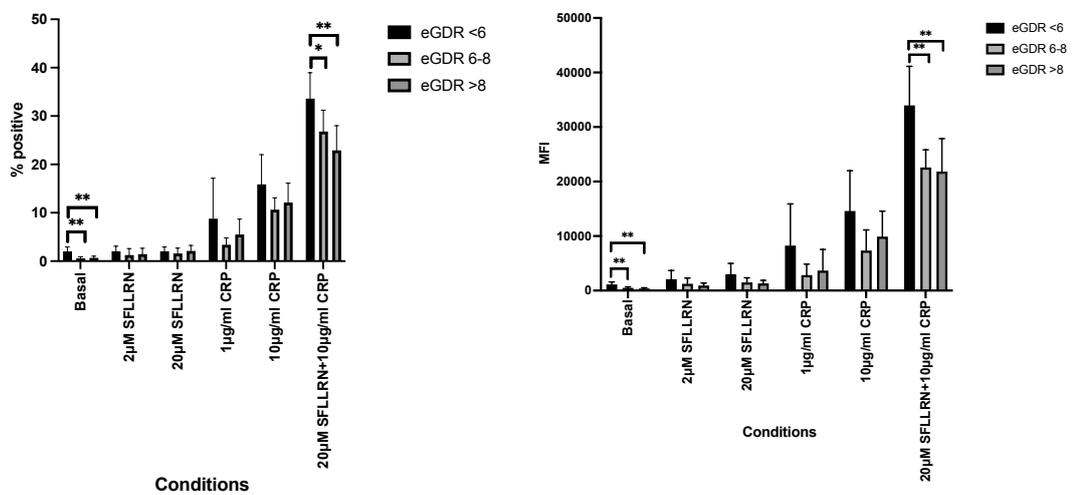
Figure 4-28: Fluorescent flow cytometry to investigate platelet expression of AnnV basally and in response to agonists in whole blood from study patients with T1D stratified according to median eGDR.

Left panel represents CD62P percent positive expression and the right panel represents the comparative median fluorescence intensity data. *p<0.05 and **p<0.01. eGDR <6 mg/kg/min n=7, 6-8 mg/kg/min n=7 and eGDR ≥8 mg/kg/min n=11.

4.3.5.5 The relationship between GV and Phosphatidylserine expression

Next, data were stratified according to median GV, 44%. Total n=19, with GV ≥44% n=10 and GV<44% n=9.

Figure 4-29 represents the data both by percent positive and MFI. In both the case of percent positive expression and MFI, there were no clear differences identified between the two groups, under basal conditions, following stimulation with single agonists (low or high concentration) and also with dual agonist stimulation.
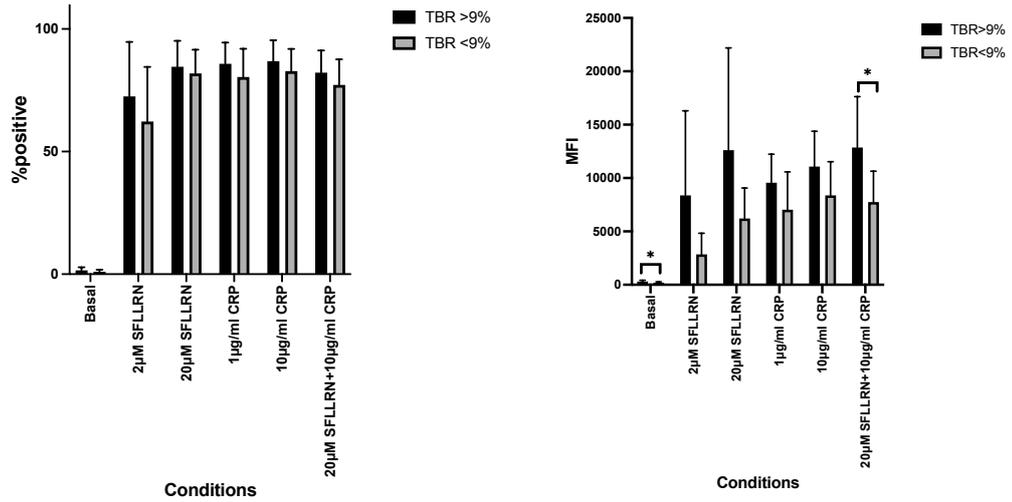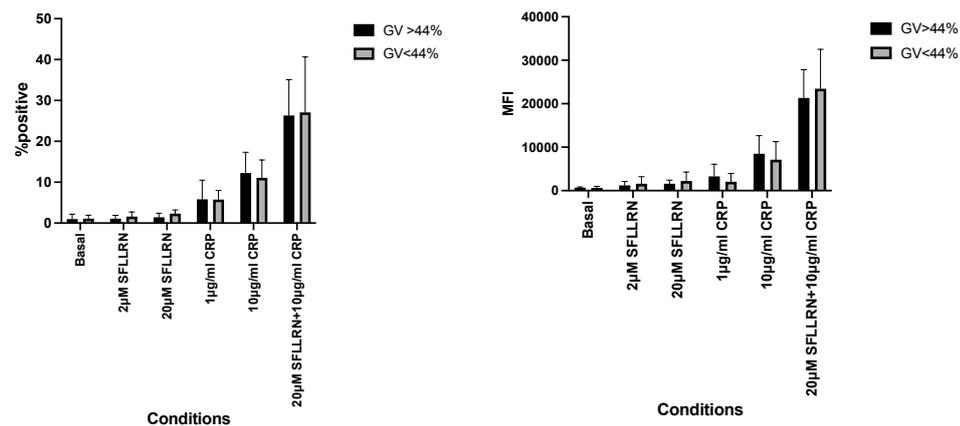

Figure 4-29: Fluorescent flow cytometry to investigate platelet expression of AnnV basally and in response to agonists in whole blood from study patients with T1D stratified according to median GV.

Left panel represents AnnV percent positive expression and the right panel represents the comparative median fluorescence intensity data. GV ≥44% n=10 and GV <44% n=9.

4.3.5.6 The relationship between hypoglycaemia and

Phosphatidylserine expression

Next, data were stratified according to hypoglycaemia, using time below range (TBR). There was a total n=19 with the groups determined by the median value for TBR which was 9%; n=10 for ≥9% and n=9 for those with TBR of <9%. Figure 4-30 represents both percent positive and MFI data.

Hypoglycaemia was not associated with significant changes in platelet activation markers, regardless whether these were studied under basal conditions or after stimulation (Figure 4-30).
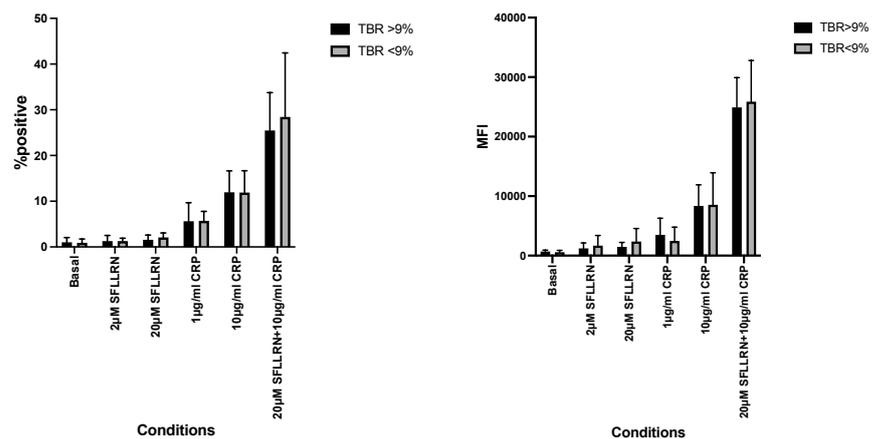
Figure 4-30: Fluorescent flow cytometry to investigate platelet expression of AnnV basally and in response to agonists in whole blood from study patients with T1D stratified according to median TBR.

Left panel represents AnnV percent positive expression and the right panel represents the comparative median fluorescence intensity data. TBR ≥9% n=10 and TBR <9% n=9.

4.4 Discussion

Platelet hyperreactivity is a key mechanism contributing to the development of cardiovascular complications in patients with diabetes [48, 49, 317]. Minimising cardiovascular risk in patients with T1D currently largely relies upon addressing the individual risk factors such as glycaemic control, hypertension and dyslipidaemia. Understanding the effects of hyperglycaemia and insulin resistance on platelet function in patients with T1D is therefore crucial to further inform this management. Whilst much of the published data have demonstrated elevated markers of platelet activation in patients with T2D or indeed mouse models, data in individuals with T1D are both limited and conflicting. This study is the first to evaluate the relationship between several important clinical parameters and platelet function in patients with T1D and is also the first to assess platelet activity in patients characterised with DD. Each of the individual markers investigated in this study evaluate different aspects of platelet activity including α-granule secretion, fibrinogen binding and procoagulant properties. Whilst previous data have shown elevated levels of each of the markers; P-selectin [320], active $\alpha_{IIb}\beta_3$ [321] and PS [322] in patients with diabetes compared with healthy controls, the impact of glycaemia and insulin resistance have not been properly explored.

We have shown that both short-term (measured by TIR over a 14-day period) and medium-term (measured by HbA1c) hyperglycaemia was associated with hyperreactivity, with elevated levels of all 3 markers shown in the cohort of patients with the higher HbA1c and lower TIR. Crucially, these differences were evident basally, indicating that in patients with sub-optimal glycaemic control platelets circulate in a partly activated rather than a truly quiescent

state. It should be stressed that patients included in this study are young (median age 24 years), without clinical macrovascular disease and therefore the platelet-driven prothrombotic milieu takes place early in the disease process, increasing vascular risk.

There were differential responses to agonist stimulation across the three markers but with a general trend towards increased expression in those with higher HbA1c and lower TIR; P-selectin expression was elevated in almost all conditions investigated, whether low or high single dose agonist stimulation or with dual agonists. Equally, active $\alpha_{IIb}\beta_3$ expression was higher with both high dose single and dual agonist stimulation in those with poorer glycaemic control. Differences in PS expression were typically only evident following dual agonist stimulation, as previously explained, but also demonstrated that those with the higher HbA1c and lower TIR had increase PS expression. These data indicate that platelets in patients with T1D with higher hyperglycaemic exposure have an increased propensity for activation response to stimuli. They have increased activation and a higher propensity towards procoagulant activity. It remains unclear whether optimising glycaemia reverses this prothrombotic environment and if so, the length of time needed for platelet function to "normalise".

In addition to glycaemia, patients with low eGDR (indicative of a higher insulin resistance) also had increased platelet activation both basally and following stimulation, across all three markers investigated. This was more evident when data were stratified in line with the landmark Nyström *et al.,* thresholds for cardiovascular risk, where those with the lowest eGDR (<6mg/kg/min) demonstrated the highest levels of all 3 markers. Once again, this has

significant implications for the risk of developing cardiovascular disease and addressing insulin resistance in this cohort must not be ignored. This constitutes a departure from current practice that takes a glycaemic focus in younger individuals with T1D. Currently, eGDR is not routinely incorporated into routine clinical practice in the UK but could be an important tool in assessing each patient's risk and informing decisions around adjunctive therapies including metformin [323]. As Table 4-1 outlined, only 2 (6%) patients from the full cohort were on metformin therapy. Such a strategy may help to identify those with insulin resistance sooner and facilitate earlier intervention. Given the data from this study suggest platelet hyperreactivity is evident even in these young patients, a 'one size fits all' approach e.g., age thresholds, to prompt intervention is unlikely to prove beneficial.

Further to the above, stratification by other clinical parameters GV, TBR and plasma glucose demonstrated no clear differences. These analyses may have been limited by the comparably lower numbers and thus more conclusive data with a longer duration and larger sample size would be required to draw more definitive conclusions. GV may not give an accurate indication of glycaemic control and in clinical practice is rarely used alone but rather in conjunction with the other glycaemic parameters. Furthermore, plasma glucose at the time of sampling provides a limited assessment of overall glycaemia, given it is a transient measurement at a specific time point of a potentially rapidly changing value. Whilst at the extreme ends of plasma glucose, differences may be seen, the vast majority of measurements were fairly similar and thus identifying any meaningful effects of transient plasma glucose on platelet activity is unlikely to result in any clear results. This may support the concept that changes in platelet function may occur over a longer-term exposure to hyperglycaemia,

potentially driving metabolic reprogramming towards hyperreactive and procoagulant platelets.

Again, limitations of these data are important to discuss. Firstly, assessing the impact of smoking status and other potential factors that could impact platelet function may be important particularly with a larger cohort of patients. Another aspect to explore would be the reliability of plasma glucose and the potential impact it would have at a particular timepoint. Undoubtedly this would be crucial to truly establish whether very short-term changes in glucose could affect glucose and a longitudinal study inducing different glucose levels would be required to do this.

Platelet dysfunction and hyperreactivity are undeniably multi-factorial, with far wider contributing factors than have been explored in this study. However, these data demonstrate that in these young adult patients with T1D, sub-optimal glycaemic control contributes to increased platelet activity both basally and with increased capacity to respond to stimuli. Furthermore, my data suggest that in young adults, DD has an additive effect and tackling IR must be at the forefront of a more holistic and individualised management of patients with T1D. As explored in chapter 1, anti-platelet therapies are not currently routinely offered as primary prevention for patients with diabetes in the UK. Given the evidence of platelet dysfunction even in these young patients with few, if any, co-morbidities this area must be addressed. Whilst clearly there are benefits of reducing hyperglycaemic exposure, increasing time in range and also tackling insulin resistance, it is unlikely that this alone will mitigate the risk. The outright benefits of anti-platelet therapies along with specific dosing regimens are equally an area of debate amongst both

diabetologists and cardiologists, with some evidence to suggest aspirin resistance as previously discussed. Understanding the impact of inhibitors on minimising this platelet hyperactivation in patients with T1D is therefore equally crucial moving forward and aiming to reduce platelet driven mechanisms contributing to cardiovascular morbidity and mortality.

Chapter 5: Sensitivity to inhibition with prostacyclin in T1D and the effects of glycaemia and insulin resistance

5.1 Introduction

Whilst platelet hyperreactivity alone is an important mechanism to understand when exploring cardiovascular risk in patients with diabetes, investigating the potential paradoxical effects of platelet inhibition in this group is also crucial to assess the extent to which platelet dysfunction may contribute to the long-term cardiovascular outcomes.

Much of the data around PGI_2 in patients with diabetes are not particularly contemporary and somewhat conflicting with regards to both the levels of PGI_2 and PGI_2 sensitivity in platelets from patients with diabetes. There is evidence to support reduced levels of both NO and PGI_2 in the setting of hyperglycaemia as a result of elevated TXA_2 [79]. Patients with diabetes have also been shown to have significantly higher TXA_2 to PGI_2 ratio than healthy controls [324]. Expression of platelet prostacyclin receptor may be decreased in patients with T2D, particularly in the setting of poorer glycaemic control and higher HbA1c [325]. Lower response to PGI_2 as determined by reduced platelet aggregation has also been shown in patients with diabetes (combination of patients with T1D and T2D) compared with healthy controls, indicating reduced sensitivity with no clear difference seen between those with T1D and T2D [326]. Further evidence to support this reduced sensitivity to PGI_2 has been reported, including in patients with T2D both with and without existing macrovascular disease [61, 327]. On the contrary, opposing data have shown that there are no clear differences in PGI_2 sensitivity in patients with diabetes and healthy controls [328] with another more recent study suggesting the reduction in PGI_2 sensitivity seen in patients with diabetes may largely be driven by dyslipidaemia rather than diabetes itself [329].

Most of the available evidence for PGI_2 sensitivity in patients with diabetes have relied upon the direct effects measured by platelet aggregation. To our knowledge, whilst conducted by this group in healthy controls [298], exploring the role of such inhibition through functional assays in patients with diabetes has not yet been conducted. As a result, the aim of this chapter was to investigate the effects of PGI_2 on different aspects of platelet activation in young adult patients with T1D and DD, through four-colour flow cytometry using the previously detailed markers P-selectin, $\alpha_{IIb}\beta_3$ and phosphatidylserine.

5.2 Methods

5.2.1 Study Population

The DEVELOP study has been detailed previously and patient data throughout this chapter are derived from the same study patients included in chapter 4, again shown in table 5-1.

Demographic and background data were taken in line with study protocols. Blood samples were taken as per study protocol, including sampling for clinical blood tests and a 1.8ml sodium citrate 3.2% tube specifically for use in the flow cytometry, which has previously been validated by the group [298].

For control data, samples were taken as previously detailed. Samples were again all drawn in a non-fasted state both from patients and volunteers and where possible sampling was from the antecubital fossa using a 21G butterfly needle with vacutainer.

5.2.2 Multiparameter flow cytometry and prostacyclin

The detailed methods used for carrying out a validated flow cytometry assay are described in chapter 2 with optimisation of conditions detailed in chapter 3. The four-colour flow cytometry assay used to assess platelet activation described in chapter 4 is again referred to throughout this chapter thus the same markers were used; CD42b-BB700 (platelet identification marker), CD62P-PE (P-selectin expression), PAC1-FITC (active $\alpha_{IIb}\beta_3$ expression) and AnnexinV-APC (PS expression).

As previously explained, data are represented as both percent positive cells and either Median Fluorescence Intensity (CD62P and PAC1) or Mean Fluorescence Intensity (AnnV binding).

In addition to the same methods for flow cytometry outlined in chapters 2 & 3, an additional step involving the addition of PGI_2 at various concentrations with whole blood for 2 minutes prior to the addition of the relevant agonists was added. The same compensation matrix as detailed in chapters 3&4 was used to account for spectral overlap between fluorophore

5.2.3 Statistical analysis

All data within this chapter were analysed using GraphPad Prism version 9.5.1 (528). For descriptive data, results are presented as median (interquartile range), mean±standard deviation (SD) and/or number (% of total). Where appropriate, normal distribution was assessed using Shapiro-Wilks. One-way ANOVA (Kruskal Wallis) was used to test multiple continuous variables. Two independent continuous variables were assessed using Student t-test or Mann-Whitney test depending on the normative distribution.

5.3 Results

5.3.1 Patient characteristics

Within this section of results, samples from the same patients in chapter 4 were used. We did not formally characterise the healthy controls but they were age and sex matched. The total number of samples from patients included from the wider DEVELOP study is therefore 34. Again, data from one further patient were excluded from all analysis due to pregnancy and thus met exclusion criteria (unaware pregnant at time of sampling). Any further data were excluded if they were clear outliers as per statistical analysis through PRISM and this has been detailed in the relevant section. Table 5-1 again summarises the characteristics of all study patients included in analyses throughout this chapter.

	Total	eGDR <8.6	eGDR ≥8.6	p-value
Number of participants	34	17	17	-
Male sex (%)	20 (59%)	10 (59%)	10 (59%)	>0.99
Age, years	24.0±3.8	23.4±3.3	24.4±4.2	0.44
Duration of diabetes, years	14.0±6.3	12.8±6.6	14.8±6.1	0.37
HbA1c, mmol/mol	65.3±14.0	73.5±12.7	57.6±10.6	**0.0006**
BMI, kg/m^2	27.6±5.6	30.1±6.7	25.3±3.2	**0.014**
Estimated glucose disposal rate (WC), mg/kg/min	8.1±2.1	6.4±1.5	9.7±0.8	**<0.0001**
Total daily insulin (U/24h)	66.0±25.7	80.0±29.0***	50.5±8.7	**0.0007**
Total daily insulin (U/kg)	0.79±0.25	0.91±0.30**	0.68±0.13	**0.008**
Family history of T2DM	4 (12%)	3 (18%)	1 (6%)	0.60
Presence of retinopathy	14 (41%)	6 (35%)	7 (41%)	>0.99
Adjunctive therapy (metformin)	2 (6%)	2 (12%)	0	0.48
Macrovascular complications	0	0	0	-

Table 5-1: Summary of DEVELOP study patient characteristics from whom samples were included in analysis.

Total cohort and comparison of those stratified according to median eGDR, 8.6mg/kg/min. Statistical analysis to compare patients with eGDR <8.6mg/kg/min and those with eGDR ≥8.6mg/kg/min. Data are representative of mean value±standard deviation excluding age which is median±standard deviation.

5.3.2 Platelet response to prostacyclin on multiparameter flow cytometry in patients with T1D

Having determined the response of each of the different markers of platelet activation in response to varying doses of PGI_2, detailed in chapter 3, it was determined that two doses of PGI_2 (10 nM and 100 nM) would be included for each of the agonist strengths previously shown in the four-colour activation assay. These were chosen as 1 nM PGI_2 showed a non-significant response at either strength agonist and 1000 nM PGI_2 induced near maximal inhibition for both PAC1 and AnnV expression, thus may make it more difficult to detect differences in sensitivity as there may be also complete inhibition in both groups. Given these data have not previously been studied in patients with T1D the results were first compared as a full cohort of T1D (n=26-32) against healthy controls (n=8) before being stratified according to HbA1c and eGDR to assess for any potential differences as a result of these important metabolic factors.

5.3.2.1 P-selectin; response to prostacyclin inhibition

The first marker assessed was P-selectin, represented by CD62P expression first by percent positive followed by MFI. This was analysed at each of the agonist doses and presented as the full data and then a comparison made as percentage reduction from expression with the agonist alone. Statistical comparison was made between the percentage reduction at each PGI_2 dose comparing response in patients with T1D and healthy controls.

First, shown in panel A of Figure 5-1, is percent positive expression following stimulation with 2μM SFLLRN. The left panel shows CD62P expression under basal conditions and following stimulation in the presence or absence of PGI$_2$. Expression was higher under each of the following conditions in those with T1D; basal (8.6±2% compared with 2.9±0.35%, *p<0.0001*), at 2 μM SFLLRN (80±9% versus 64±7%, p=*0.0012*), at 10 nM PGI$_2$ (9.6±9% versus 5±2%, *p=0.02*) and 100 nM PGI$_2$ 8±7% versus 4 0.6%, *p=0.01*). However, as shown in panel B of Figure 5-1, the percentage reduction in expression in the presence of PGI$_2$ at either dose were almost identical when comparing those with T1D and healthy controls.

The same data were then analysed by MFI CD62P expression and is shown in panels C&D of Figure 5-1. Basal data showed those with T1D had a higher mean expression, 541±241 versus 345±64, *p=0.0012*. There was not a significant difference at 2 μM SFLLRN but in the presence of both 10 nM PGI$_2$ (3676±4780 versus 915±464) and 100 nM PGI$_2$ (2397±3737 versus 481±203) differences were both significant, *p=0.006* and *p=0.01* respectively. In terms of percentage reduction from expression at 2μM SFLLRN shown in panel D of Figure 5-1, there was similar pattern to that described for percent positive expression however with the more sensitive MFI scale, there appeared a greater reduction in CD62P expression in the controls than T1D both at 10 nM PGI$_2$ (92±4.0% versus 77±27%, *p=0.01*) and 100 nM PGI$_2$ (95±2.0% versus 84±19%, *p=0.008*), though of note is the much greater variability with wide standard deviation in the T1D cohort.

Figure 5-1: Multiparameter flow cytometry CD62P expression at basal, stimulated with 2uM SFLLRN with and without PGI₂ (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive CD62P expression and panel B as percentage reduction comparative to expression at 2 μM SFLLRN alone. Data presented in panel C as total MFI CD62P expression and panel D as MFI percentage reduction comparative to expression at 2 μM SFLLRN alone. Statistical analysis refers to T1D versus controls. *p<0.05, **p<0.01 and ****p<0.0001. For T1D n=31 and n=8 for healthy volunteers.

Next, the effects of PGI_2 at 20 μM SFLLRN were examined. In terms of percent positive expression, basal differences have already been described. There were no differences seen with 20 μM SFLLRN alone, with likely maximal percent positive expression in both groups. Only in the presence of 100 nM PGI_2 was there a significant difference between the two groups, with higher expression in those with T1D, 61±21% versus 46±14% in controls, *p=0.04* as shown in panel A of Figure 5-2. In terms of percentage reduction, shown in panel B of Figure 5-2, there appeared to be a greater reduction in the controls compared with the patients with T1D however the differences were non-significant.

The comparative data for MFI expression demonstrated similar results to those described for percent positive. Only in the presence of 10 nM PGI_2 was there a significant difference with higher expression in those with T1D, 18999±9756 versus 13951±3841 in controls, *p=0.049* shown in panel C of Figure 5-2. Again, although there appeared to be a greater percentage reduction in the controls compared to those with T1D neither were significantly different, likely owing to the large amount of variation and wide standard deviation shown in panel D of Figure 5-2.

Figure 5-2: Multiparameter flow cytometry CD62P expression at basal, stimulated with 20 μM SFLLRN with and without PGI2 (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive CD62P expression and panel B as percentage reduction comparative to expression at 20 μM SFLLRN alone. Data presented in panel C as total MFI CD62P expression and panel D as MFI percentage reduction comparative to expression at 20 μM SFLLRN alone. Statistical analysis refers to T1D versus controls. *p<0.05, **p<0.01 and ****p<0.0001. For T1D n=31 and n=8 for healthy volunteers.

At both 1 μg/ml CRP-XL and 10 μg/ml CRP-XL, for both percent positive and MFI CD62P expression, there were no clear differences between those with T1D and the healthy controls in the presence of either dose of PGI2. Relatively low inhibition was seen in line with the earlier optimisation experiment data. Particularly when analysing MFI expression, there was wide variation evident within each category. These data are represented in Figures 5-3 and 5-4.
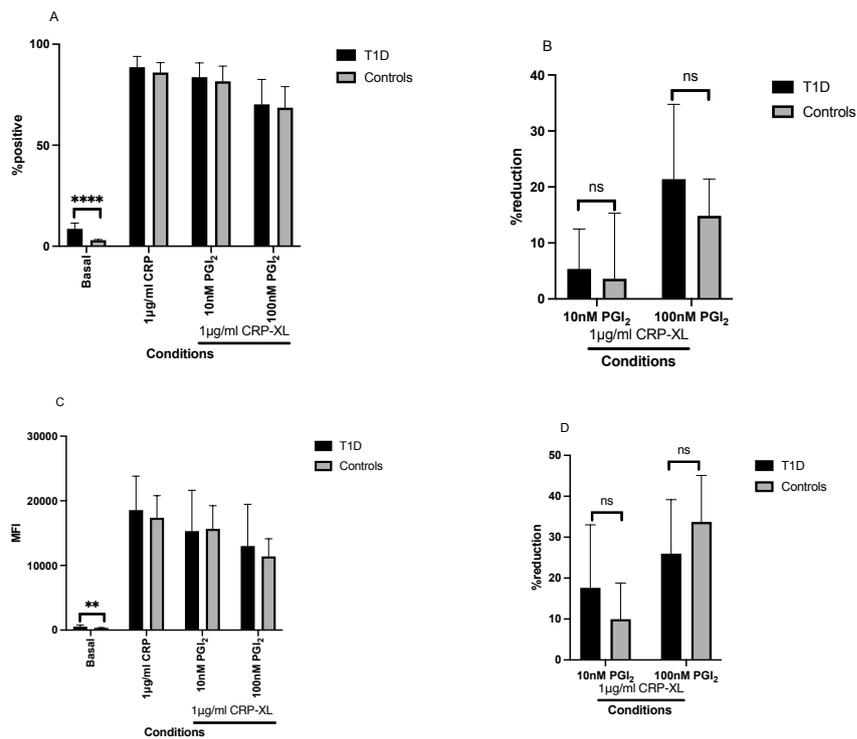
Figure 5-3: Multiparameter flow cytometry CD62P expression at basal, stimulated with 1 μg/ml CRP-XL with and without PGI2 (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive CD62P expression and panel B as percentage reduction comparative to expression at 1 μg/ml CRP-XL alone. Data presented in panel C as total MFI CD62P expression and panel D as MFI percentage reduction comparative to expression at 1 μg/ml CRP-XL alone. Statistical analysis refers to T1D versus controls. **p<0.01 and ****p<0.0001. For T1D n=31 and n=8 for healthy volunteers.

Figure 5-4: Multiparameter flow cytometry CD62P expression at basal, stimulated with 10 μg/ml CRP-XL with and without PGI2 (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive CD62P expression and panel B as percentage reduction comparative to expression at 10 μg/ml CRP-XL alone. Data presented in panel C as total MFI CD62P expression and panel D as MFI percentage reduction comparative to expression at 10 μg/ml CRP-XL alone. Statistical analysis refers to T1D versus controls. **p<0.01 and ****p<0.0001For T1D n=31 and n=8 for healthy volunteers.

Finally, for CD62P expression, the same data were analysed following stimulation with dual agonists with both doses of PGI$_2$. The results again showed minimal inhibition regardless of analysis by MFI or percent positive with no discernible differences between those with T1D and the controls at either dose of PGI$_2$. These data are represented in Figures 5-5 as both overall expression (percent positive and MFI) and percentage reduction comparative to the expression with agonist alone.
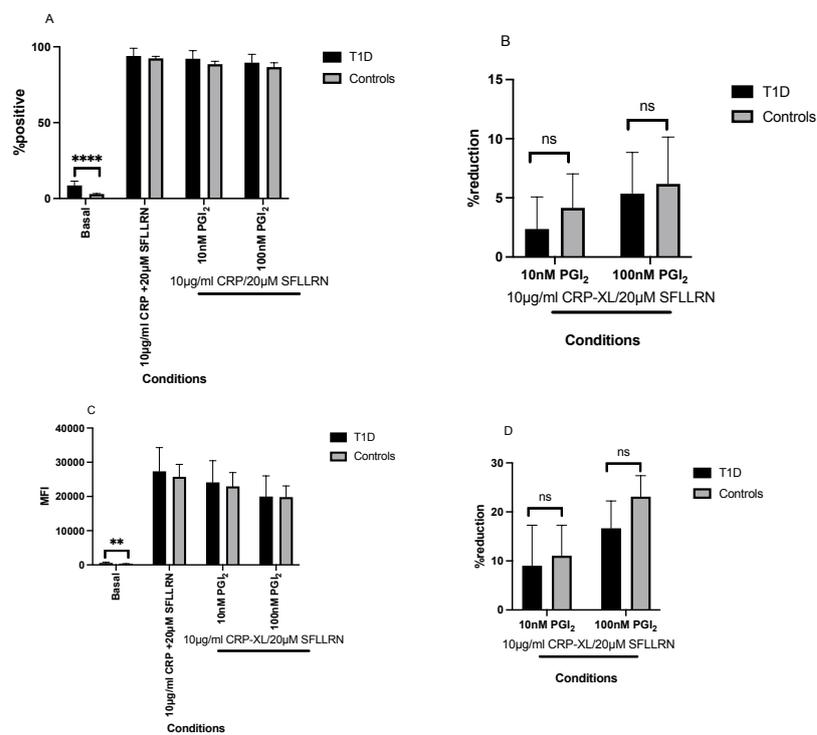
Figure 5-5: Multiparameter flow cytometry CD62P expression at basal, stimulated with dual agonists with and without PGI2 (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive CD62P expression and panel B as percentage reduction comparative to expression with dual agonists alone. Data presented in panel C as total MFI CD62P expression and panel D as MFI percentage reduction comparative to expression with dual agonists alone. Statistical analysis refers to T1D versus controls. **$p < 0.01$ and ****$p < 0.0001$. For T1D n=31 and n=8 for healthy volunteers.

5.3.2.2 Active $\alpha_{IIb}\beta_3$; response to prostacyclin inhibition

The same data were next analysed for PAC1, representative of active $\alpha_{IIb}\beta_3$ expression. Firstly, shown in Figure 5-6 is total expression (percent positive and MFI) at 2 μM SFLLRN along with percentage reduction comparative to the expression with agonist alone. Under basal conditions, patients with T1D had higher PAC1 expression compared with healthy controls measured both by percent positive ($1.1\pm0.9\%$ versus $0.3\pm0.1\%$, *p=0.0001*) and MFI (371 ± 165 versus 132 ± 87, *p=0.01*) shown in panel A and panel C of Figure 5-6.

With 2 μM SFLLRN alone, for percent positive only a numerical difference was detected for PAC1 expression in T1D compared with controls ($68\pm21\%$ versus $59\pm17\%$; p>0.1) but this difference became significant when MFI measurement was employed (5583 ± 4967 in those with T1D versus 2155 ± 487 in control, *p=0.001*). In terms of response to PGI_2 when measured by percent positive patients with T1D had higher expression compared with controls in the presence of both 10 nM PGI_2 ($0.63\pm0.6\%$ versus $0.16\pm0.1\%$, *p=0.003*) and 100 nM PGI_2 ($0.56\pm0.9\%$ versus $0.15\pm0.1\%$, *p=0.03*). Similarly for MFI, PAC1 expression was higher in the patients with T1D at both PGI_2 doses, 1073 ± 653 versus 445 ± 150, p<0.0001 and 362 ± 148 versus 241 ± 86, *p=0.02* respectively. When analysing the data by percentage reduction there were no clear differences identified using either percent positive or MFI, shown in panels B and D of Figure 5-6 with almost complete inhibition in both cases.

Figure 5-6: Multiparameter flow cytometry PAC1 expression at basal, stimulated with 2 μM SFLLRN, with and without PGI2 (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive PAC1 expression and panel B as percentage reduction comparative to expression at 2 μM SFLLRN alone. Data presented in panel C as total MFI PAC1 expression and panel D as MFI percentage reduction comparative to expression at 2 μM SFLLRN alone. Statistical analysis refers to T1D versus controls. *p<0.05, ***p<0.001 and ****p<0.0001. For T1D n=32 and n=8 for healthy volunteers.

At 20 μM SFLLRN, shown in panel A of Figure 5-7, patients with T1D had higher percent positive expression than healthy controls (84$\pm$7% versus 76$\pm$5%, *p=0.003*). The same pattern was seen in the presence of both 10 nM PGI$_2$ (29$\pm$24% versus 9$\pm$11%, *p=0.003*) and 100 nM (2 $\pm$2% versus 0.5$\pm$0.3%, *p=0.005*). When analysed by percentage reduction from expression with 20 μM SFLLRN alone, shown in panel B of Figure 5-7, at 10 nM PGI$_2$ the smaller reduction was seen in patients with T1D, 66$\pm$29% versus 89$\pm$13% in healthy controls (*p=0.003*). At 100nM PGI$_2$ there was almost complete reduction in both groups and thus no clear difference was present.

MFI expression, shown in panel C of Figure 5-7, was significantly higher in T1D than healthy controls with 20 μM SFLLRN alone, 8982$\pm$6150 versus 6961$\pm$2065, *p=0.03*. In the presence of 10 nM PGI$_2$ there was also higher expression in those with T1D, 2740$\pm$3505 versus 796$\pm$642, *p=0.02*. At 100 nM PGI$_2$, similar to the percent positive expression, with almost complete inhibition there was no discernible difference between the groups. When represented by percentage reduction, at 10 nM PGI$_2$ there was smaller reduction seen in the T1D group, 71$\pm$21% versus 87$\pm$13% in the healthy control group, *p=0.03*. At 100 nM PGI$_2$ again with nearly complete inhibition there was no clear difference between groups.
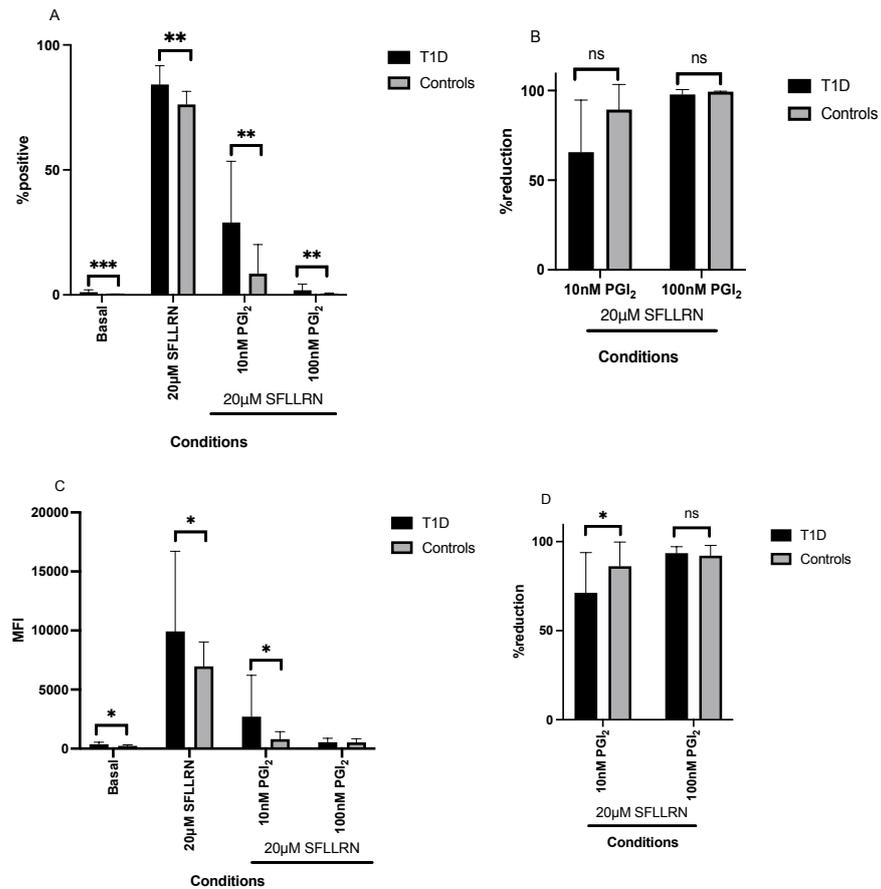
Figure 5-7: Multiparameter flow cytometry PAC1 expression at basal, stimulated with 20 μM SFLLRN, with and without PGI2 (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive PAC1 expression and panel B as percentage reduction comparative to expression at 20 μM SFLLRN alone. Data presented in panel C as total MFI PAC1 expression and panel D as MFI percentage reduction comparative to expression at 20 μM SFLLRN alone. Statistical analysis refers to T1D versus controls. *p<0.05, **p<0.01 and ***p<0.001. For T1D n=32 and n=8 for healthy volunteers.

At 1 μg/ml CRP-XL alone, for percent positive (panel A of Figure 5-8) there was greater PAC1 expression in those with T1D than healthy controls, 84$\pm$7% versus 77$\pm$4%, *p=0.006*. In the presence of 10 nM PGI$_2$, patients with T1D also had higher expression, 68$\pm$14% versus 36$\pm$23%, *p=0.004* and also at 100 nM PGI$_2$, 15$\pm$10% versus 7$\pm$5%, *p=0.02*. By percentage reduction, in the presence of both doses of PGI$_2$, there was a greater reduction seen in healthy controls (55$\pm$28% versus 21$\pm$15%, *p=0.01* at 10nM PGI$_2$ and 80$\pm$13% versus 90$\pm$6%, *p=0.01* at 100nM PGI$_2$) shown in panel B of Figure 5-8.

For MFI, similar was seen; with 1 μg/ml CRP-XL alone patients with T1D had higher PAC1 expression than healthy controls, 8975$\pm$5780 versus 5469$\pm$2533, *p=0.02.* In the presence of 10 nM PGI$_2$ PAC1 expression was also higher in patients, 4108$\pm$3764 versus 1853$\pm$411, *p=0.006* but at 100 nM PGI$_2$, there was no difference as shown in panel C of Figure 5-8. When represented by percentage reduction, shown in panel D of Figure 5-8, with 10 nM PGI$_2$ there was smaller reduction in PAC1 expression in the patients with T1D, 57$\pm$34% versus 78$\pm$6% in healthy controls *p=0.005*, but at 100 nM PGI$_2$ the percentage reduction was similar.
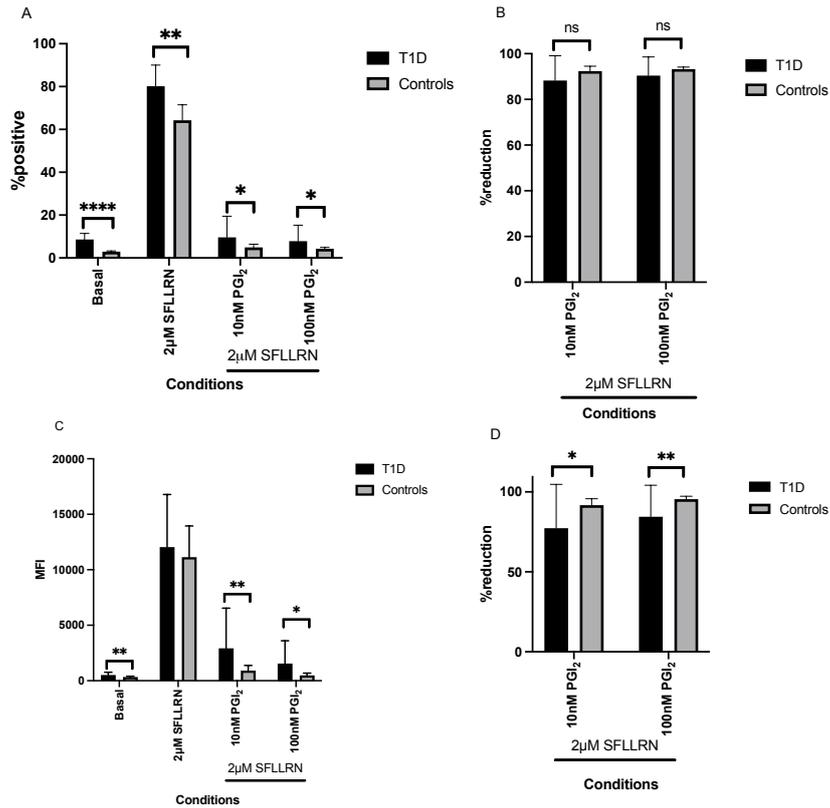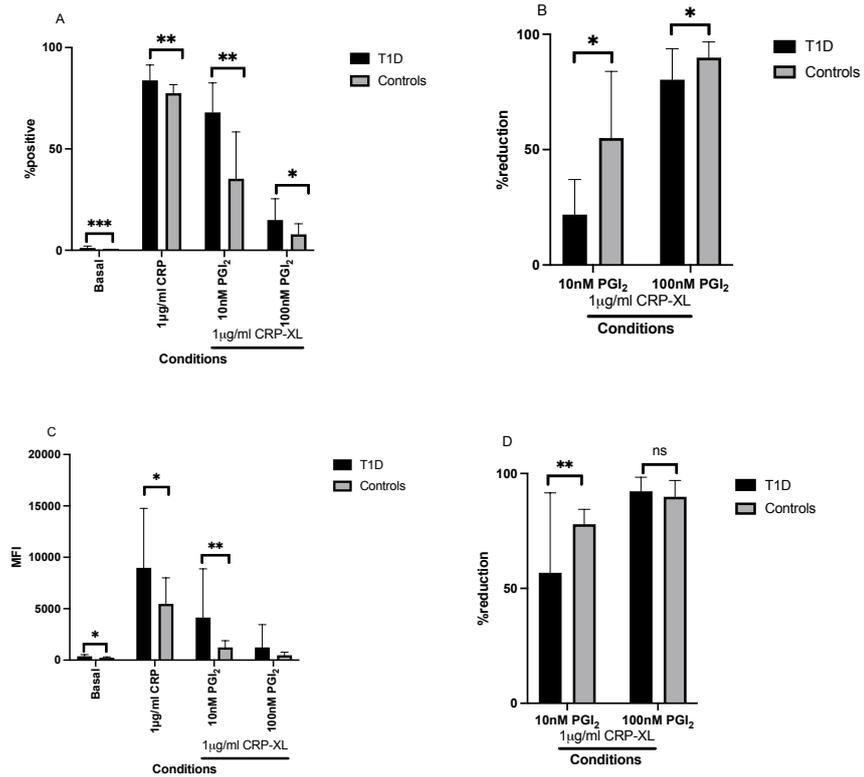
Figure 5-8: Multiparameter flow cytometry PAC1 expression at basal, stimulated with 1 μg/ml CRP-XL, with and without PGI2 (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive PAC1 expression and panel B as percentage reduction comparative to expression at 1 μg/ml CRP-XL alone. Data presented in panel C as total MFI PAC1 expression and panel D as MFI percentage reduction comparative to expression at 1 μg/ml CRP-XL alone. Statistical analysis refers to T1D versus controls. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. For T1D n=32 and n=8 for healthy volunteers.

At 10 μg/ml CRP-XL alone, shown in panel A of Figure 5-9, patients with T1D had higher PAC1 expression (84$\pm$10%) than healthy controls (76$\pm$5%), *p=0.02.* With 10 nM PGI$_2$, there was no difference but at 100nM PGI$_2$, patients again had higher expression 30$\pm$13% versus 20$\pm$7%, *p=0.01.* By percentage reduction, shown in panel B of Figure 5-9, in the presence of only 100 nM PGI$_2$, was there a significant difference, with a smaller reduction seen in patients with T1D than healthy controls (62$\pm$18% versus 74$\pm$8%, *p=0.02*).

For MFI, with 10 μg/ml CRP-XL, patients with T1D had higher expression than healthy controls, 11335$\pm$5824 versus 8419$\pm$1942, *p=0.03.* In the presence of 10 nM PGI$_2$ PAC1 expression was also higher in the T1D group, 7757$\pm$5645 versus 3635$\pm$886, *p=0.001* and also at 100nM PGI$_2$, 2089$\pm$2653 versus 449$\pm$323, *p=0.004* shown in panel C of Figure 5-9.

When represented by percentage reduction, shown in panel D of Figure 5-9, there was no significant difference at 10 nM PGI$_2$ but at 100 nM PGI$_2$ the percentage reduction was smaller in T1D, 79$\pm$27% versus 94$\pm$3% in healthy controls, *p=0.01.*
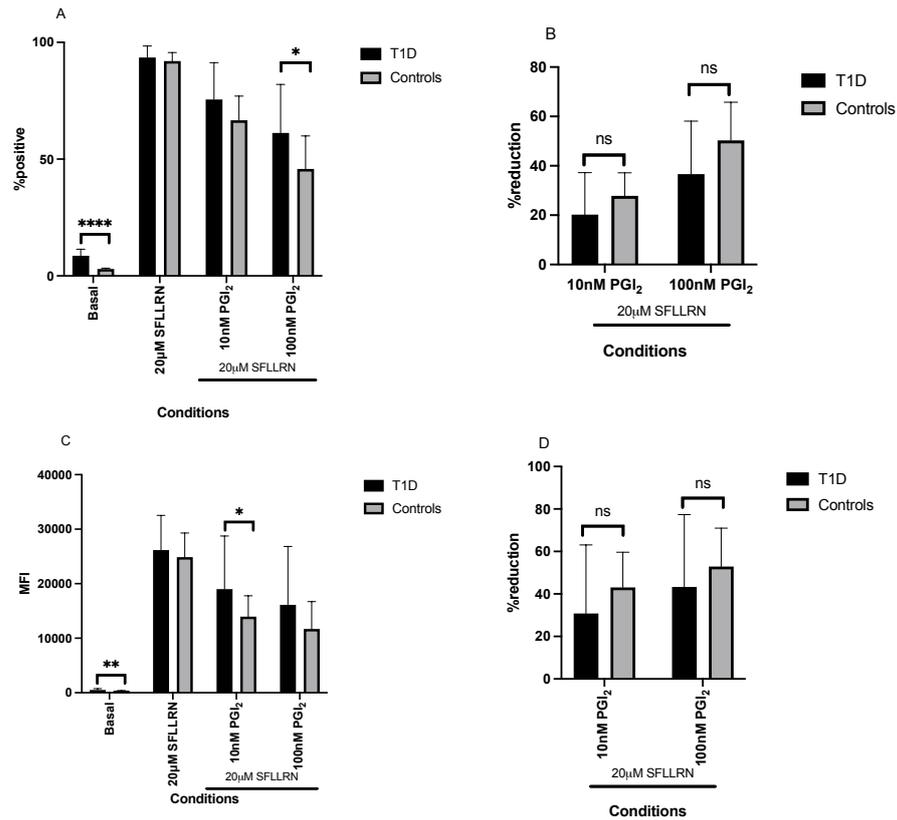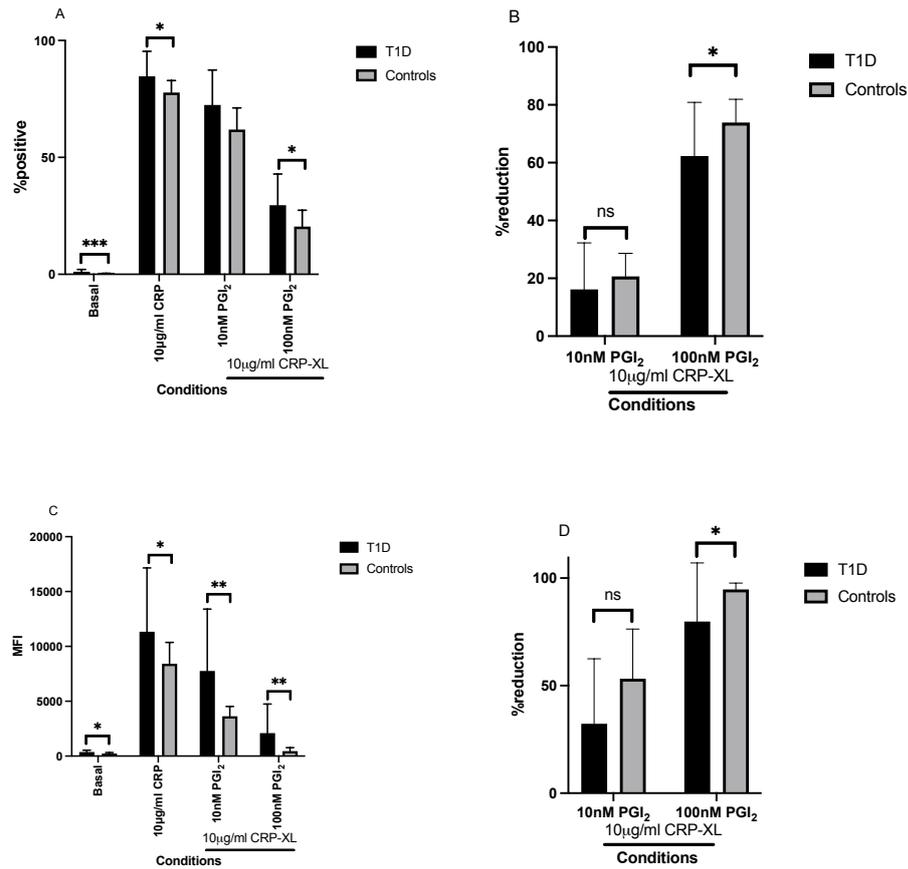
Figure 5-9: Multiparameter flow cytometry PAC1 expression at basal, stimulated with 10 μg/ml CRP-XL, with and without PGI2 (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive PAC1 expression and panel B as percentage reduction comparative to expression at 10 μg/ml CRP-XL alone. Data presented in panel C as total MFI PAC1 expression and panel D as MFI percentage reduction comparative to expression at 10 μg/ml CRP-XL alone. Statistical analysis refers to T1D versus controls. *p<0.05, **p<0.01 and ***p<0.001. For T1D n=32 and n=8 for healthy volunteers.

Finally, with dual agonists, there was significantly higher PAC1 expression in patients with T1D compared with controls, 82±6% versus 72±6%, *p=0.004.* The same was the case in the presence of both 10 nM (77±12% versus 62±5%, *p<0.0001*) and 100 nM PGI_2 (44±9% versus 26±6%, *p<0.0001*) as shown in panel A of Figure 5-10. By percentage reduction, there was no difference at 10 nM PGI_2 but in the presence of 100 nM PGI_2 patients with T1D had a lower reduction in expression, 45±14% versus 63±8% in healthy controls *p=0.0007* (panel B, Figure 5-10).

The comparative MFI data were similar, with higher expression in patients with T1D following stimulation with dual agonists alone (11375±6680 versus 7861±1458, p=0.01) and in the presence of both doses of PGI_2 (10329±5768 versus 6378±737, p=0.001 with 10 nM PGI_2 and 5662±4195 versus 1272±839, p<0.0001 with 100 nM PGI_2), panel C of Figure 5-10. By percentage reduction, at both doses of PGI_2 there was a significantly smaller reduction in patients with T1D compared with healthy controls (4±15% versus 16±7%, *p=0.006* at 10 nM PGI_2 and 47±29% versus 84±8% *p<0.0001* at 100 nM PGI_2), panel D of Figure 5-10.
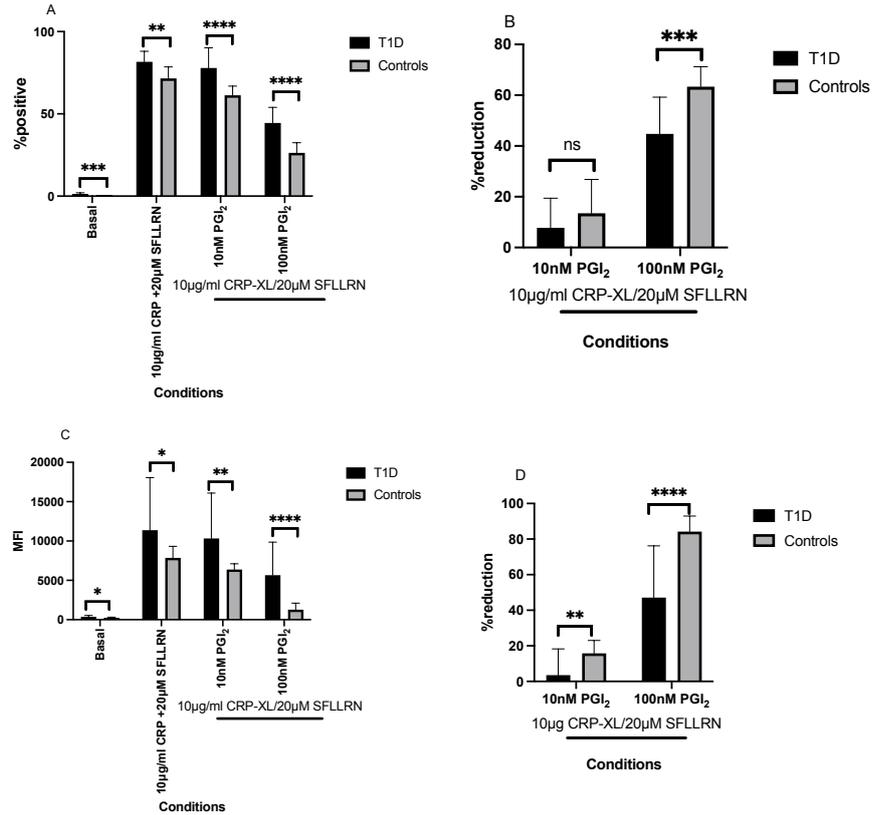
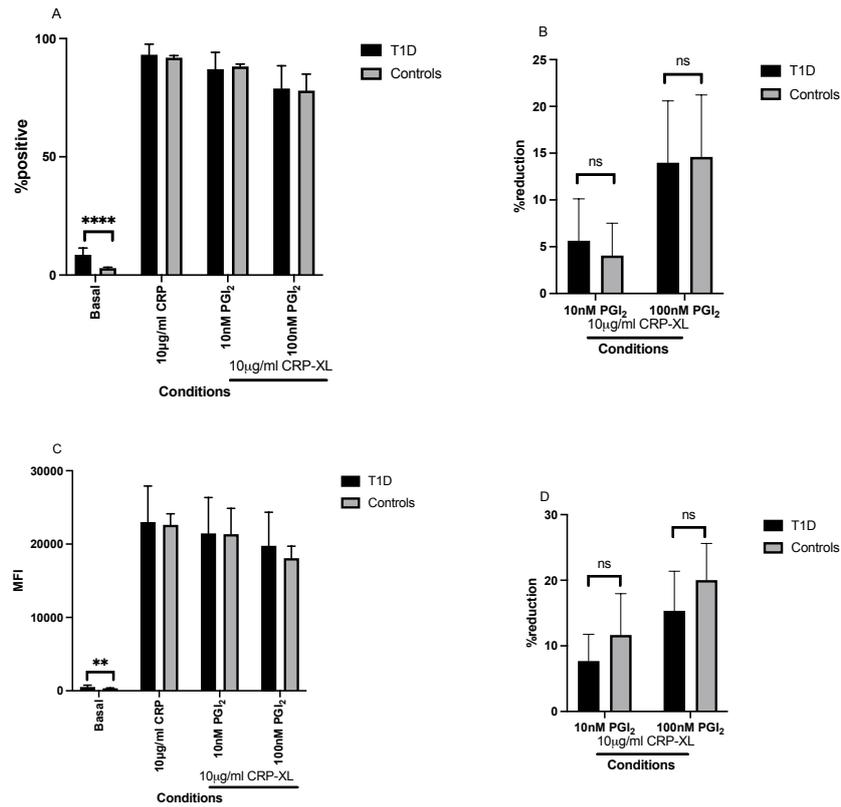**Figure 5-10: Multiparameter flow cytometry PAC1 expression at basal, stimulated with dual agonists, with and without PGI2 (10 nM and 100 nM) in whole blood.**

Data presented in panel A as total percent positive PAC1 expression and panel B as percentage reduction comparative to expression with dual agonists alone. Data presented in panel C as total MFI PAC1 expression and panel D as MFI percentage reduction comparative to expression with dual agonists alone. For T1D n=32 and n=8 for healthy volunteers. Statistical analysis refers to T1D versus controls. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. For T1D n=32 and n=8 for healthy volunteers.

5.3.2.3 Phosphatidylserine; response to prostacyclin inhibition

For the analysis of AnnV expression, representing PS exposure, as per data from the prior chapter, the total number of patients was slightly lower at n=26, with again n=8 for healthy controls. Under basal conditions, patients with T1D had higher AnnV expression than healthy controls, both when expressed as percent positive, $1\pm0.9\%$ versus $0.3\pm0.1\%$, *p=0.001* and also MFI, 869 ± 862 versus 314 ± 110, *p=0.004*.

As with previous data, single agonists 2 μM SFLLRN and 20 μM SFLLRN alone, as well as low dose CRP-XL (1 μg/ml) showed minimal increase in expression with no discernible differences found between groups. I have therefore focussed on 10 μg/ml CRP-XL and dual agonists for the purposes of analysis of AnnV expression.

With 10 μg/ml CRP-XL stimulation alone, there was no difference between the two groups when analysed by percent positive. However, patients with T1D had higher expression in the presence of both 10 nM PGI_2 ($8\pm5\%$ versus $4\pm2\%$, *p=0.003*) and 100nM PGI_2 ($3\pm1.5\%$ versus $1.1\pm0.3\%$, *p<0.0001*). Similarly, when analysed by percentage reduction, there was lower sensitivity to PGI_2 in patients with T1D compared with healthy controls both to 10 nM PGI_2 ($44\pm33\%$ versus $73\pm16\%$, *p=0.003*) and to 100 nM PGI_2 ($77\pm14\%$ versus $91\pm5\%$, *p=0.0005*).

The comparative MFI expression data are similar, but with a significantly higher expression in patients with T1D compared with healthy controls with agonist stimulation alone (10561 ± 6181 versus 6340 ± 2192, *p=0.006*). In the presence of 10 nM PGI_2 alone there was no difference in expression between the groups but with 100 nM PGI_2 there was higher AnnV expression in patients

with T1D than healthy controls, 2530±2865 versus 801±231, *p=0.005.* By percentage reduction, there was a greater reduction seen in T1D patients compared with controls at 10 nM PGI$_2$ (55±28% versus 41±11%, *p=0.04*), however this was reversed at 100nM PGI$_2$ (70±29% versus 86±5%, *p=0.01*). These data are shown in Figure 5-11.
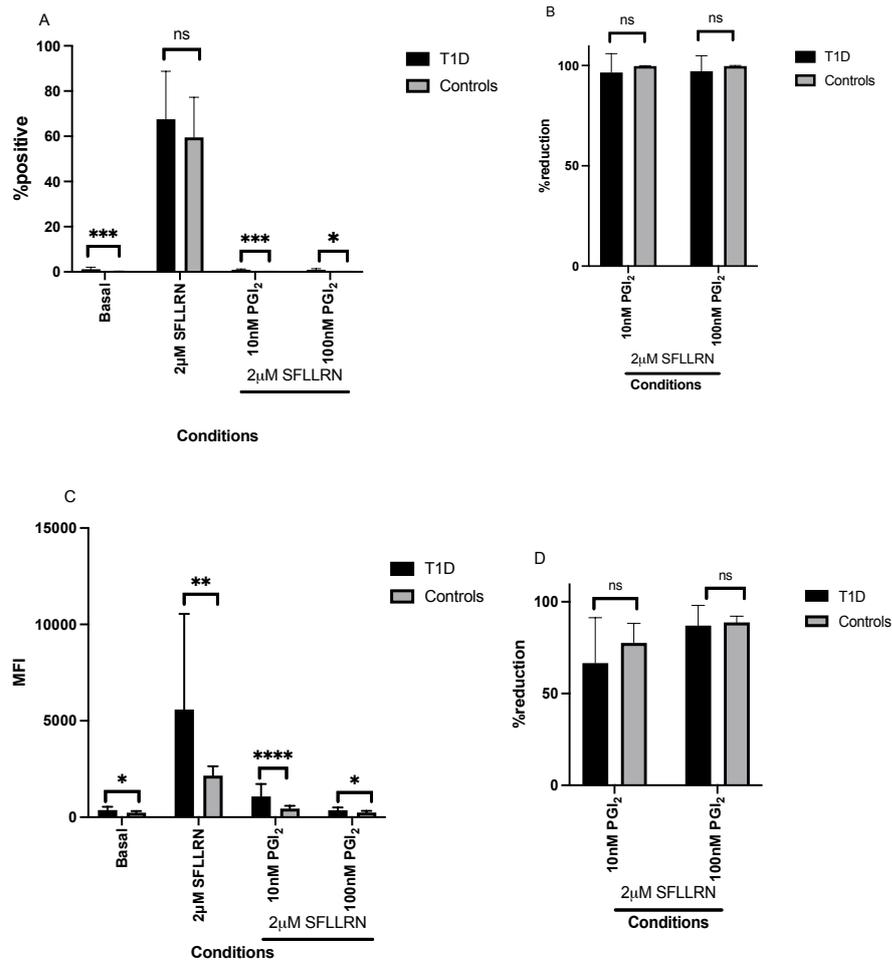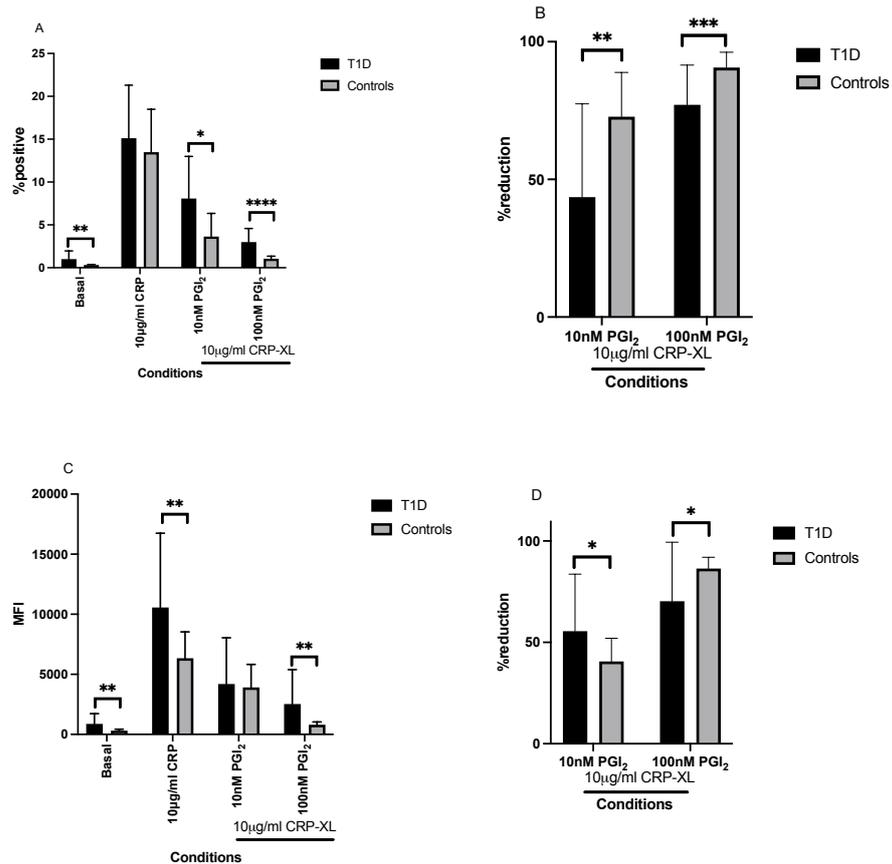

Figure 5-11: Multiparameter flow cytometry AnnV expression at basal, stimulated with 10 µg/ml CRP-XL, with and without PGI2 (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive AnnV expression and panel B as percentage reduction comparative to expression at 10 µg/ml CRP-XL alone. Data presented in panel C as total MFI AnnV expression and panel D as MFI percentage reduction comparative to expression at 10 µg/ml CRP-XL alone. Statistical analysis refers to T1D versus controls. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. For T1D n=26 and n=8 for healthy volunteers.

Following stimulation with dual agonists, those with T1D had higher expression measured by percent positive, 33±10% versus 27±4%, *p=0.02*. In the presence of 10 nM PGI_2 there was also higher expression seen in the T1D group, 17±6% versus 9±3%, *p<0.0001* and also with 100 nM PGI_2 (5±2% versus 3±0.8%, *p<0.0001*). By percentage reduction, at 10 nM PGI_2 and 100 nM PGI_2, T1D samples had lower reduction in AnnV expression compared with controls using 10 nM PGI_2 (46±14% versus 67±6%, *p<0.0001)* and 100 nM PGI_2 (83±6% versus 90±2%, *p<0.0001*). These data are shown in panels A and B of Figure 5-12.

For MFI, again the T1D cohort had greater AnnV expression with dual agonists alone (27124±10105 versus 19948±1577, *p=0.002*) as well as with both 10 nM PGI_2 (14653±7773 versus 9350±1987*, p=0.004*) and 100 nM PGI_2 (4782±3878 versus 2168±776, *p=0.004*). By percentage reduction, at 10 nM $PGI_2,$ the difference was non-significant but at 100 nM PGI_2 patients with T1D had lower reduction in AnnV expression than healthy controls (81±12% versus 89±4%, *p=0.007*) with these data shown in panels C and D of Figure 5-12.
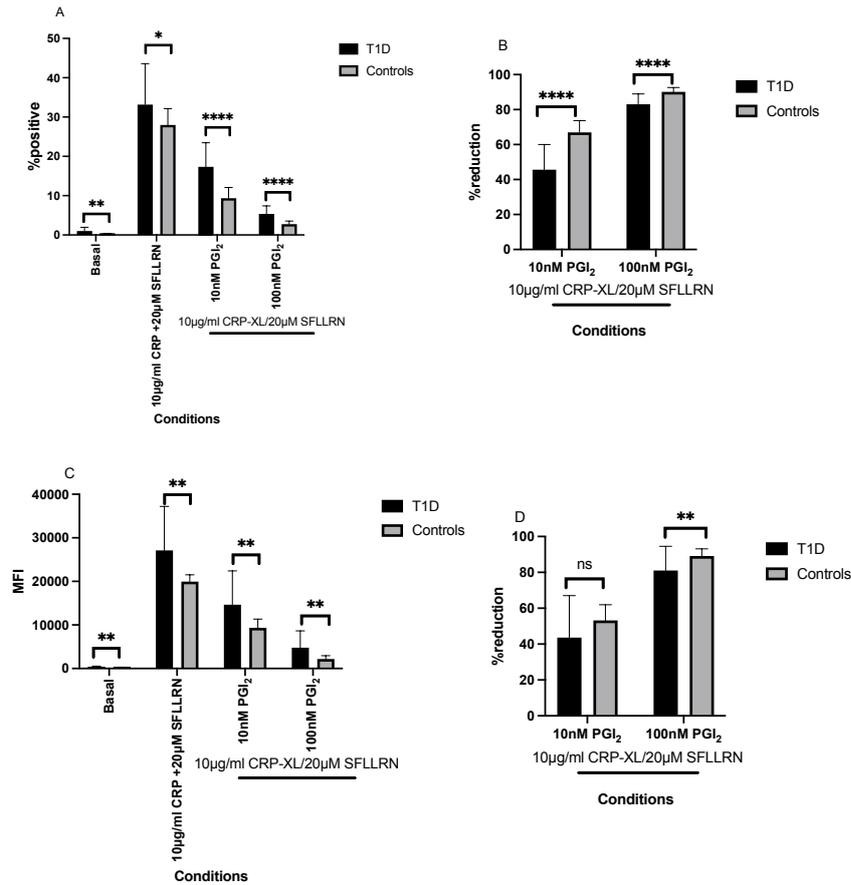
Figure 5-12: Multiparameter flow cytometry AnnV expression at basal, stimulated with dual agonists, with and without PGI2 (10 nM and 100 nM) in whole blood.

Data presented in panel A as total percent positive AnnV expression and panel B as percentage reduction comparative to expression with dual agonists alone. Data presented in panel C as total MFI AnnV expression and panel D as MFI percentage reduction comparative to expression with dual agonists alone. Statistical analysis refers to T1D versus controls. *p<0.05, **p<0.01 and ****p<0.0001. For T1D n=26 and n=8 for healthy volunteers.

5.3.2.4 Effects of glycaemic control on prostacyclin sensitivity in T1D/DD

To further assess response to inhibition, data from the cohort of patients with T1D were then stratified according to glycaemic control (measured by HbA1c). For clarity of analysis, data included were expression following single agonist stimulation with 1 μg/ml CRP-XL alone and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) again with and without 10 nM and 100 nM PGI$_2$. All previous markers were again measured including CD62P (P-selectin), PAC1 (active $\alpha_{IIb}\beta_3$) and AnnV (PS). Data represent the percentage reduction with both doses of PGI$_2$ compared to maximum expression with agonist(s) alone. For CD62P percent positive expression, panel A in Figure 5-13 represents the expression under basal conditions, following stimulation (1 μg/ml CRP-XL or dual agonists) and in the presence of 10 nM/100 nM PGI$_2$ at each agonist concentration. When analysing percentage reduction in the presence of PGI$_2$, this was again relatively low particularly with dual agonist stimulation. Shown in panel B in Figure 5-13, at 1 μg/ml CRP-XL, the difference in the presence of 10nM PGI$_2$ was non-significant but there was a significantly greater percentage reduction in CD62P expression in those with HbA1c <64 mmol/mol in the presence of 100 nM PGI$_2$, 53$\pm$14% versus 40$\pm$18%, *p=0.03*. At dual agonist strength there was no clear difference between the two groups at either dose of PGI$_2$, shown in panel C Figure 5-13.

For MFI expression, again the full data are shown in panel A, Figure 5-14. When analysing the data by percentage reduction, as shown in panel B and panel C, the differences seen between groups at neither 1 μg/ml CRP-XL nor dual agonists were significant.

Figure 5-13: Multiparameter flow cytometry percent positive CD62P expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists with and without PGI$_2$ (10 nM and 100 nM) in whole blood stratified according to median HbA1c.

Panel A represents total percent positive expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with dual agonists. *p<0.05 and ***p<0.001. Total n=15 for HbA1c $\geq$64mmol/mol and n=16 <64mmol/mol.
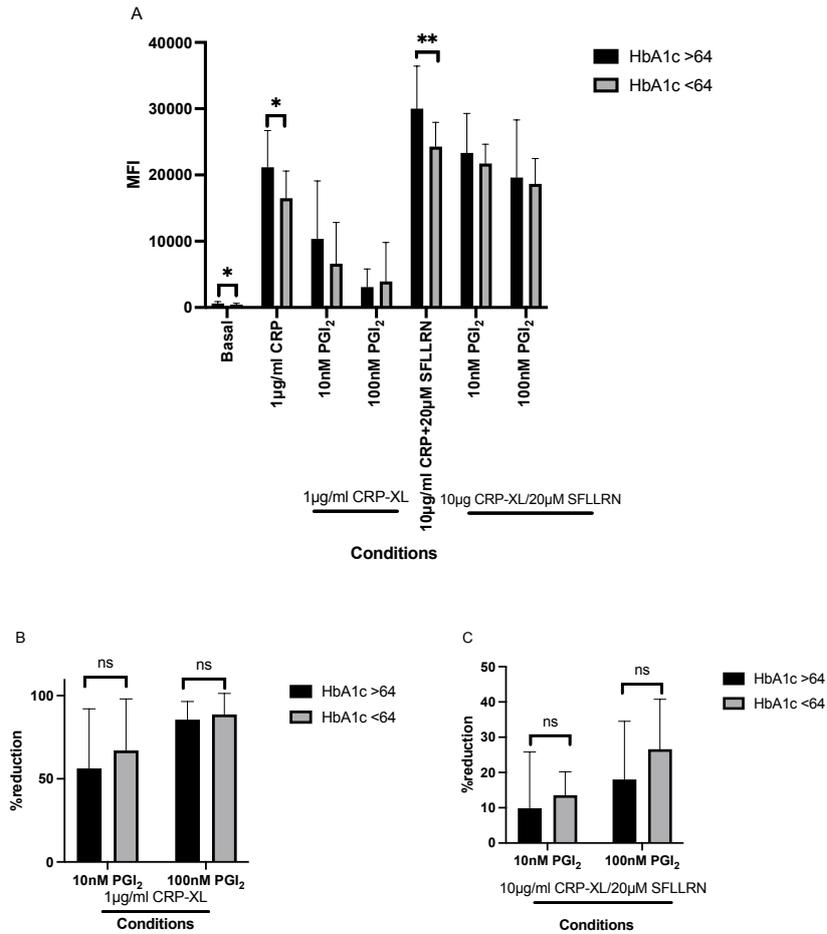
Figure 5-14: Multiparameter flow cytometry MFI CD62P expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists with and without PGI2 (10 nM and 100 nM) in whole blood stratified according to median HbA1c.

Panel A represents total MFI expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with dual agonists. *p<0.05, **p<0.01. Total n=15 for HbA1c ≥64mmol/mol and n=16 <64mmol/mol.

Next, we assessed PAC1 percent positive expression again in the same conditions, shown in panel A of Figure 5-15. When analysed by percentage reduction, at 1 μg/ml CRP-XL, those with the lower HbA1c <64. mmol/mol had greater response to inhibition both at 10 nM PGI_2 (34$\pm$11% versus 22$\pm$13%, *p=0.01*) and at 100 nM PGI_2 (64$\pm$12% versus 52$\pm$10%, *p=0.008*), shown in panel B, Figure 5-15. With dual agonist stimulation, there was again greater reduction seen in those with lower HbA1c in the presence of both 10 nM PGI_2 (15$\pm$6% versus 8$\pm$10%, *p=0.04*) and 100 nM PGI_2 (47$\pm$9% versus 34$\pm$14%, *p=0.005*).

For MFI, shown in Figure 5-16, at 1 μg/ml CRP-XL, there was greater reduction seen in patients with HbA1c<64 mmol/mol in the presence of 10 nM PGI2 (64$\pm$26% versus 43$\pm$25%, *p=0.03*) and also at 100 nM PGI_2 (88$\pm$10% versus 68$\pm$19%, *p=0.001*). Following dual agonist stimulation, a significantly greater reduction was seen in patients with the lower HbA1c only in the presence of the higher 100 nM PGI_2 dose (66$\pm$15% versus 49$\pm$16%, *p=0.008*), as shown in panel C Figure 5-16.

Figure 5-15: Multiparameter flow cytometry percent positive PAC1 expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) with and without PGI$_2$ (10nM and 100nM) in whole blood stratified according to median HbA1c.

Panel A represents total percent positive expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with dual agonists. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. n=16 for both HbA1c $\geq$64mmol/mol and <64mmol/mol.

Figure 5-16: Multiparameter flow cytometry MFI PAC1 expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) with and without PGI$_2$ (10 nM and 100nM) in whole blood stratified according to median HbA1c.

Panel A represents total MFI expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with dual agonists. *p<0.05 and **p<0.01. n=16 for both HbA1c $\geq$64 mmol/mol and <64 mmol/mol.

We also assessed AnnV percent positive expression under the same described conditions, shown in panel A of Figure 5-17 again grouped by median HbA1c of 64 mmol/mol. By percentage reduction, there were no clear differences between the two groups, largely owing to the low expression even without PGI_2. With dual agonist stimulation, there was far higher expression and thus it was apparent that there was greater reduction in those with HbA1c <64 mmol/mol in the presence of both 10 nM PGI_2 (60±8% versus 46±14%, *p=0.008*) and 100nM PGI_2 (87±5% versus 74±7%, *p<0.0001*).

For MFI, at 1 μg/ml CRP-XL, with 10 nM PGI_2, there was no apparent difference in percentage reduction but with 100 nM PGI_2 patients with lower HbA1c had greater reduction in expression (71±18% versus 51±19%, *p=0.01*). Following dual agonist stimulation, again there was no significant difference between the two groups but in the presence of 100 nM PGI_2 again there was a far greater reduction in those with HbA1c <64 mmol/mol, 86±8% versus 72±9%, *p=0.001*, shown in Figure 5-18.

Figure 5-17: Multiparameter flow cytometry percent positive AnnV expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) with and without PGI2 (10 nM and 100 nM) in whole blood stratified according to median HbA1c.

Panel A represents total percent positive expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI2 compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI2 compared to expression following stimulation with dual agonists. *p<0.05, **p<0.01 and ****p<0.0001. n=13 for both HbA1c ≥64mmol/mol and <64mmol/mol.

Figure 5-18: Multiparameter flow cytometry MFI AnnV expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) with and without PGI$_2$ (10 nM and 100 nM) in whole blood stratified according to median HbA1c.

Panel A represents total MFI expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI2 compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI2 compared to expression following stimulation with dual agonists. *p<0.05 and **p<0.01. n=13 for both HbA1c $\geq$64mmol/mol and <64mmol/mol.

5.3.2.5 Effects of insulin resistance on prostacyclin sensitivity in T1D

In addition to assessing the effects of glycaemic control on PGI_2 sensitivity, we then stratified the same data according to insulin resistance, using eGDR as previously detailed in earlier chapters. Groups were categorised into three, eGDR <6 mg/kg/min (n=8), 6-8 mg/kg/min (n=9) and ≥8 mg/kg/min (n=14). As was the case for assessing the effects of glycaemia, for clarity data included were expression by both percent positive and MFI under basal conditions, with 1 μg/ml CRP-XL and dual agonist stimulation as well as the two agonist strengths in the presence of 10 nM PGI_2 and 100 nM PGI_2, shown in panel A on each Figure. Again, each of the markers of platelet activation included in the four-colour panel were measured and analysed, detailed below.

Firstly, CD62P expression by percent positive was assessed (Figure 5-19 panel A). The data were analysed by percentage reduction at each agonist strength in the presence of both PGI_2 doses, shown in panel B of Figure 5-19. At 1 μg/ml CRP-XL, there was no difference in percentage reduction between the three groups at 10 nM PGI_2 but in the presence of 100 nM PGI_2 patients with eGDR ≥8 mg/kg/min had significantly higher percentage reduction than those with eGDR <6 mg/kg/min, 76±23% versus 40±9%, *p=0.0002* with no difference seen comparing the other groups. Following stimulation with dual agonists, in the presence of 10 nM PGI_2 those with eGDR ≥8 mg/kg/min had significantly greater reduction than those with eGDR <6 mg/kg/min (10±7% versus, 4±4%, *p=0.04*), again with no difference seen when comparing the other groups. Similarly, the same pattern was seen in the presence of 100nM PGI_2, with eGDR ≥8 mg/kg/min having significantly higher percentage

reduction than those in the lowest eGDR category, 17±8% versus 8±5%, *p=0.0098*.
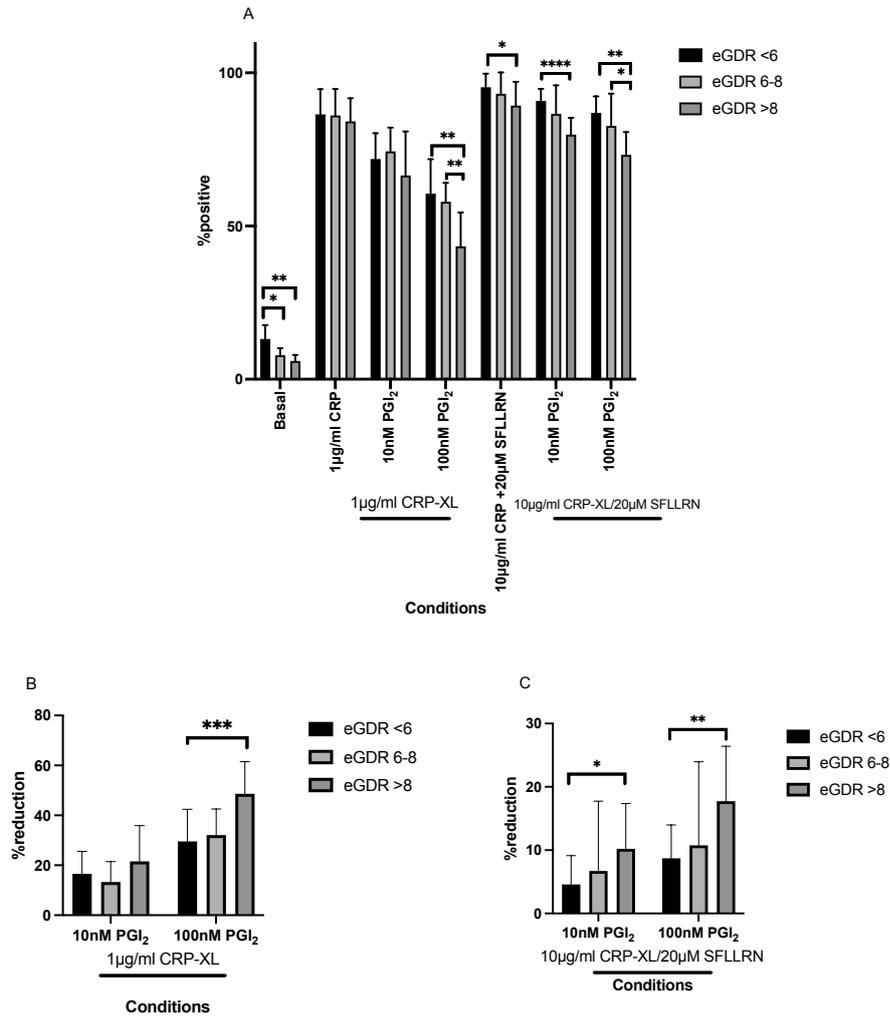
Figure 5-19: Multiparameter flow cytometry percent positive CD62P expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) with and without PGI$_2$ (10 nM and 100 nM) in whole blood stratified according to eGDR.

Panel A represents total percent positive expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with dual agonists. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. eGDR <6 mg/kg/min n=8, eGDR 6-8 mg/kg/min n=9 and eGDR ≥8 mg/kg/min n=14.

The comparative MFI data are shown in panel A of Figure 5-20. By percentage reduction, at 1 μg/ml CRP-XL patients with the highest eGDR had greater percentage reduction than those with the lowest eGDR both at 10 nM PGI_2 (60$\pm$25% versus 22$\pm$11%, *p=0.0008*) and 100nM PGI_2, (82$\pm$14% versus 40$\pm$9%, *p<0.0001*) which was also significantly higher than those with eGDR 6-8 mg/kg/min (56$\pm$23%, *p=0.01*). With dual agonist stimulation, at 10 nM PGI_2 there were no differences between the three groups. With the higher 100 nM PGI_2, those with the highest eGDR had greater percentage reduction than those with the lowest, 32$\pm$12% versus 17$\pm$11%, *p=0.01* with no differences seen when comparing the other groups, shown in panel C Figure 5-20.
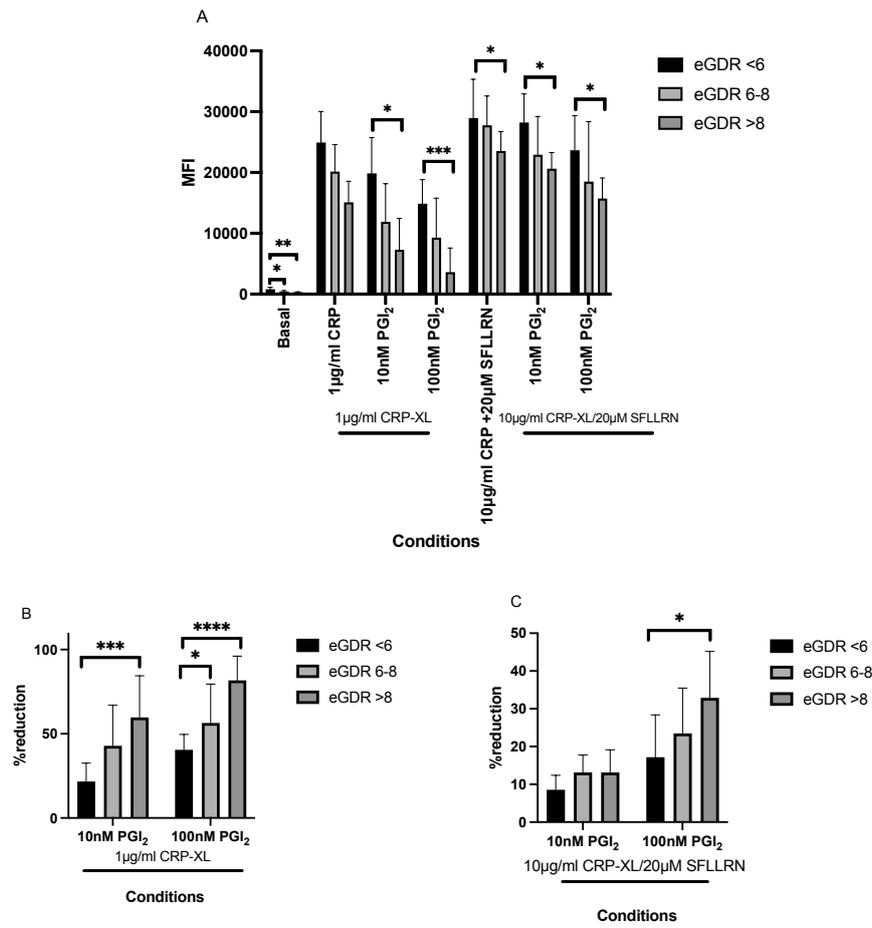
Figure 5-20: Multiparameter flow cytometry MFI CD62P expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) with and without PGI2 (10 nM and 100 nM) in whole blood stratified according to eGDR.

Panel A represents total MFI expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI2 compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI2 compared to expression following stimulation with dual agonists. *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$. eGDR <6 mg/kg/min n=8, eGDR 6-8 mg/kg/min n=9 and eGDR $\geq$8 mg/kg/min n=14.

The same data were then analysed for PAC1 expression as shown in Figures 5-21 and 5-22. First looking at percent positive expression, by percentage reduction, at 1 μg/ml CRP-XL, there was a significantly greater reduction in those with eGDR $\geq$8 mg/kg/min than those patients with eGDR <6 mg/kg/min with 10 nM PGI_2 (42$\pm$7% versus 20$\pm$7%, *p=0.0002*). With 100 nM PGI_2 those with eGDR $\geq$8 mg/kg/min had greater reduction in response to the PGI_2 than those with eGDR <6 mg/kg/min (73$\pm$5% versus 50$\pm$11%, *p=0.0008*) and those with eGDR 6-8 mg/kg/min (57$\pm$9%, *p=0.0006*) as shown in panel B, Figure 5-21. With dual agonists, in the presence of both 10nM and 100nM PGI_2 there were no clear differences between the three groups, again shown in panel C of Figure 5-21.

For MFI, at 1 μg/ml CRP-XL, at 10 nM PGI_2 patients with eGDR $\geq$8 mg/kg/min had greater percentage reduction (52$\pm$12%) compared with both patients with eGDR 6-8 mg/kg/min (39$\pm$12%, *p=0.03*) and those with eGDR <6 mg/kg/min (29$\pm$9%, *p=0.0001*). At the higher 100 nM PGI_2, findings were similar with patients in the highest eGDR category having greater reduction (76$\pm$6%) than both patients with eGDR 6-8 mg/kg/min (63$\pm$13%, *p=0.01*) and with eGDR <6 mg/kg/min (51$\pm$12%, *p=0.0004*). With dual agonists, in the presence of 10 nM PGI_2 there were no differences seen between groups but at the higher 100 nM PGI_2, patients with eGDR $\geq$8 mg/kg/min had an increased percentage reduction (75$\pm$7%) compared with those with eGDR 6-8 mg/kg/min (59$\pm$15%, *p=0.01*) and eGDR <6 mg/kg/min (48$\pm$17%, *p=0.003*) as shown in panel C Figure 5-22.
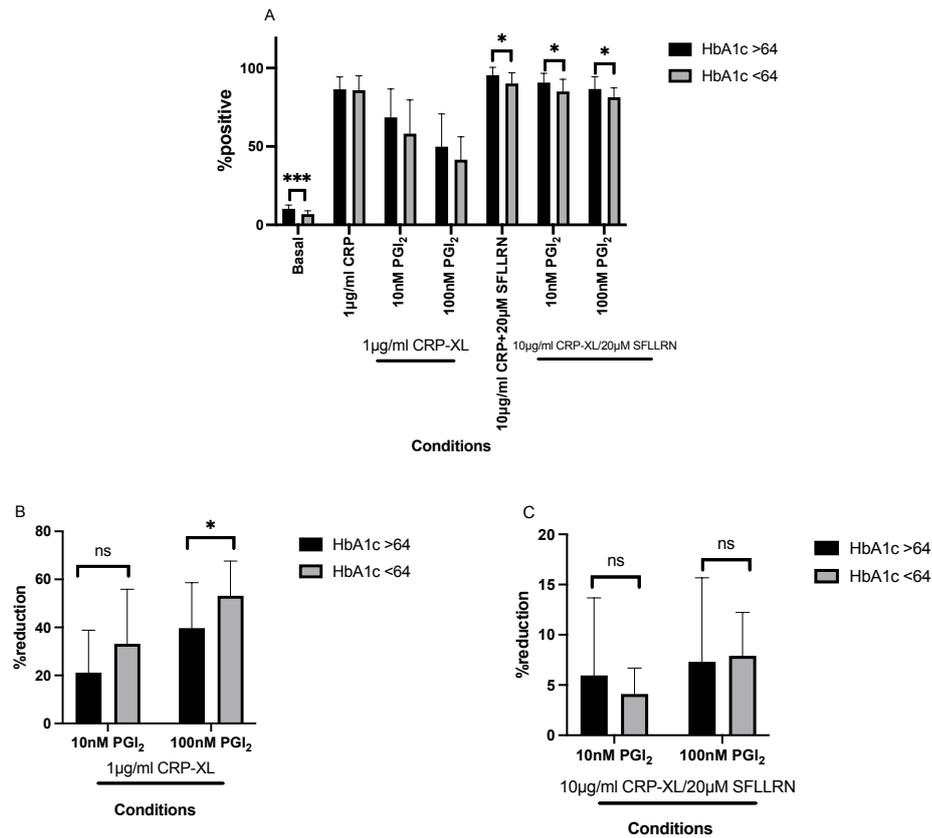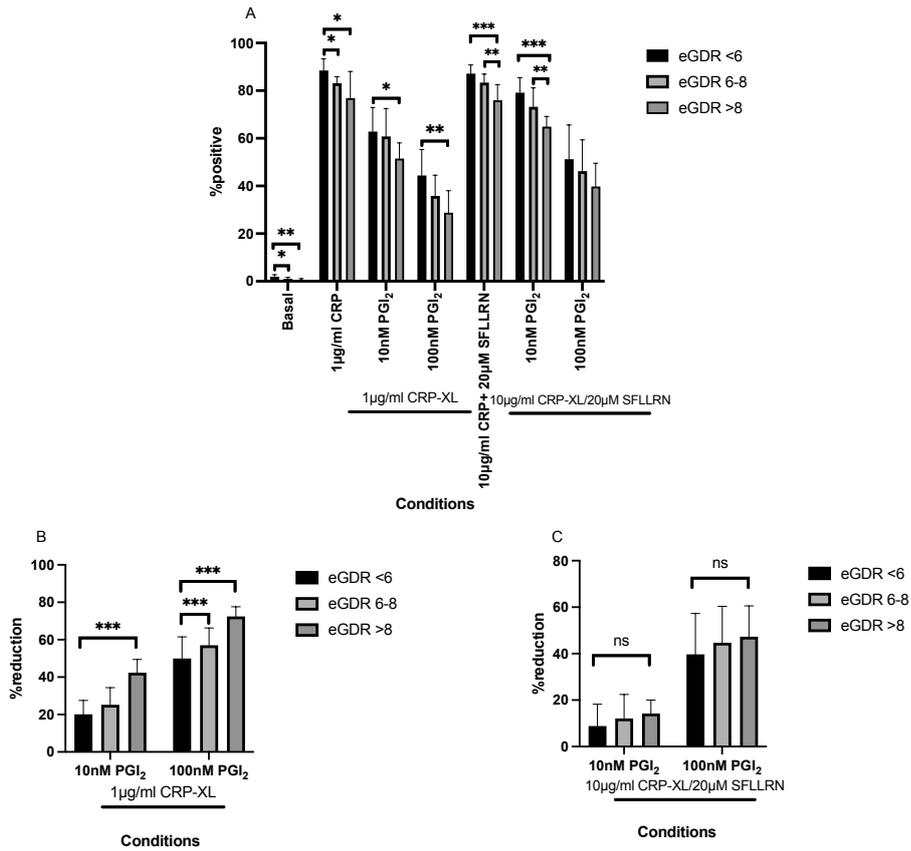
Figure 5-21: Multiparameter flow cytometry percent positive PAC1 expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) with and without PGI$_2$ (10nM and 100nM) in whole blood stratified according to eGDR.

Panel A represents total percent positive expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI2 compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI2 compared to expression following stimulation with dual agonists. *p<0.05, **p<0.01 and ***p<0.001. eGDR <6 mg/kg/min n=8, eGDR 6-8 mg/kg/min, n=9 and eGDR $\geq$8 mg/kg/min n=15.
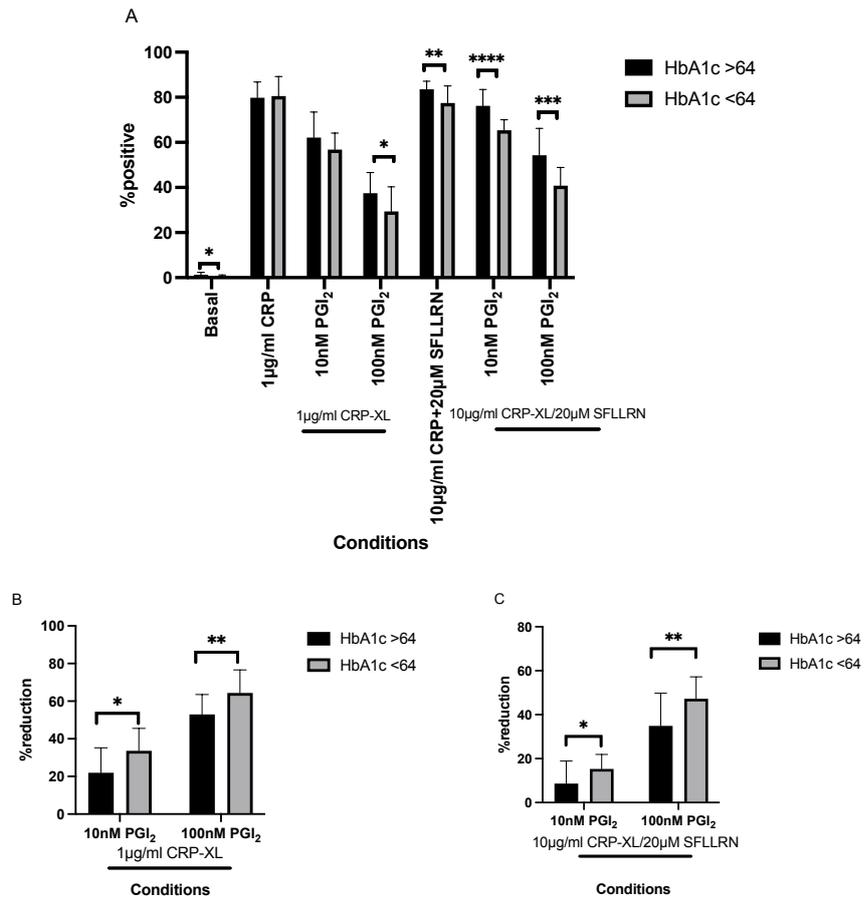
Figure 5-22: Multiparameter flow cytometry MFI PAC1 expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) with and without PGI₂ (10 nM and 100 nM) in whole blood stratified according to eGDR.

Panel A represents total MFI expression. Panel B represents percentage reduction in the presence of 10/100nM PGI2 compared to expression following stimulation with agonist alone at 1μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI2 compared to expression following stimulation with dual agonists. *p<0.05, **p<0.01 and ***p<0.001. eGDR <6 mg/kg/min n=8, eGDR 6-8 mg/kg/min n=9 and eGDR ≥8 mg/kg/min n=15.

Finally, AnnV expression was measured under the same conditions. As per all previous AnnV data, expression at 1 μg/ml CRP-XL was low and thus difficult to draw any true conclusions from differences in percentage reduction in the presence of PGI_2, with no clear differences between the three groups identified at either dose for percent positive expression, shown in panel B Figure 5-23. At the dual agonist strength stimulation, in the presence of 10 nM PGI_2, those with eGDR $\geq$8 mg/kg/min had greater reduction than those with eGDR <6 mg/kg/min (59$\pm$10% versus 43$\pm$15%, *p=0.04*) and also greater than those with eGDR 6-8 mg/kg/min (46$\pm$10%, *p=0.02*). At 100nM PGI_2, those with eGDR $\geq$8 mg/kg/min had significantly greater response to PGI_2 (84$\pm$5%) than those with eGDR 6-8 mg/kg/min (73$\pm$6%, *p=0.002*) and eGDR<6mg/kg/min (71$\pm$8%, *p=0.007*), shown in panel C Figure 5-23.

The data for MFI were similar. With 1 μg/ml CRP-XL, there was no difference seen in percentage reduction with either 10 nM PGI_2 nor 100 nM PGI_2, again likely due to the overall low expression, shown in panel B, Figure 5-24. At the dual agonist dose, in the presence of 10 nM PGI_2, patients with eGDR $\geq$8 mg/kg/min had the greatest response (59$\pm$10%) compared with eGDR 6-8 mg/kg/min (46$\pm$10%, *p=0.02*) and eGDR <6 mg/kg/min (42$\pm$15%, *p=0.03*). With the higher PGI_2 dose, the difference was even greater, with those with the highest eGDR again having greatest reduction in expression, 84$\pm$5%, versus 73$\pm$5% (*p=0.002*) in those with eGDR 6-8mg/kg/min and 70$\pm$8% (*p=0.005*) in those with the lowest eGDR, with these data again shown in panel C, Figure 5-24.
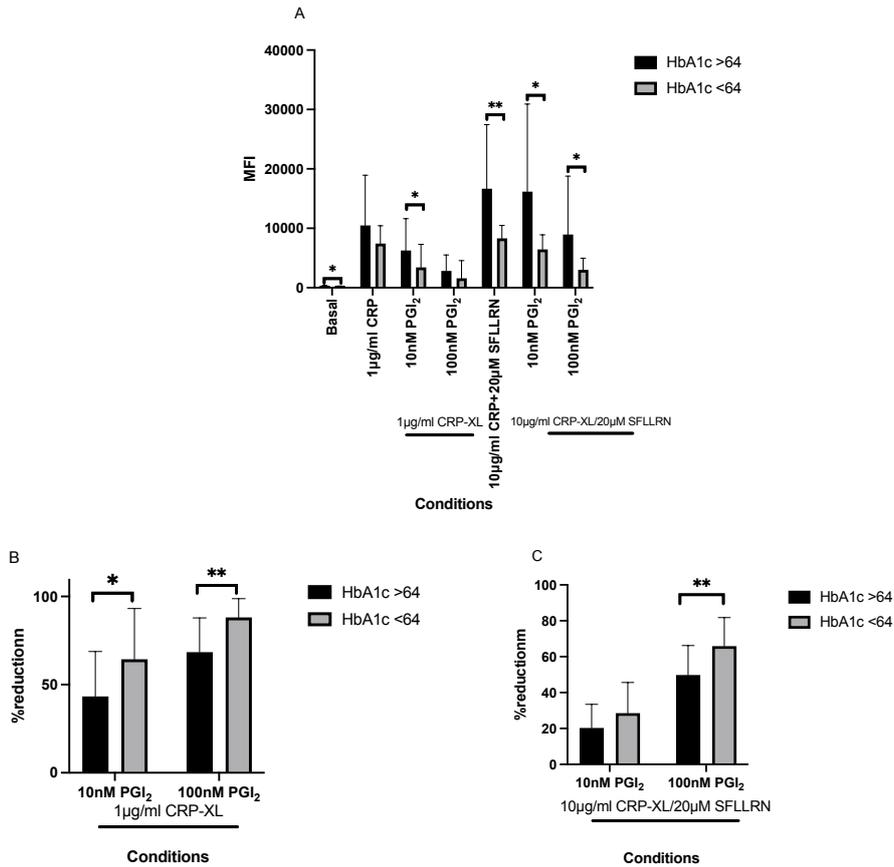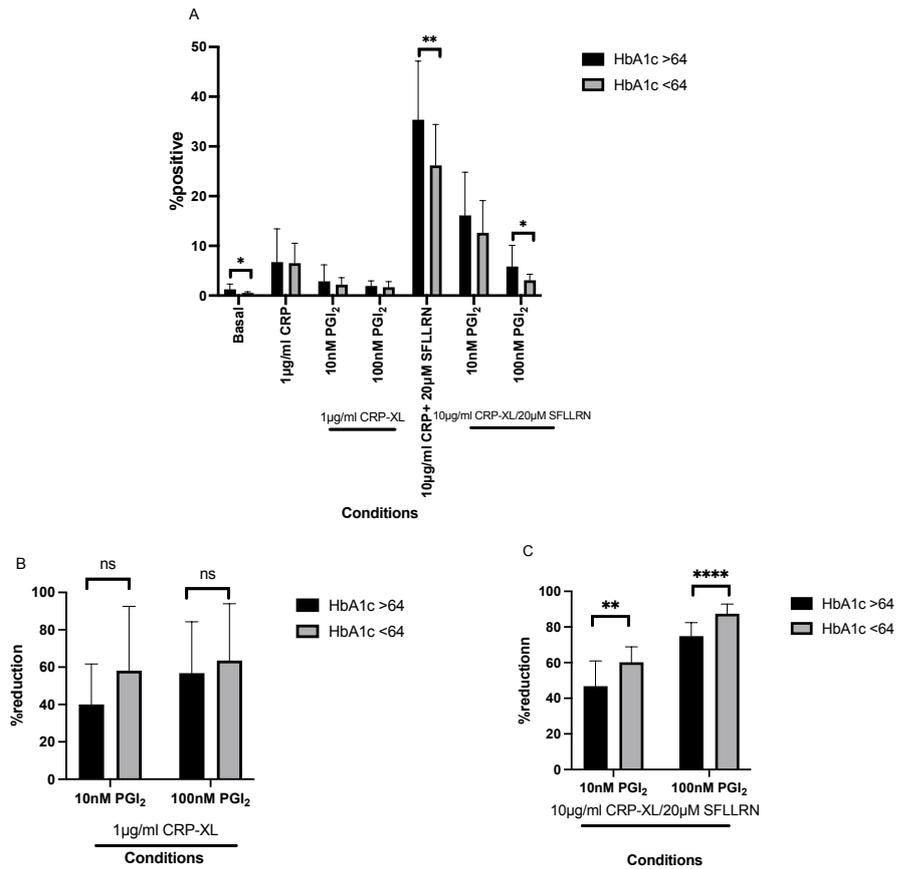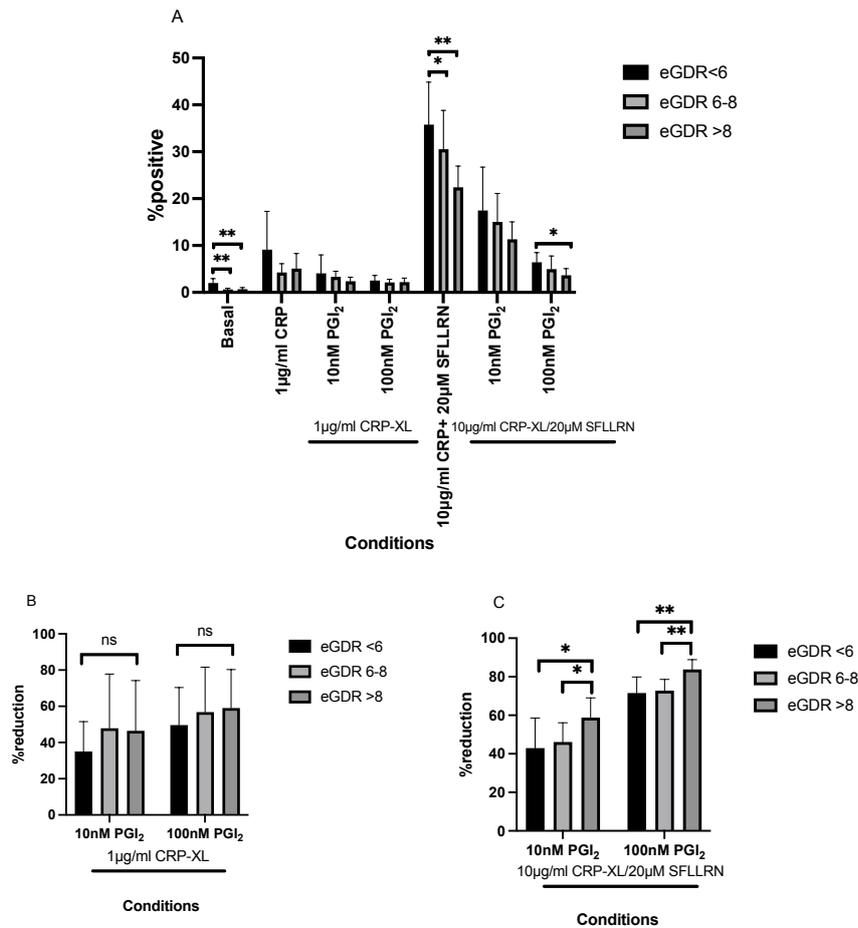
Figure 5-23: Multiparameter flow cytometry percent positive AnnV expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) with and without PGI$_2$ (10 nM and 100 nM) in whole blood stratified according to eGDR.

Panel A represents total percent positive expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI$_2$ compared to expression following stimulation with dual agonists. *p<0.05 and **p<0.01. eGDR <6 mg/kg/min n=7, eGDR 6-8 mg/kg/min n=7 and eGDR ≥8 mg/kg/min n=11.
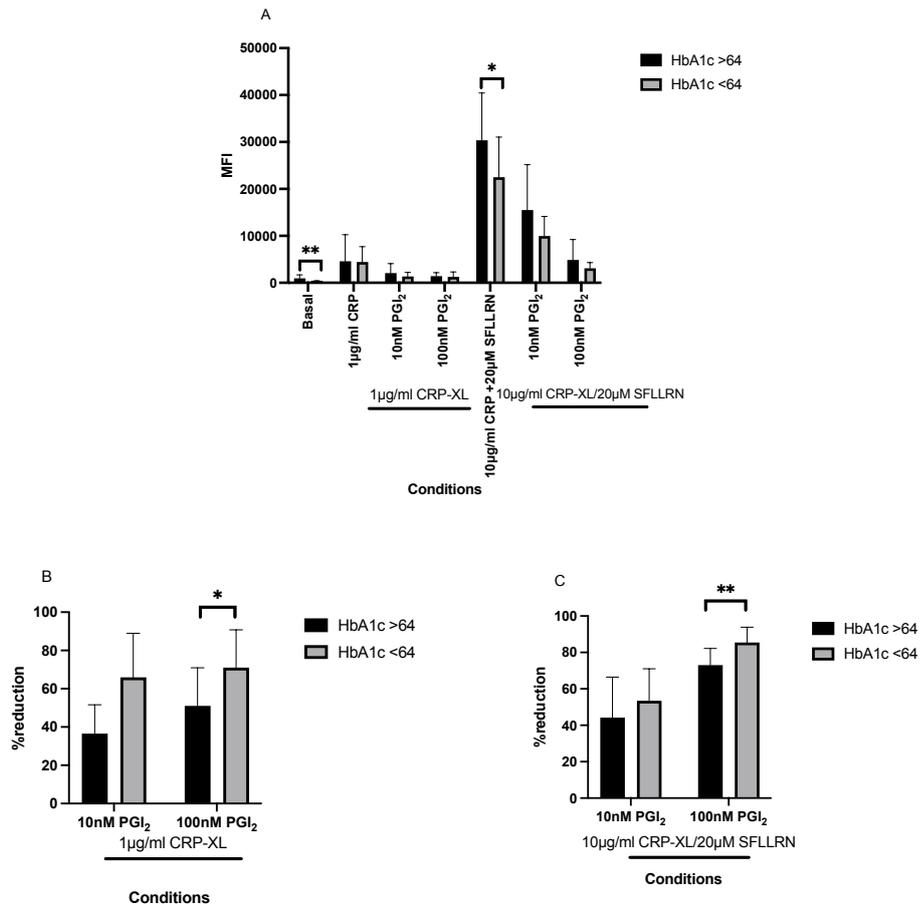
Figure 5-24: Multiparameter flow cytometry MFI AnnV expression at basal and stimulated with 1 μg/ml CRP-XL and dual agonists (10 μg/ml CRP-XL and 20 μM SFLLRN) with and without PGI₂ (10 nM and 100 nM) in whole blood stratified according to eGDR.

Panel A represents MFI expression. Panel B represents percentage reduction in the presence of 10/100 nM PGI₂ compared to expression following stimulation with agonist alone at 1 μg/ml CRP-XL. Panel C represents percentage reduction in the presence of 10/100 nM PGI₂ compared to expression following stimulation with dual agonists. *p<0.05 and **p<0.01. eGDR <6 mg/kg/min n=7, eGDR 6-8 mg/kg/min n=7 and eGDR ≥8 mg/kg/min n=11.

5.4 Discussion

Understanding increased cardiovascular risk in patients with diabetes must not only focus on the increase in activation and hyperreactivity but also look at the converse factors that should, under normal physiological conditions, provide opposition and reduce platelet activation.

My data are the first to assess response to prostacyclin simultaneously across a variety of markers representing different aspects of platelet activation. Importantly, this is also the first study to investigate the response to prostacyclin in the setting of DD; albeit previous evidence pointed towards a reduced sensitivity to PGI_2 in obese individuals without diabetes, with a degree of improved sensitivity following weight loss which also correlated with improved insulin sensitivity [330], which may indeed be relevant to our patient cohort with the lowest eGDR and thus highest IR.

Under basal conditions, these data have shown that patients with T1D have increased expression of CD62P, PAC1 and AnnV-binding compared with healthy controls, again suggesting that platelets in patients with T1D circulate in an activated rather than truly quiescent state. Furthermore, the platelets from patients with T1D appeared hyperreactive in response to stimulation with platelet agonist when compared with healthy controls. Based on the optimisation data, CD62P expression reached maximal with almost all the strengths of agonist and thus it was only possible to detect differences with low dose 2 μM SFLLRN. Differences between those with T1D and healthy controls were more evident with PAC1 expression, with greater expression seen in patients T1D at all strengths of agonists. AnnV binding was shown to

be greater in patients with T1D following dual agonist stimulation, suggesting a greater procoagulant propensity.

These data also showed a differential response to PGI_2 between patients with T1D and healthy controls. At any dose of PGI_2, particularly in the setting of potent agonist stimulation, there is diminished CD62P response comparative to both PAC1 and AnnV. Thus, it was only with the lower strength agonist stimulation that differences were detected between those with T1D and healthy controls in terms of CD62P expression. In terms of PAC1 expression, there was a decreased sensitivity to PGI_2 in patients with T1D, more evident with the higher dose of PGI_2 in the setting of stronger agonist stimulation, with similar results seen in AnnV expression.

Beyond these initial data comparing patients with T1D and healthy controls, this study also assessed the relationship between sub-optimal glycaemic control/ insulin resistance and PGI_2 response. CD62P expression again did not show a particularly strong response to PGI_2, particularly in the setting of dual agonist stimulation and so clear differences in those with elevated HbA1c and lower HbA1c were not apparent. Yet, when comparing expression in those without insulin resistance (eGDR ≥ 8 mg/kg/min) to those with the highest insulin resistance (eGDR <6 mg/kg/min) there was evidence of a diminished sensitivity to PGI_2 amongst the latter. Although there was a degree of variation in the data, both sub-optimal glycaemic control and higher insulin resistance appeared to correlate with a smaller reduction in PAC1 expression following treatment with PGI_2. For AnnV expression, mostly based on the results following dual agonist stimulation, patients with higher HbA1c and lower

eGDR had a lower reduction in expression in the presence of PGI$_2$ than those with lower HbA1c and no insulin resistance.

Collectively, these data therefore support previously published data suggesting diminished sensitivity to inhibition in patients with T1D and furthermore, given effects were seen across all three markers these changes are not preferentially driven by one mechanism of platelet activation. Beyond this, these changes may be further exacerbated by hyperglycaemia and also insulin resistance. This is an important consideration when assessing a patient's risk of developing cardiovascular complications. Again, this is a young cohort of patients without pre-existing macrovascular disease and it is possible these differences could be even greater in older populations in the presence of co-morbidities. Furthermore, it suggests the conversation about anti-platelet therapies in the setting of primary prevention should still be considered, particularly in patients with low-risk of bleeding complications. It does however support the well-recognised benefits of tackling individual risk factors such as improving glycaemic control and reducing insulin resistance, as suggested by the Russo *et al.,* study showing improved sensitivity to PGI$_2$ following weight loss and simultaneous reduction in insulin resistance [330].

One caveat to highlight is that whilst there appears to be an association between both IR and sub-optimal glycaemia with the changes in platelet function it is difficult to assess for true causality through this data alone. It may be the case both are manifestations of other disease processes and further data would be required to characterise this more. Further to this, another limitation of these data is the lack of thorough characterisation of the healthy control cohort. We did not take a detailed medical history nor smoking/alcohol

status which may affect platelet function (although volunteers were asked not to participate if they had chronic health conditions, long-term medication or on anti-platelet/anti-coagulant medications).

This chapter further characterises the functional phenotype of platelets in patients with T1D and DD. The results and those from the previous chapter demonstrate that not only are platelets potentially circulating in a pre-activated state, they are hyperreactive following stimulation and hyporesponsive to inhibition. This phenotype appears even stronger in those with greater hyperglycaemic exposure and in the presence of insulin resistance. Having now illustrated some of these functional differences, understanding what drives and influences these changes is an integral part of identifying potential mechanisms. Furthermore, this may help to identify areas to target in an attempt to ultimately develop novel therapies that can reduce these functional changes and thereby mitigate the potential contribution to the increased cardiovascular risk in this patient cohort.

Chapter 6: Bioenergetic profiling of platelets in T1D

6.1 Introduction

Whilst exploring changes in platelet function in the setting of both T1D and DD are important, it is next crucial to explore the potential mechanisms that are driving this altered functional phenotype. As explored in chapter one, platelets have a myriad of functions that extend far beyond haemostasis and most of these functions require rapid activation driven by access to efficient and abundant energy sources. Platelet mitochondrial oxidative phosphorylation and glycolysis are crucial processes facilitating the switch from quiescent to an activated state through ATP generation [331]. Evidence has shown that platelets possess a high degree of metabolic flexibility that allows them to respond adequately in situations of thrombosis or haemorrhage [332]. Ultimately, functional changes are driven by this metabolic shift and platelets are able to switch seamlessly between glycolysis and oxidative phosphorylation using glucose or free fatty acids (FFA) as their energy source [186]. With further supporting evidence of a switch from predominantly oxidative phosphorylation at rest to aerobic glycolysis following stimulation, mediated in part by pyruvate dehydrogenase (PDH) [333].

It has now become apparent that changes in metabolic flux and cellular bioenergetics are signatures associated with many diseases. Given the key role of glycolysis and oxidative phosphorylation in fuelling platelet activity, alterations in these processes can result in disordered platelet function and this has been implicated in a number of disease processes [334]. Further to this, mitochondrial dysfunction has been especially implicated in the

upregulation of PS expression in the setting of a procoagulant subpopulation of platelets [335] as seen in our study patients with poor glycaemic control and/or presence of insulin resistance in chapters 4 and 5. Whilst mitochondrial dysfunction and associated platelet function abnormalities have been documented in a number of acute pathological processes, including ischaemia and sepsis, patients with T2D have also shown altered ATP synthesis and reduced mitochondrial membrane potential [197]. Similarly, though there is comparatively less evidence, altered platelet glycolysis has been implicated in disease states including pulmonary arterial hypertension as well as atherosclerosis [336, 337].

Beyond the roles of both glycolysis and mitochondrial respiration in meeting energy demand in platelets, understanding the sources of fuel in the setting of both normal physiology and in patients with diabetes may help to explain any bioenergetic as well as the functional changes described in earlier chapters.

Glucose has long been considered the predominant substrate for both glycolysis and oxidative phosphorylation within the platelet. Glucose is taken up by platelets via glucose transporters or GLUTs [338]. It was first established more than 30 years ago that platelets express GLUTs on their surface, with more in depth evidence showing they specifically possess GLUT-1 and GLUT-3 [339]. GLUT-1 is thought to be predominantly responsible for basal uptake of glucose, though far less abundant than GLUT-3 which has been shown to account for about 85% of GLUT expression in platelets [340]. Upon platelet activation there is now convincing evidence to support glucose uptake predominantly through GLUT-3 [341]. At rest, GLUT-3 is found located within

α-granules themselves, unlike GLUT-1, which is expressed constitutively on the cell surface. Following activation, GLUT-3 is mobilised from within the α-granules and translocated to the membrane surface to facilitate transport of glucose [342]. GLUT-3 knockout mice have been shown to have reduced markers of platelet activation involved in α-granule secretion, whilst some other activation markers remained unaffected, suggesting that glucose is not the sole source of fuel in these cells [343]. Platelets have also been shown to have glycogen stores and thus have an endogenous glucose source that has also been directly linked to platelet activation and mitochondrial respiration [344]. Furthermore, evidence supports that glycogenolysis, even in the absence of glucose, can sufficiently support platelet activation and play a role in various platelet functions [186].

Despite the focus on glucose, there is evidence of the role of alternative substrates dating back to the 1970s including FFA and glutamine which have also been linked to platelet aggregation and activation [345-347]. However, these pathways are comparatively poorly characterised. One study demonstrated that in the absence of glucose, platelets possess the metabolic flexibility to switch from glucose to FFA to respond to energy demands, which occurred both basally and following stimulation [186]. The data also supported the notion that in the absence of glucose, access to FFA is essential for maintaining oxidative phosphorylation with minimal effect on oxygen consumption rate (OCR) measurement in the absence of glucose alone, yet significantly reduced OCR in the absence of glucose and presence of β-oxidation (FA oxidation) inhibitors [186]. Again, this suggests that platelets possess the ability to switch substrates depending on the respective

availability of nutrients, whilst seemingly maintaining these important metabolic processes.

In the setting of T1D, evidence of glucose uptake is once again conflicting. Whilst limited data exist in humans with T1D, mice/rat models of diabetes have demonstrated both decreased and increased glucose uptake in platelets and macrophages [187, 189]. It is therefore imperative that further studies, particularly in people with T1D, are conducted.

Thus, whilst the use of bioenergetics as a research tool in disease states is still a relatively novel area, studies examining these processes in patients with T1D are particularly lacking. This chapter therefore first explores potential alterations in the bioenergetic profiles of platelets in patients with T1D and whether these may be a contributory mechanism driving the functional changes already explored in the previous chapters. Following this, we aimed to examine glucose uptake in patients with T1D compared with healthy controls both under basal conditions and following stimulation as well as the relative contribution of FFA as a substrate in patients with T1D.

6.2 Methods

6.2.1 Study Population

The DEVELOP study has been detailed in prior chapters including the inclusion/exclusion criteria. Isolated platelets were prepared (chapter 2, section 2.4.1) [348] from both study patients and healthy controls.

For healthy control data, samples were again taken from healthy volunteers as described in previous chapters. The same platelet isolation protocol was used as the patients with T1D.

6.2.2 Seahorse real-time cell metabolic analysis

Detailed methods for carrying out real time cell metabolic analysis using the Seahorse XFe96 Analzyer (Agilent Technologies, Inc) can be found in chapter 2, section 2.4.5. Experiments were conducted in line with the standard Cell Mito Stress test and Glycolysis Stress test using the Agilent supplied kits. Oxygen consumption rate (OCR) as a measure of mitochondrial respiration and Extracellular Acidification Rate (ECAR) as a measure of glycolysis were included. Data were collected and interpreted in line with Agilent validated parameters (Figure 6-1) [349, 350] and Table 6-1 summarises the calculations used to derive these data. For the Mito Stress Test, basal respiration is made up of ATP-linked respiration (ATP-production) and proton leak. The addition of oligomycin, an inhibitor of ATP-synthase allows calculation of ATP-linked respiration, as the drop from basal respiration [351]. The remainder excluding non-mitochondrial respiration is therefore equivalent to proton leak. Increase proton leak is considered multifactorial but in part may be due damage to inner membranes of the mitochondria in the presence of mitochondrial dysfunction [352].
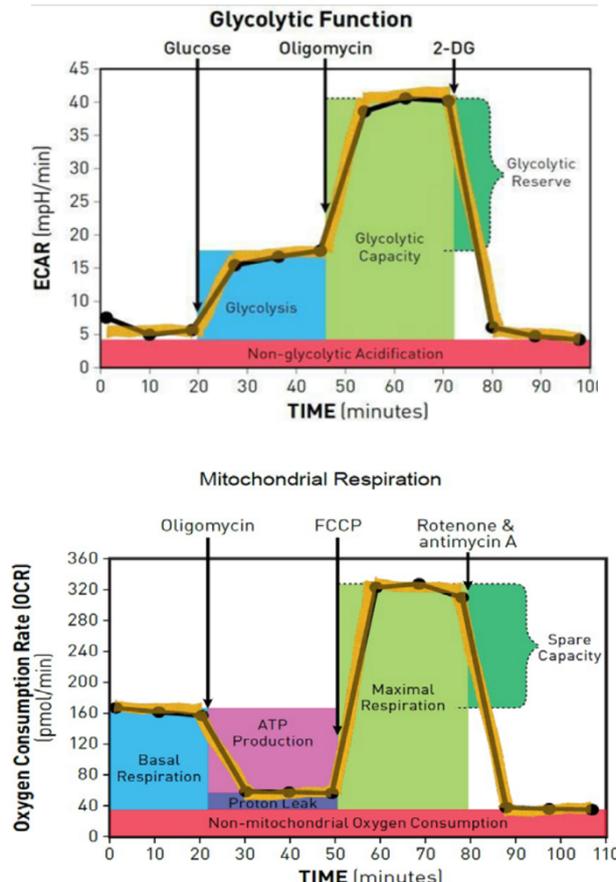
Figure 6-1: Agilent Technologies standard profiles for Glycolysis and Mito Stress Test.

Top panel, Glycolysis Stress Test, measuring Extracellular Acidification Rate and bottom panel Mito Stress Test, measuring Oxygen Consumption Rate. Oligomycin acts on ATP synthase to block electronic flow and decrease mitochondrial respiration (conversely increasing glycolysis), 2-DG (2-deoxy-glucose) binds to hexokinase blocking the glycolysis pathway, FCCP (carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone), is an uncoupling agent which disrupts mitochondrial membrane potential facilitating maximal mitochondrial respiration. Antimycin A/rotenone act on complex III and complex I of the ETC respectively to completely block mitochondrial respiration.

Produced from Agilent Technologies materials.

Parameter	Calculation
Basal Glycolysis	(Rate following glucose, before oligomycin)-(rate prior to glucose injection)
Non-Glycolytic Acidification	Rate prior to glucose injection
Glycolytic capacity	(Rate following oligomycin injection, before 2-DG)-(measurement prior to glucose injection)
Glycolytic reserve	(Glycolytic capacity) – (Glycolysis)
Non-mitochondrial respiration	Minimum rate following Rotenone/Antimycin A
Basal Respiration	(Rate prior to oligomycin) – (non-mitochondrial respiration)
ATP-production	(Rate prior to oligomycin) – (rate following oligomycin)
Proton leak	(Rate following oligomycin) – (non-mitochondrial respiration)
Maximal Respiration	(Rate following FCCP) - (non-mitochondrial respiration)
Spare (Reserve) Capacity	(Maximal Respiration) - (Basal Respiration)

Table 6-1: Agilent Technologies standard calculations for Glycolysis Stress Test [349] and Mito Stress Test [350].

6.2.3 Glucose uptake in platelets

Glucose uptake experiments were conducted using previously established protocols within the group using 2-[N-(7-nitrobenz-2-ox-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose or 2-NBDG) (Abcam), followed by flow cytometric analysis using the Cytoflex S. 2-NBDG is a glucose analogue and is labelled with a fluorophore, 7-nitrobenzanfurazan. Data were collected at rest and following stimulation with the platelet agonist, SFLLRN (20μM). For stimulated glucose uptake, the washed platelets were incubated with SFLLRN for 10 minutes prior to addition of the 2-NBDG fluorophore as per protocol. No isotype control was required and platelet identification was established

through FFC and SSC given the use of washed platelets and thus low interference from other cells.

6.2.4 Platelet GLUT analysis

Flow cytometric analysis data were collected using a two-colour assay. For both GLUT-1 and GLUT-3, commercial antibodies were available conjugated to Alexa Fluor 405 along with APC-CD42b as the platelet identification marker. These were used at variable concentrations described later in detail. As per chapter 2, antibodies were incubated with blood or washed platelets for 20 minutes at room temperature whilst kept in the dark. Again, all cytometric analysis was conducted using the Cytoflex S. Compensation was conducted as required where spectral overlay between fluorophores existed. GLUT analysis was initially carried out in whole blood and later in isolated platelets using standard protocols already described.

6.2.5 β-oxidation measured by Seahorse Analyzer

An adapted Mito Stress Test described in detail in the previous chapter was performed to investigate the role of fatty acid oxidation in platelet mitochondrial respiration. This involved the addition of a β-oxidation inhibitor, etomoxir, at a concentration chosen based on established protocols which have been used in previously published work by the group [186]. Only rotenone/antimycin-A from the standard Mito Stress Test was then included to allow calculation of non-mitochondrial respiration given FA oxidation was the focus of these data.

6.2.6 Statistical analysis

All data within this chapter were analysed using GraphPad Prism version 9.5.1 (528). For descriptive data, results are presented as median (interquartile range), mean±standard deviation (SD) and/or number (% of total). Where appropriate normal distribution was assessed using Shapiro-Wilks. One-way ANOVA (Kruskal Wallis) was used to test multiple continuous variables. For comparison of two independent continuous variables using Student t-test or Mann-Whitney test depending on the normative distribution and relevant standard deviation of the two groups. Correlation was used to test for a linear relationship between two continuous variables.

6.3 Results

6.3.1 Patient characteristics

The total number of patients from whom data were included in this chapter are summarised in table 6-2. From the wider DEVELOP study there were a total of 10 patients from whom data have been included for analysis, these are a separate cohort to previous chapters. Data were excluded from 1 patient at data points requiring oligomycin and FCCP due to reagent issues, but basal data have still been included. The patients from whom data were included for glucose uptake in this chapter are a subset of those who were included in the Glycolysis/Mito Stress Tests and are summarised in table 6-3. Patients who underwent the adapted Mito Stress Test with etomoxir are summarised in table 6-4 and are separate from those included in the standard Mito Stress and Glycolysis Stress tests, n=4 underwent the adapted Mito Stress Test with

etomoxir. A total of n=6 underwent glucose uptake experiments under basal conditions with 4 out of the 6 undergoing additional glucose uptake following stimulation. Formal characterisation was not done for healthy volunteers.

	Total
Number of participants	10
Male sex (%)	6 (60%)
Age, years	23.3±2.7
Duration of diabetes, years	11.2±5.3
HbA1c, mmol/mol	62±11
BMI, kg/m^2	27.4±6.5
Estimated glucose disposal rate (WC), mg/kg/min	8.3±2.4
Total daily insulin (U/24h)	56±16.8
Total daily insulin (U/kg)	0.7±0.2
Family history of T2DM	1 (10%)
Presence of retinopathy	2 (20%)
Adjunctive therapy (metformin)	1 (10%)
Macrovascular complications	0

Table 6-2: Summary of DEVELOP study patient characteristics from whom samples were included in Seahorse data analysis.

Data are representative of mean value±standard deviation excluding age which is median±standard deviation.

	Total
Number of participants	6
Male sex (%)	3 (50%)
Age, years	22.9±3.1
Duration of diabetes, years	11.7±5.4
HbA1c, mmol/mol	67±9.7
BMI, kg/m^2	28.3±7.9
Estimated glucose disposal rate (WC), mg/kg/min	8.1±2.4
Total daily insulin (U/24h)	61.2±18.2
Total daily insulin (U/kg)	0.7±0.2
Family history of T2DM	0
Presence of retinopathy	2 (33%)
Adjunctive therapy (metformin)	1 (17%)
Macrovascular complications	0

Table 6-3: Summary of DEVELOP patient characteristics from whom samples were taken and included in glucose uptake data analysis.

Data are representative of mean value±standard deviation excluding age which is median±standard deviation.

	Total
Number of participants	4
Male sex (%)	2 (50%)
Age, years	22.5±3.2
Duration of diabetes, years	9.8±4.5
HbA1c, mmol/mol	61.3±15.2
BMI, kg/m^2	29.3±9.7
Estimated glucose disposal rate (WC), mg/kg/min	8.6±2.6
Total daily insulin (U/24h)	60.9±27.6
Total daily insulin (U/kg)	0.7±0.1
Family history of T2DM	1 (25%)
Presence of retinopathy	1 (25%)
Adjunctive therapy (metformin)	1 (25%)
Macrovascular complications	0

Table 6-4: Summary of patient characteristics from whom samples were taken and included in Mito Stress Test with etomoxir data.

Data are representative of mean value±standard deviation excluding age which is median±standard deviation.

6.3.2 Basal glycolysis and mitochondrial respiration in patients with T1D

First, basal glycolysis was analysed for the cohort of patients with T1D (n =10), compared with the healthy controls (n=10). Patients with T1D had a significantly lower basal glycolysis ECAR than healthy controls [ECAR (mean±SD) of 0.24±0.4 versus 1.5±0.6mpH/min, respectively, *p<0.0001*], shown in Figure 6-2. Furthermore, although stratifying data according to clinical parameters was limited by group sizes, linear regression of HbA1c and eGDR against ECAR was conducted to investigated for any correlation. HbA1c demonstrated no correlation, r^2=0.02 *(p=0.83),* though it should be noted that the cohort had relatively good glycaemic control with a mean of 62mmol/mol and only 3 out of the 10 patients (30%) with HbA1c ≥64mmol/mol which was the median threshold used in the stratification of activation panel data as well as response to PGI$_2$ in the previous two chapters. The data were

also stratified according to eGDR(waist circumference). There was evidence of a correlation between higher eGDR, that is, lower insulin resistance and higher glycolysis, $r^2=0.5$, *p=0.02*. These data are shown in Figure 6-3, with the left sided panel A representative of HbA1c and the right (panel B) representative of eGDR.
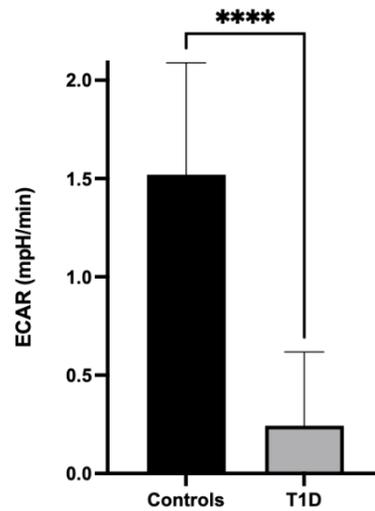
Figure 6-2: Basal glycolysis measured through ECAR (mpH/min) on Seahorse XFe96 Analyzer (Agilent Tech Inc) conducted on isolated platelets in patients with T1D and healthy controls.

Statistical analysis compares patients with T1D and controls. ****$p<0.0001$. n=10 in both groups.
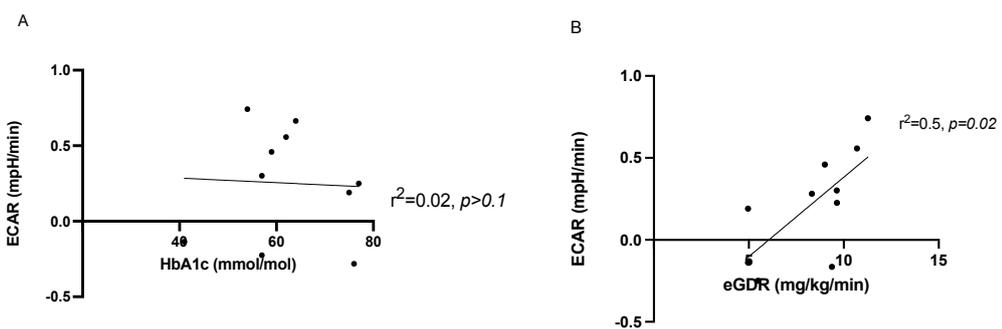
Figure 6-3: Basal glycolysis, ECAR (mpH/min), on Seahorse XFe96 Analyzer (Agilent Tech Inc) conducted on isolated platelets in patients with T1D stratified by HbA1c and eGDR.

Data are stratified according to HbA1c in panel A and by eGDR in panel B. Statistical analysis was conducted by linear regression analysis. n=10.

Basal mitochondrial respiration, indicative of oxidative phosphorylation was also measured. Data are shown in Figure 6-4. Patients with T1D had a statistically higher basal mitochondrial respiration than healthy controls [11±5pmol/min versus 5±2 pmol/min, respectively, *p=0.005*]. Again, data were correlated against both HbA1c and eGDR values within the cohort of patients with T1D and no correlations were found (r^2=0.03, *p=0.66* and r^2=0.2, *p=0.19,* respectively)*.* These data are shown in Figure 6-5 with panel A representing correlation with HbA1c and panel B representing the same for eGDR.
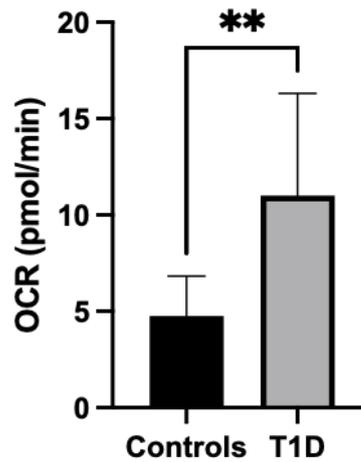
Figure 6-4: Basal mitochondrial respiration, measured through OCR (pmol/min) on Seahorse XFe96 Analyzer (Agilent Tech Inc) conducted on isolated platelets in patients with T1D and healthy controls.

Statistical analysis compares patients with T1D and controls. *$**p<0.01$*. n=10 in both groups.
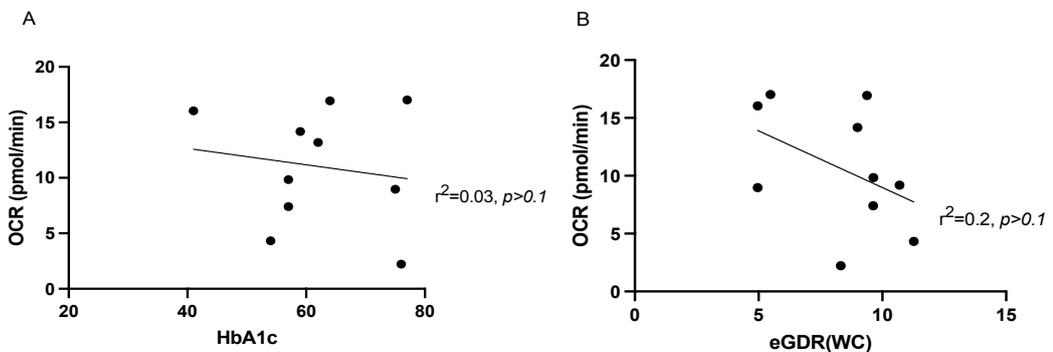
Figure 6-5: Mitochondrial respiration, OCR (pmol/min), on Seahorse XFe96 Analyzer (Agilent Tech Inc) conducted on isolated platelets in patients with T1D stratified according to HbA1c and eGDR.

Data are stratified according to HbA1c in panel A and eGDR in panel B. Statistical analysis was conducted by correlation analysis between parameters. n=10.

6.3.3 Glycolytic Stress Test in patients with T1D

Further to the basal glycolysis measurement, analysis can be done to provide insight into the potential effects of T1D on different aspects of glycolysis using the Glycolysis Stress Test. Non-glycolytic acidification is the ECAR measurement prior to injection with glucose and refers to processes beyond glycolysis that contribute to the cellular acidification rate. There was no difference between the two groups in this parameter (Figure 6-6). Furthermore, glucose refers to the total ECAR measured following addition of glucose and although numerically higher in the patients with T1D, the difference failed to reach statistical significance. Glycolysis refers to the total measurement following addition of glucose minus the non-glycolytic acidification rate and this is where a difference was identified, with significantly lower basal glycolysis in patients with T1D. Glycolytic capacity refers to the maximal possible rate of glycolysis i.e., conversion of glucose into either pyruvate or lactate and is essentially a surrogate measure of the capacity of ATP generation. Here, both patients with T1D and healthy controls had very similar maximum glycolytic capacity, with no difference detected comparing the two groups (5.6±3mpH/min in healthy controls versus 5.2±2mpH/min in T1D, *p=0.67*). Finally, glycolytic reserve, calculated through glycolytic capacity minus glycolysis and referring to the response of platelets to an energy demand showed no differences between the two groups (4.1±3mpH/min in healthy controls versus 4.6.0±2mpH/min in T1D, *p=0.59).* There was a relatively high level of variation amongst both patients with T1D and healthy

controls suggesting greater numbers are needed to identify potential differences between groups.

Figure 6-6: ECAR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls.

Panel A represents the combined ECAR measurements against time (minutes) with an indication of the time points for each injection of reagents representing the typical Glycolysis Stress Test. Panel B represents the same data directly analysed for each of the relevant parameters with statistical analysis carried out for patients with T1D versus healthy controls. ****$p < 0.0001$. 2-DG= 2-deoxy-glucose. n=10 in both groups.

In addition to each of the individual parameters being compared between the two groups, data were also analysed comparing parameters against one another and are presented below.

6.3.3.1 Assessing the relationship between basal glycolysis and glycolytic capacity

First comparing basal glycolysis and glycolytic capacity. The data are largely shifted left in the patients with T1D, driven by the lower basal glycolysis already discussed. However, there was no clear correlation with the samples studied; r^2=0.28 in patients with T1D, *p=0.12* and r^2= 0.03 in controls, *p=0.65.* These data are shown in Figure 6-7.

Figure 6-7: ECAR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; comparison of basal glycolysis and glycolytic capacity.

Data are plotted as basal glycolysis (mpH/min) on the x-axis against glycolytic capacity (mpH/min) on the y-axis. Statistical analysis assessed linear regression for each group. n=10 in both groups.

6.3.3.2 Assessing the relationship between basal glycolysis and glycolytic reserve

Next, we assessed the correlation between basal glycolysis and glycolytic reserve, as shown in Figure 6-8. A trend towards a correlation between basal glycolysis and glycolytic reserve was evident in the T1D group (r^2=0.38, *p=0.08*) but not controls r^2=0.15, *p=0.26*).

Figure 6-8: ECAR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; comparison of basal glycolysis and glycolytic reserve.

Data are plotted as basal glycolysis (mpH/min) on the x-axis against glycolytic reserve (mpH/min) on the y-axis. Statistical analysis assessed linear regression for each group. n=10 in both groups.

6.3.3.3 Assessing the relationship between glycolytic capacity and glycolytic reserve

Finally, a comparison was made between glycolytic capacity and the glycolytic reserve. As glycolytic capacity is included in the algorithm for calculating glycolytic reserve the two were closely correlated in both groups, $r^2=0.98$ $p<0.0001$ in patients with T1D and $r^2=0.94$, $p<0.0001$ in healthy controls. There was a tendency towards higher glycolytic reserve with a left and upwards shift in the patients with T1D compared with health controls. The data are shown In Figure 6-9.

Figure 6-9: ECAR data from Seahorse xFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; **comparison of glycolytic capacity and glycolytic reserve.**

Data are plotted as basal glycolysis (mpH/min) on the x-axis against glycolytic reserve (mpH/min) on the y-axis. Statistical analysis assessed as linear regression for each group. n=10 in both groups.

6.3.4 Mito Stress Test in patients with T1D

Similar analysis can be conducted on the data from the Mito Stress test to investigate other aspects of mitochondrial respiration in patients with T1D. The various parameters of interest and the algorithms by which they are calculated were summarised in table 6-1. Figure 6-10 provides a summary of all the OCR readings for each group with all of the relevant time points at which reagents are injected during a typical Mito Stress test labelled.

In patients with T1D there was a significantly higher non-mitochondrial respiration compared with healthy controls [(mean±SD) 5.0±2mpH/min versus 2.2±2mpH/min, respectively *p=0.02*; Figure 6-10]. Patients with T1D had higher proton leak compared with controls (5.1±4 versus 0.7±1pmol/min, *p=0.006*; Figure 6-10) with a trend towards higher ATP-linked respiration (7.2±4 versus 4.4±2pmol/min, *p=0.08*). As a % of total basal mitochondrial respiration, patients with T1D therefore had a higher proportion attributable to proton leak, 36±24% compared with 12±18% in healthy controls, *p=0.03*.

Maximal respiratory capacity is measured following the addition of an uncoupler, FCCP, which simulates a physiological energy demand and triggers oxidation of substrates in response and thus equates to the cell's ability to meet energy demands physiologically. As shown earlier this is the maximal OCR measured minus the non-mitochondrial respiration. Across the two groups maximal respiratory capacity appeared very similar, with a mean of 14±5mpH/min in patients with T1D compared with 13±5mpH/min in healthy controls, *p=0.76.* Further to this, the reserve capacity, calculated as the maximal capacity minus the basal respiration was also analysed. As a result of the higher basal mitochondrial respiration and comparable maximal

capacity, there was a trend for patients with T1D to have a diminished reserve capacity compared with healthy controls (4.1±5 versus 8.5±5, *p=0.06*).

Figure 6-10: OCR data from Seahorse xFe96 Analyzer using isolated platelets from patients with T1D and healthy controls.

Panel A represents combined OCR measurements against time (minutes) with an indication of the time points for each injection of reagents. Panel B represents the same data directly analysed for each of the relevant parameters with statistical analysis carried out for patients with T1D versus healthy controls. *p<0.05, **p<0.01. FCCP= (carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone), Rot/AA= Rotenone/Antimycin A. n=10 in both groups.

Each of the individual parameters described were then correlated against one another to assess for potential correlations and differences between the patients with T1D and the healthy controls.

6.3.4.1 Assessing the relationship between basal mitochondrial respiration and maximal respiratory capacity

First a comparison between basal mitochondrial respiration (x-axis) and maximal respiratory capacity (y-axis) was made. While a trend of a correlation between basal mitochondrial respiration and maximal respiratory capacity was evident in the diabetes group, no such relationship was found in controls (r^2= 0.24, *p=0.09* versus r^2=0.07, *p=0.36*; Figure 6-11).

Figure 6-11: OCR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; comparison of basal mitochondrial respiration and maximal respiratory capacity.

Data are plotted as basal mitochondrial respiration (pmol/min) on the x-axis against maximal respiratory capacity (pmol/min) on the y-axis. Statistical analysis assessed as correlation for each group. n=10 in both groups.

6.3.4.2 Assessing the relationship between basal mitochondrial respiration, ATP-linked respiration and proton leak

Next, basal mitochondrial respiration (x-axis) was correlated against ATP-linked respiration. There was a strong correlation between higher basal mitochondrial respiration and increased ATP-linked respiration in both T1D and controls ($r^2 = 0.88$, *p<0.0001* and $r^2 = 0.90$ *p<0.0001*; Figure 6-12).
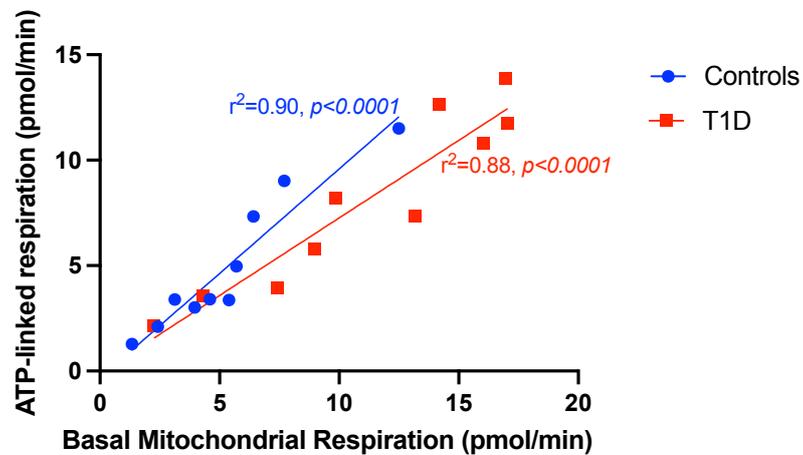
Figure 6-12: OCR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; comparison of basal mitochondrial respiration and ATP-linked respiration.

Data are plotted as basal mitochondrial respiration (pmol/min) on the x-axis against ATP-linked respiration (pmol/min) shown on the y-axis. Statistical analysis assessed as correlation for each group. n=10 in both groups.

Basal mitochondrial respiration was also correlated against proton leak. A correlation between basal respiration and proton leak was identified in T1D but only a trend was observed in controls (r^2=0.6, *p=0.008* versus r^2=0.21, *p=0.08;* Figure 6-13).
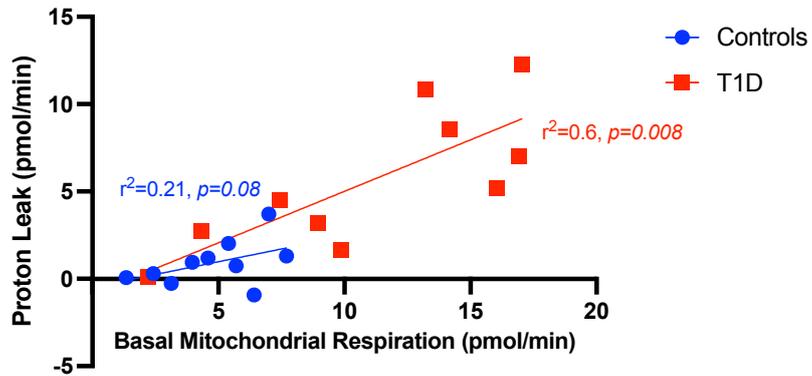
Figure 6-13: OCR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; comparison of basal mitochondrial respiration and proton leak.

Data are plotted as basal mitochondrial respiration (pmol/min) on the x-axis against proton leak (pmol/min) shown on the y-axis. Statistical analysis assessed as linear regression for each group. n=10 in both groups.

6.3.4.3 Assessing the relationship between maximal respiratory capacity, ATP-linked respiration and reserve capacity

Maximal respiratory capacity and ATP-linked respiration were then compared. There was a correlation between the two parameters in both T1D and controls (r^2=0.80, *p=0.0005* versus r^2=0.57, *p=0.01*; Figure 6-14).
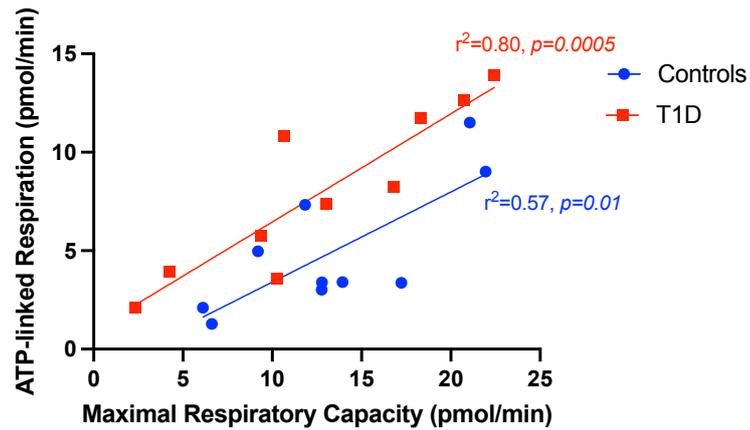
Figure 6-14: OCR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; comparison of maximal respiratory capacity and ATP-linked respiration.

Data are plotted as maximal respiratory capacity (pmol/min) on the x-axis against ATP-linked respiration (pmol/min) shown on the y-axis. Statistical analysis assessed as linear regression for each group. n=10 in both groups.

Finally, a comparison was made between maximal respiratory capacity and reserve capacity. T1D and healthy controls showed a similar correlation between maximal respiratory capacity and ATP-linked respiration ($r^2=0.54$, *p=0.02* versus $r^2=0.71$, *p=0.002;* Figure 6-15).
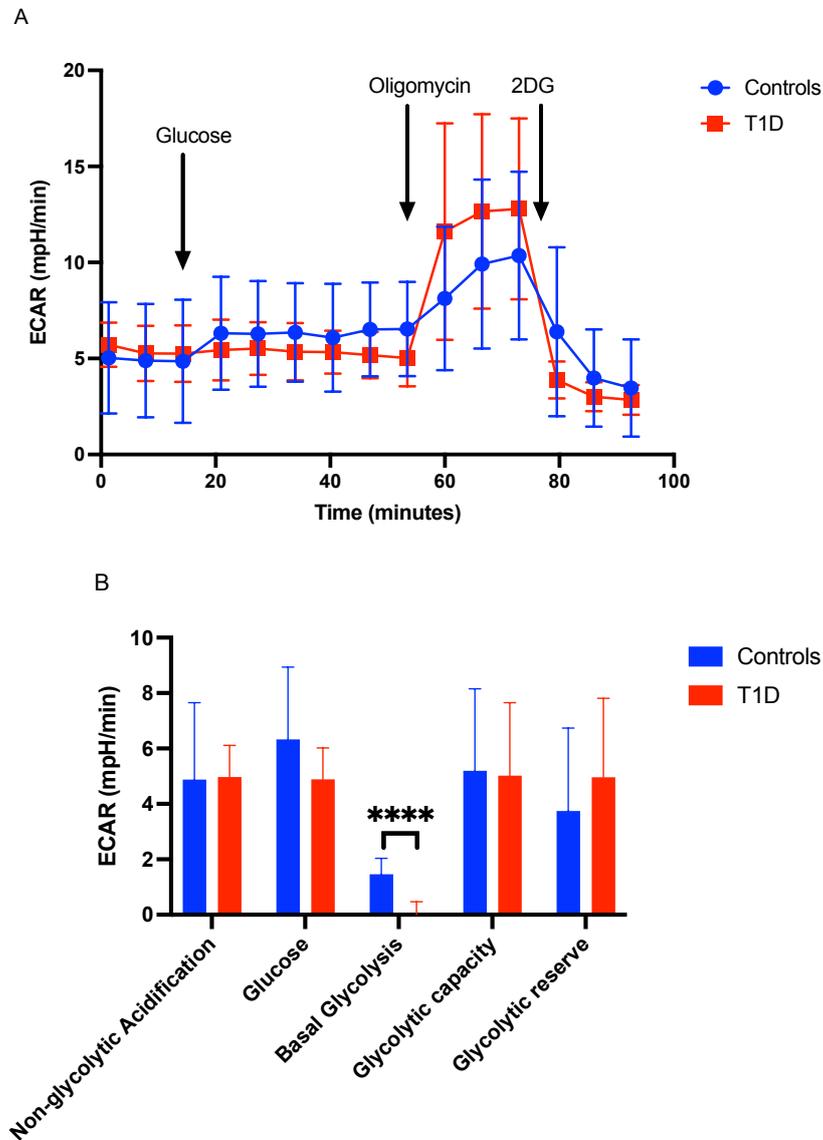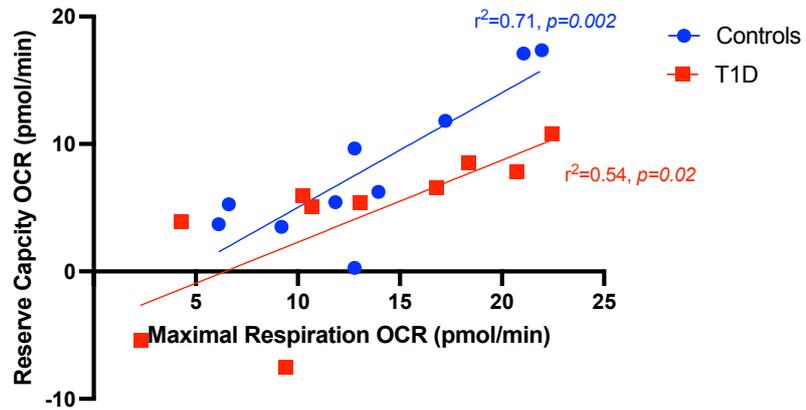

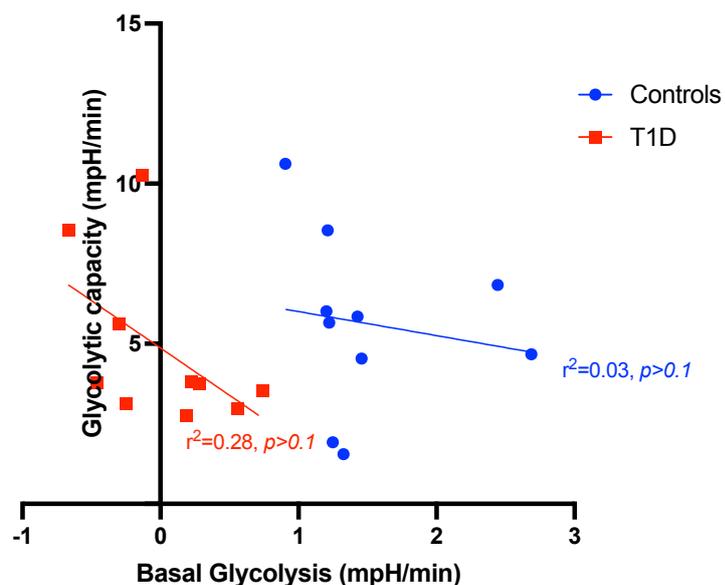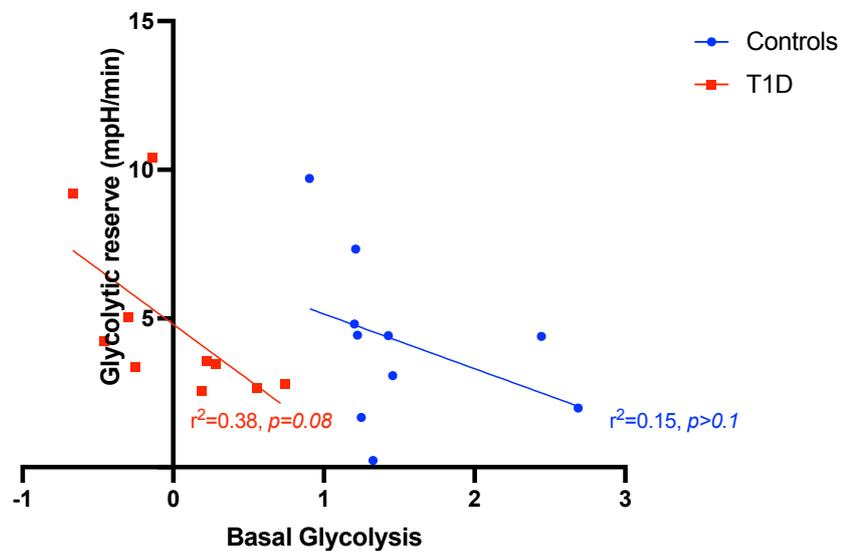**Figure 6-15: OCR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; comparison of maximal respiration and reserve capacity,**

Data are plotted as maximal respiratory capacity (pmol/min) on the x-axis against ATP-linked respiration (pmol/min) shown on the y-axis. Statistical analysis assessed as linear regression for each group. n=10 in both groups.

6.3.5 Agonist-stimulated glycolysis and mitochondrial respiration in T1D

In addition to measuring basal glycolysis and mitochondrial respiration, measurements were taken for both ECAR and OCR following addition of two different platelet agonists, CRP-XL as per previous chapters along with thrombin. Both agonists were added prior to the reagents for both the Glycolysis stress test (but after glucose) and Mito stress test i.e. prior to oligomycin in both cases. Additional measurements for basal glycolysis and basal mitochondrial respiration were taken independently of the data presented above.

Data were first analysed for ECAR and OCR separately to assess impact of agonists on each of the bioenergetic parameters and then a comparison was made between thrombin and CRP-XL to investigate any differential effects. Thrombin was included as a second agonist as these data were novel and the lab group had previous experience of thrombin in similar experiments but not CRP-XL. Given the use of CRP-XL in the previous functional assays, this was kept for comparison.

As shown, there is a sharp, and similar, rise in ECAR in response to injection with CRP-XL (at 36 minutes) in both groups (Figure 6-16, panel A). The change in ECAR was measured from prior to injection with CRP-XL to the maximal ECAR measurement following injection of CRP-XL prior to addition of oligomycin, again indicated on the graph. Patients with T1D had a mean rise of 12.2±2mpH/min compared with 10.0±3mpH/min in healthy controls,

demonstrating a marginal difference between the two groups ($p=0.07$; Figure 6-16, panel C).

Following injection with CRP-XL, there was a greater increase in OCR in T1D compared with healthy controls (23.1 ± 6 versus 16.7 ± 5pmol/min, respectively, $p=0.02$; Figure 6-16, panel B). Again, the change in OCR was measured as the maximal OCR measured following CRP-XL administration prior to oligomycin minus the OCR measurement prior to CRP-XL injection. Only a trend for a difference was observed comparing T1D with controls 10.6 ± 2 versus 8.7 ± 2pmol/min, $p=0.07$; Figure 6-16, panel D).

The same analysis was conducted with the injection of the platelet agonist, thrombin. Figure 6-17 represents the combined data for ECAR and OCR in both healthy controls (blue) and patients with T1D (red). Following the thrombin injection, there was a rapid rise in ECAR in both groups without a difference detected comparing T1D with controls (15.2 ± 3 versus 13.9 ± 4mpH/min, $p=0.57$). However, given the lower ECAR in patients with T1D prior to administration of thrombin, there was an overall greater rise in ECAR in the T1D cohort compared with controls (10.3 ± 3 versus 7.7 ± 1mpH/min, $p=0.03$; Figure 6-17, panel C).

Measurement of OCR is summarised in panel B of Figure 6-17 for both healthy controls (blue) and T1D (red) in response to thrombin stimulation. Patients with T1D had a higher maximal mean OCR measurement following injection with thrombin, 23 ± 5pmol/min when compared with healthy controls, 15.4 ± 7pmol/min, ($p=0.01$). The change in OCR following injection with thrombin was measured. There was, a trend towards a higher rise in OCR in the patients with T1D compared with controls (7.7 ± 2 versus 5.5 ± 2pmol/min, $p=0.06$; Figure 6-17, panel D).
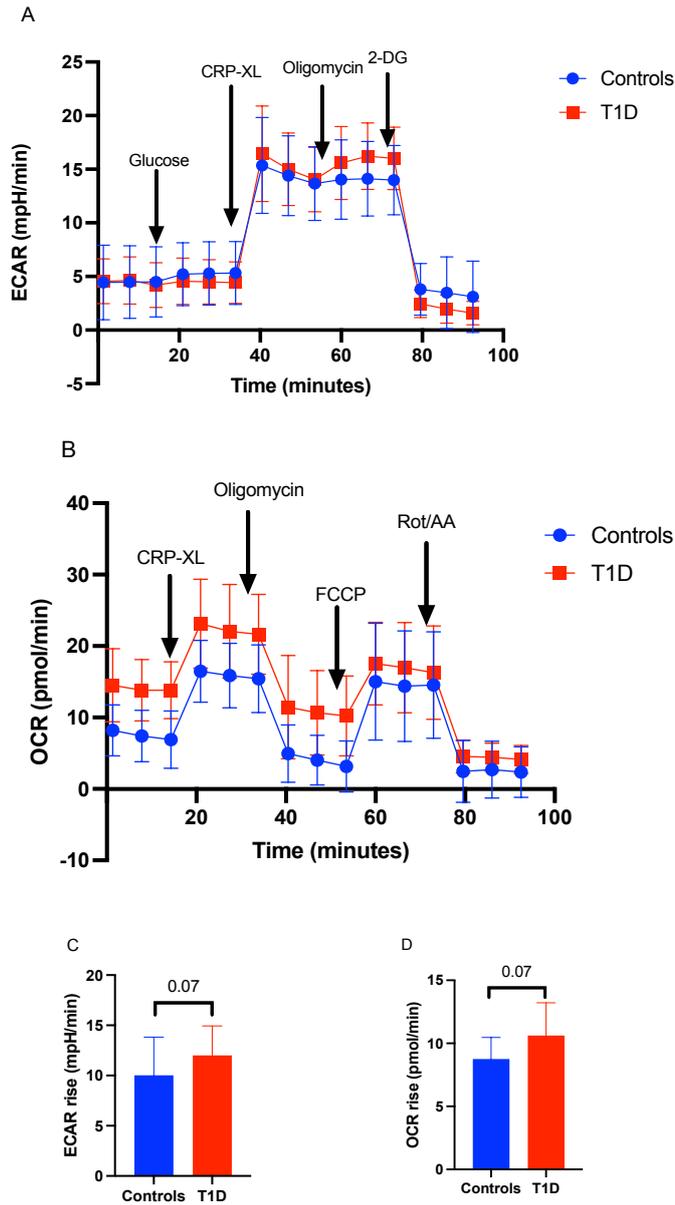
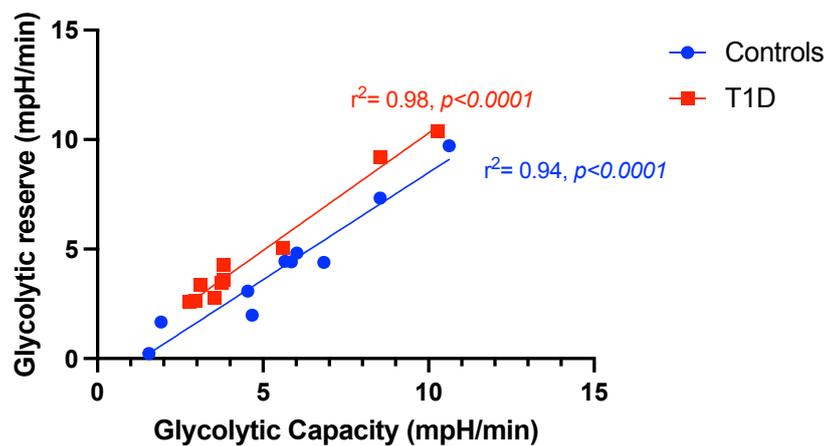**Figure 6-16: ECAR and OCR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; assessment of response to agonist stimulation with CRP-XL.**

Panel A represents combined ECAR data from Glycolysis Stress tests in healthy controls (blue) and patients with T1D (red), including following injection with CRP-XL. Panel B represents combined OCR data from Mito Stress tests in healthy controls (blue) and patients with T1D (red), again including following injection with CRP-XL. Panel C represents the change in ECAR following injection with CRP-XL and panel D represents the same for OCR data. CRP-XL= Collagen-related peptide-cross linked, 2-DG= 2-deoxy-glucose, FCCP= carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone, Rot/AA= Rotenone/Antimycin A. n=10 in both groups.
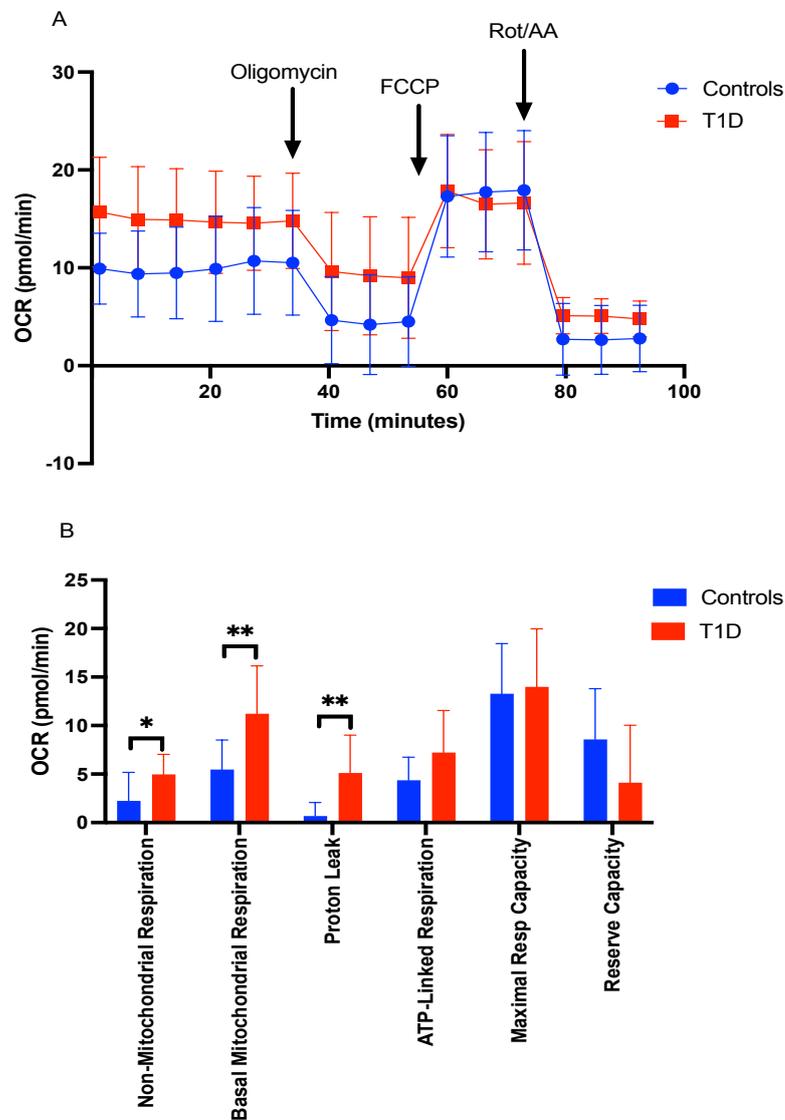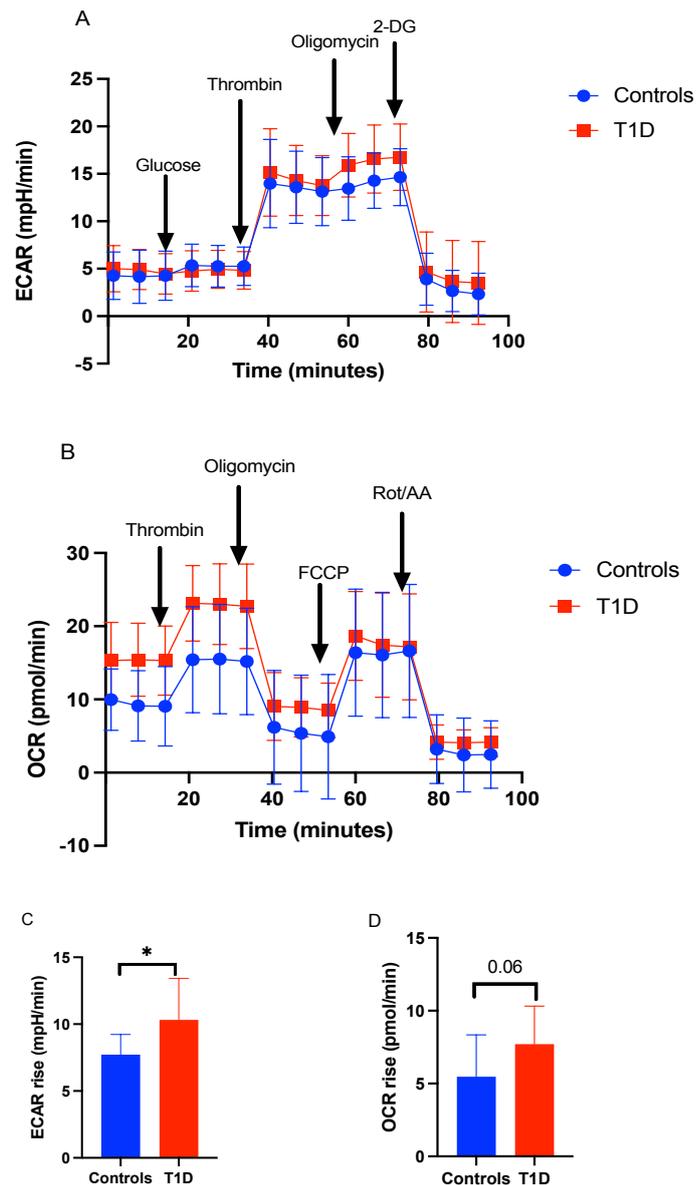
Figure 6-17: ECAR and OCR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; assessment of response to agonist stimulation with thrombin.

Panel A represents combined ECAR data from Glycolysis Stress tests in healthy controls (blue) and patients with T1D (red), including following injection with thrombin. Panel B represents combined OCR data from Mito Stress tests in healthy controls (blue) and patients with T1D (red), again including following injection with thrombin. Panel C represents the change in ECAR following injection with thrombin and panel D represents the same for OCR data. *$p<0.05$. CRP-XL= Collagen-related peptide-cross linked, FCCP=carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone, Rot/AA= Rotenone/Antimycin A. n=10 in both groups.

Finally, a comparison between the two agonists was made to investigate potential differential response to one agonist over the other in either of the two cohorts. In the patients with T1D, there was a mean ECAR rise of 12.2±2mpH/min in response to CRP-XL while the rise after thrombin was 10.3±3mpH/min (*p=0.21*). In terms of OCR, there was a greater rise of 10.6±2pmol/min in response to CRP-XL compared to thrombin, 7.7±2pmol/min, *p=0.02* (Figure 6-18, panels A and B). In healthy controls, the ECAR rise in response to CRP-XL was 10.0±3mpH/min compared to a mean rise of 7.7±1mpH/min following injection with thrombin, *p=0.09*. In terms of OCR, the rise following CRP-XL was 8.7±2pmol/min and after thrombin was 5.5±2pmol/min, *p=0.007*. These data are shown in panel C and panel D of Figure 6-18. These data suggest that there may be a greater response to CRP-XL in both T1D and healthy controls albeit the difference was only significant when comparing rise in OCR.
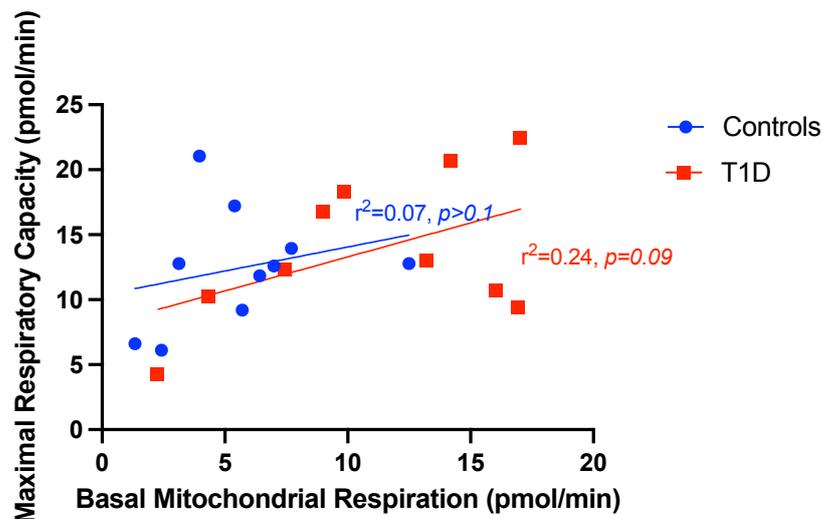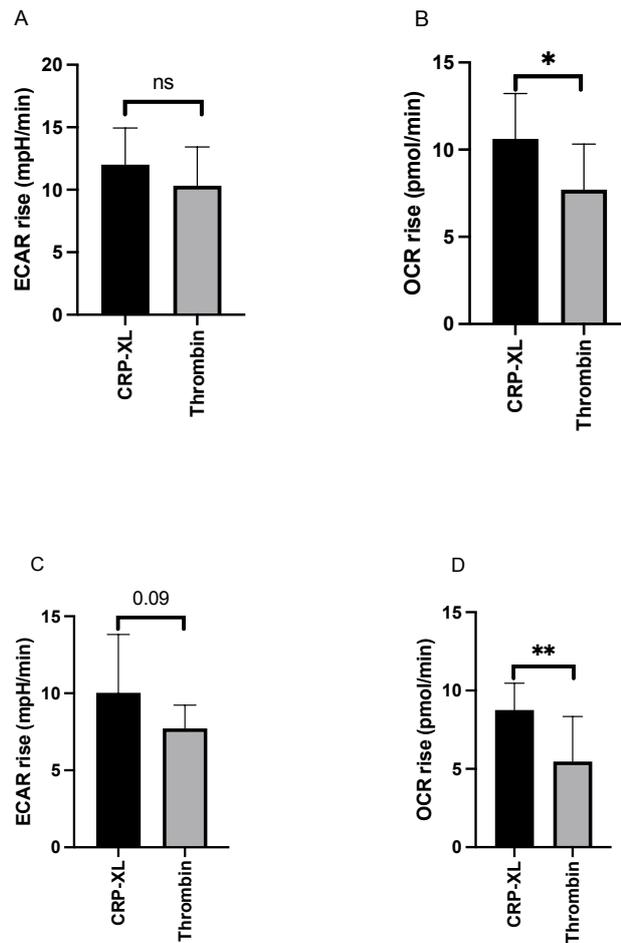
Figure 6-18: ECAR and OCR data from Seahorse XFe96 Analyzer using isolated platelets from patients with T1D and healthy controls; comparison of relative response to CPR-XL and thrombin.

Panel A represents rise in ECAR in response to both CRP-XL and thrombin in patients with T1D. Panel B represents rise in OCR in response to both CRP-XL and thrombin in T1D. Panel C represents rise in ECAR in response to both CRP-XL and thrombin in healthy controls. Panel D represents rise in OCR in response to both CRP-XL and thrombin in healthy controls. *p<0.05 and **p<0.01. *p=0.09* in panel C included to demonstrate trend given relatively limited numbers. CRP-XL= collagen-related peptide cross-linked. n=10 in both groups.

Finally, data were plotted as OCR measurements (y-axis) against ECAR (x-axis) at basal and following agonist treatment. Patients with T1D had higher basal OCR and lower basal glycolysis compared with controls with these differences maintained following injection with CRP-XL (Figure 6-19). Thus, the overall difference between the two groups is maintained, with the patients with T1D having more 'energetic' platelets. These data were also analysed as the ratio of increase in ECAR vs increase in OCR for both cohorts and both agonists. In the presence of CRP-XL, there was no difference in ECAR rise in T1D compared with controls (1.1±0.3 versus 1.2±0.7, *p=0.81,* Figure 6-19, panel C). A similar pattern is shown in response to thrombin (Figure 6-19, panels B and D)*.*
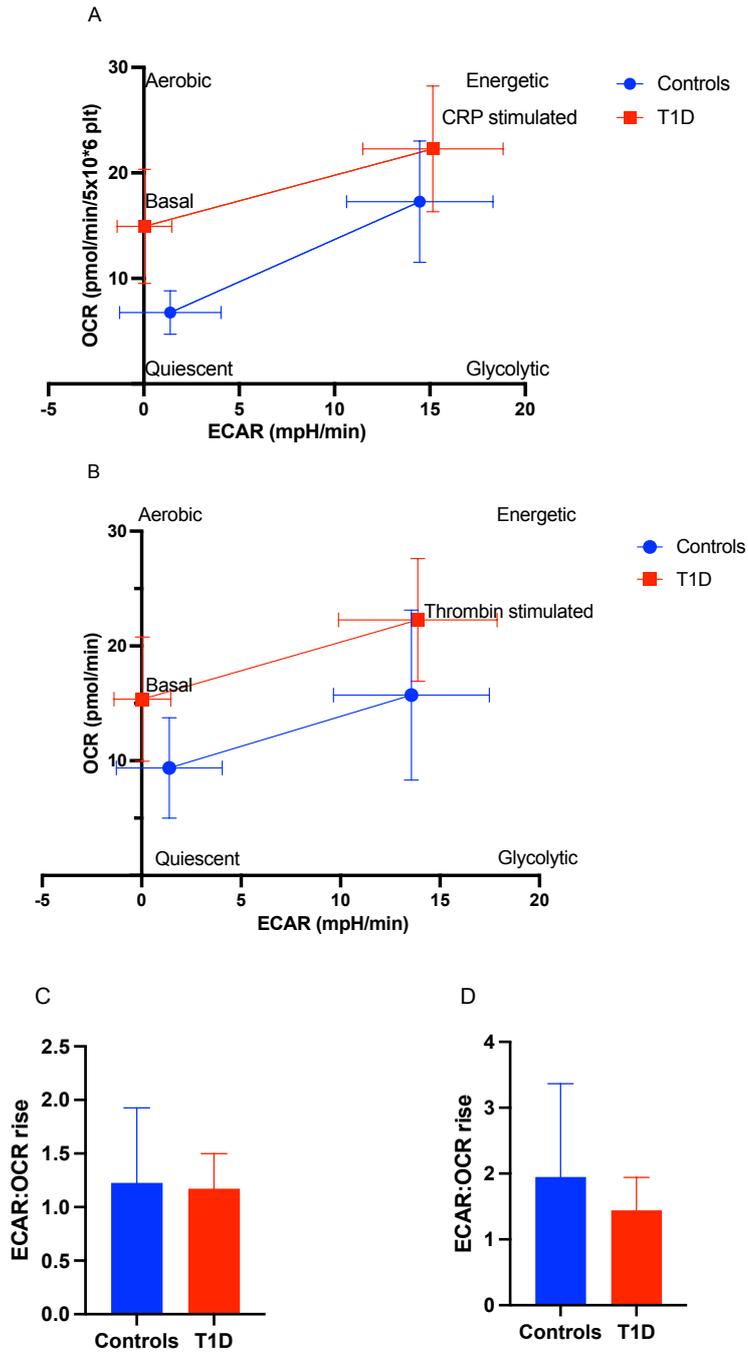
Figure 6-19: OCR (y-axis) against ECAR (x-axis) plotted for both T1D and healthy controls basally and following stimulation conducted in isolated platelets using standard Glycolysis Stress test and Mito Stress test Agilent Technologies Inc.

Panel A represents OCR (y-axis) against ECAR (x-axis) basally and in response to CRP-XL. Panel B represents OCR (y-axis) against ECAR (x-axis) basally and in response to thrombin. Panel C represents the ratio of ECAR rise: OCR rise in response to CRP-XL injection. Panel D represents the ratio of ECAR rise: OCR rise in response to thrombin injection. CRP= collagen-related peptide cross-linked. n=10 in both groups.

6.3.6 Basal and stimulated platelet glucose uptake in T1D

Data collected for glucose uptake were analysed as MFI only, given the lack of valid commercially available background fluorescence isotype control as a reference for percent positive. Initial experiments were conducted to assess basal glucose uptake in both patients with T1D and healthy controls for comparison. In patients with T1D, there was a mean MFI of 13037±2023 compared to 16598±2100 in the healthy controls, *p=0.008*, shown in panel A of Figure 6-20. The numbers included in these pilot data were too small to sub-categorise by clinical parameters, but suggested reduced glucose uptake.

Data were next analysed following stimulation with the platelet agonist, SFLLRN (20μM). Both patients with T1D and healthy controls showed significant increase in glucose following stimulation, shown in panel B of Figure 6-20. Patients with T1D had a mean MFI of 16667±1053 following stimulation, a significant increase compared with basal uptake, *p=0.006*. Similarly, healthy controls also had a significant increase in MFI, rising to a mean MFI of 21144±2077 following stimulation, *p=0.01*. Therefore, patients with T1D had a significantly lower mean stimulated glucose uptake than healthy controls, *p=0.01,* shown in panel C of Figure 6-20. Importantly, the data were then analysed as fold over basal, to adjust for the lower basal glucose uptake described in patients with T1D and no difference was demonstrated (1.36±0.2 fold increase in T1D versus 1.25±0.1 in healthy controls, *p=0.69*), shown in panel D of Figure 6-20.
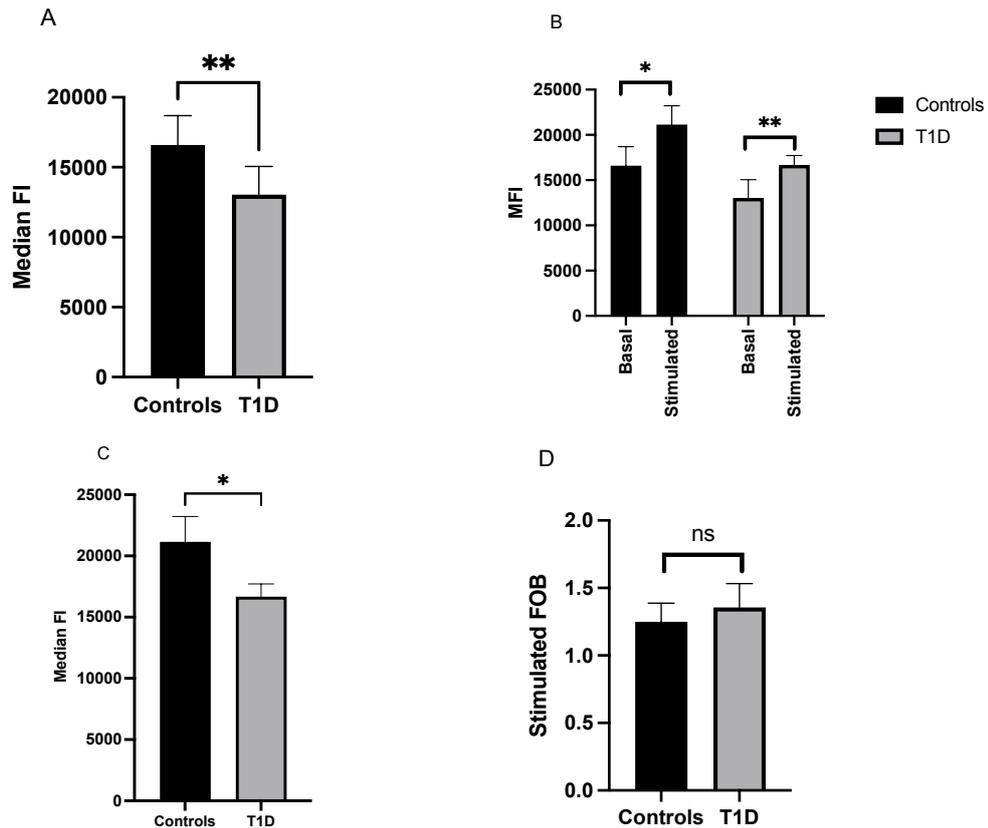
Figure 6-20: Basal and stimulated glucose uptake as expressed by Median Fluorescence Intensity for 2-NBDG, conducted in washed platelets in both T1D and healthy controls.

Panel A represents basal glucose uptake as mean MFI for T1D and healthy controls. Panel B represents the basal versus stimulated glucose uptake for both patients with T1D and controls. Panel C represents MFI data following stimulation with 20uM SFLLRN. Panel D represents data expressed as fold over basal (FOB), using basal glucose uptake data already presented. *$p<0.05$, **$p<0.01$. n=6 in both groups.

6.3.7 Platelet GLUT expression

Having identified a reduction in basal glucose uptake, the potential mechanisms influencing this were explored. As described in the introduction to this chapter, glucose transporters are integral to glucose uptake in platelets. Commercial fluorescently labelled antibodies for both GLUT-1 and GLUT-3 were therefore chosen. A series of optimisation experiments using a range of

fluorophore concentrations was designed to determine the optimal concentration for inclusion in an assay. This was first conducted in whole blood followed by washed platelets. As shown in panel A of Figure 6-21, fluorophore concentrations from 0.25μg/ml up to 5μg/ml were included with negative isotype controls included for reference at matched concentrations. The median fluorescence intensity (MFI) did not increase significantly at any dose of GLUT-1 fluorophore, with an MFI of 13.4 at 5μg/ml and the lowest MFI of 8.6 at 1μg/ml. The negative control MFI was higher than the GLUT-1 fluorophore at almost all doses, with the greatest difference seen at 4μg/ml, 10.7 with the GLUT-1 fluorophore versus 23.8 with the negative control. Panel B of Figure 6-21 shows the same experiment using the GLUT-3 fluorophore. Once again increasing doses from 0.25μg/ml to 5μg/ml were used. Again, there was only a very small rise in the MFI with the GLUT-3 fluorophore, from 2.3 at 0.25μg/ml to 15.6 at 5μg/ml. The negative control MFI was higher, reaching a peak of 128.2 at 5μg/ml. These data again suggested no binding of the GLUT-3 fluorophore to platelets.

Having conducted the experiments in whole blood, with no evidence of binding of either the GLUT-1 or GLUT-3 fluorophores, the same experiments were then conducted in washed platelets to ensure there was no interference with binding to other cells in whole blood.

For GLUT-1 expression, shown in panel C of Figure 6-21, there was minimal change irrespective of fluorophore concentration, with a peak of 190 at 4μg/ml, however this still remained comparable to the concentration matched control again indicating no expression/uptake. For GLUT-3 expression, fluorophore binding did appear to increase slightly at the higher concentrations with a peak

MFI of 110.9 at 5μg/ml but this remained lower than the negative control, which was the case across all concentrations as shown in panel D, Figure 6-21.
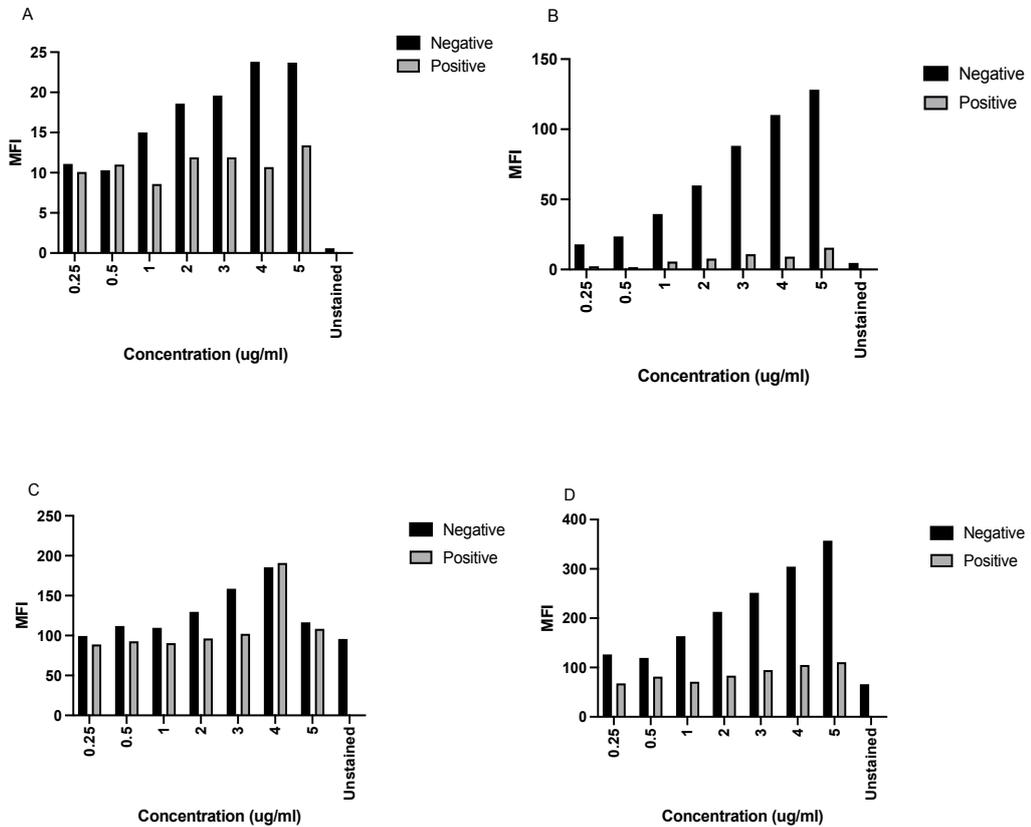
Figure 6-21: GLUT-1 and GLUT-3 fluorophore concentration optimisation conducted in whole blood and washed platelets from a healthy volunteer.

Panel A represents expression by MFI for a concentration range 0.25μg/ml to 5μg/ml for the GLUT-1 fluorophore and matched concentrations for the control in whole blood. Panel B represents MFI expression for the GLUT-3 fluorophore in whole blood with matched controls. Panel C and panel D represent the same experiments conducted in washed platelets.

6.3.8 β-oxidation in mitochondrial respiration in T1D

In order to investigate the relative contribution of FA oxidation to the elevated basal mitochondrial respiration demonstrated in patients with T1D in the previous chapter, etomoxir was added to an adapted Mito Stress Test which has been previously detailed. Following the initial measurements of basal respiration, etomoxir was injected at a standardised dose, (40μM). Following this, rotenone/antimycin A was added as per an adapted Mito Stress Test [186], with the focus on OCR response to etomoxir as a measure of FA oxidation. Panel A of Figure 6-22 shows the summarised results from patients with T1D (n=4) and also healthy controls (n=3).

As reported in the previous chapter there was a significantly higher basal mitochondrial respiration (basal respiration minus non-mitochondrial respiration) in the patients with T1D compared with healthy controls (14.9±2pmol/min versus 8.4±2pmol/min, respectively; *p=0.02*). Following injection with etomoxir, 3 measurements were taken. At this point, the mean OCR measurement for T1D was significantly lower than basal respiration, 11.0±2pmol/min compared with mean 17.6±2pmol/min basally, *p=0.03*. As shown, there was also a reduction in OCR seen in healthy controls, however this was non-significant, 6.8±1pmol/min versus the 10.2±2pmol/min, *p=0.1*.
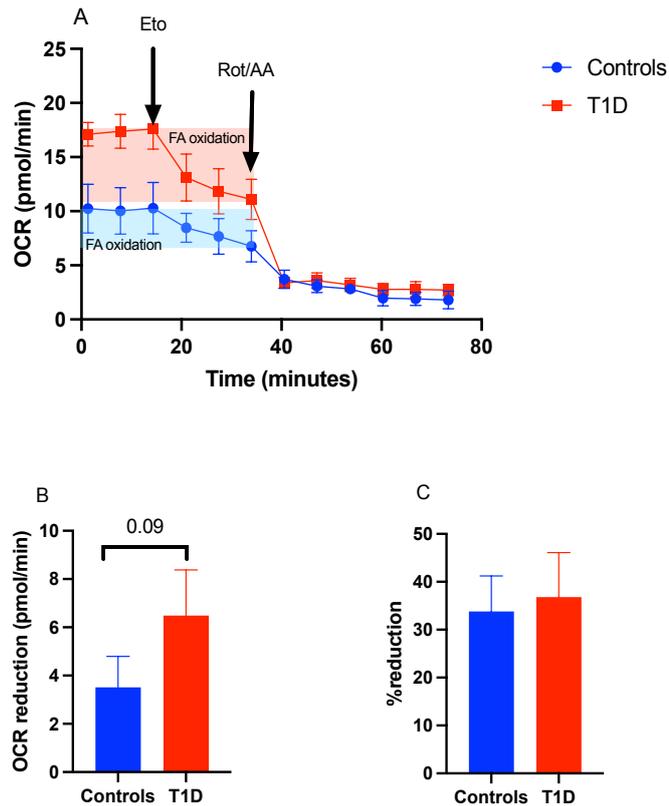
Figure 6-22: Adapted Mito Stress Test including etomoxir to measure FA oxidation.

OCR was measured in isolated platelet from both patients with T1D and healthy controls. Panel A shows the summary of OCR measurements. Panel B shows the relative absolute reduction of OCR in the presence of etomoxir as a surrogate measure of FA oxidation. Panel C shows this reduction as a percentage of the basal respiration. Eto=etomoxir, Rot/AA= Rotenone/antimycin A. n=4 in T1D and n=3 in controls.

Data were then analysed to assess the reduction in OCR following addition of etomoxir, representative of FA oxidation as shown in Figure 6-22, panel A. The difference in OCR measurement was then calculated as an absolute figure, shown in panel B of Figure 6-22, as well reduction as a percentage of basal respiration shown in panel C. Patients with T1D had a trend towards a higher mean reduction of 6.5±2pmol/min compared with 3.5±1pmol/min in healthy controls, *p=0.09.* As a percentage of basal respiration, patients with T1D had a mean 37±9% reduction compared with 33±7% in healthy controls which failed to reach statistical significance, *p=0.67*.

6.4 Discussion

Cell bioenergetics are a growing area of interest in the assessment and understanding of a variety of disease states including development of cardiovascular disease [352, 353]. Many platelet functions require a rapid response to stimuli and these processes are highly energy demanding, with the key metabolic processes of glycolysis and mitochondrial respiration facilitating these demands [354]. Thus it is reasonable to assume that platelet bioenergetics are a key area of importance and have been implicated in disease processes with links to clinical conditions, such as Alzheimer's dementia and sickle cell disease, as already shown [355, 356]. Furthermore, important metabolic milieu of diabetes outlined in previous chapters including hyperglycaemia and increased oxidative stress have been linked to changes in bioenergetic profiles [357]. Platelet bioenergetics in patients with T1D is an area poorly researched and our study provides novel patient data exploring changes in both glycolysis and mitochondrial respiration.

Data presented in this thesis demonstrate that patients with T1D display a shift in basal platelet glycolysis when compared with healthy controls. Patients had a lower ECAR with minimal variation with an elevated OCR compared with controls, indicative of increased basal mitochondrial respiration in the patient cohort. There are very limited existing data for comparison, with studies to date conducted in animal models of diabetes or individuals with T2D [189, 358], some of which support my findings particularly of reduced basal glucose uptake and reduced basal glycolysis. The profound changes seen are in the context of a relatively young cohort (median age 23 years) with reasonable glycaemic control (mean HbA1c 62 mmol/mol) represent novel and clinically

important findings, particularly as these changes may be further exacerbated in the context of increased age, other cardiovascular co-morbidities or poorer glycaemic control. Whilst I found no correlation between glycaemia and changes in these metabolic processes, there was a suggestion that the presence of greater insulin resistance, measured as lower eGDR, may further exacerbate the reduced glycolysis seen when comparing the full cohort of patients with T1D and healthy controls, although greater numbers would be required to thoroughly characterise this.

Beyond basal alterations in the bioenergetic signature of patients with T1D, our data also explored response to energy demand through both Glycolytic and Mito stress tests. In terms of changes in glycolysis in response to stress, there were no clear differences identified between the two cohorts. The patients with T1D 'overcame' the deficit in basal glycolysis to gain comparable glycolytic capacity and glycolytic reserve. This suggests the defect in glucose metabolism is predominantly seen prior to activation and the platelets from patients with T1D are sufficiently able to meet energy demands in this setting. In the patients with T1D, we observed significantly higher non-mitochondrial respiration compared to the healthy control cohort. This essentially refers to processes occurring within the platelet that are oxygen consuming but non-mitochondrial and typically linked to inflammatory enzymes such as NADPH oxidases and cyclo-oxygenases [352]. Non-mitochondrial respiration has been shown to be higher in states of increased oxidative stress [359]. . Similarly, there was elevated proton leak in the patients with T1D which could be indicative of increased mitochondrial dysfunction and mitochondrial membrane impairment [188]. Patients also had a tendency towards an elevated ATP-linked respiration, but greater numbers are required to draw

more definitive conclusions. Such findings have been associated with increased oxidative stress which may be one of the driving mechanisms in patients with T1D [352]. Maximal respiratory capacity, in response to stress was comparable in the two groups but as a result of the higher basal respiration in the patients with T1D, there was a trend towards a lower respiratory capacity in the patient cohort. Overall, drawing on the data from both ECAR and OCR, there appears to be a greater alteration in basal glycolysis and mitochondrial respiration, with a relatively intact ability to respond to energy demands facilitating platelet activation and functional processes.

Beyond the expected correlation between glycolytic capacity and glycolytic reserve, there were no clear correlations identified for any of the parameters of glycolysis in either cohort. Interestingly, ATP-linked respiration and basal respiration were strongly correlated in both cohorts and although a weaker correlation, the same was true for proton leak. Whilst to an extent this may be expected given both parameters contribute to the overall basal mitochondrial respiration measurement, the correlation of each suggests a degree of flexibility within human platelet metabolism both in normal physiological circumstances and in T1D, a notion that has previously been reported elsewhere with a variation between the contribution of each reported in patients with sickle cell disease [188, 356].

Finally, these data suggest that despite the discussed altered platelet metabolism, there were no definitive differences following platelet stimulation with CRP-XL comparing T1D cohort and healthy controls. In response to thrombin, there was a marginally greater rise in ECAR in the patients with T1D

but again greater numbers would be required to draw more absolute conclusions. Of note, there was a greater response to CRP-XL than thrombin in terms of OCR in both healthy controls and T1D, with an additional trend towards greater response in ECAR only in the healthy controls suggesting possible greater sensitivity in healthy controls.

Collectively, these data show that patients with T1D, even at a young age and with no existing macrovascular disease, have a bioenergetic shift which may contribute to the altered functional phenotype with increased platelet hyperreactivity explored in earlier chapters. This area needs greater exploration with increased numbers and including patients with more variable glycaemia and/or in the presence of more micro/macrovascular complications, but these novel data may provide some insight into platelet bioenergetics in this understudied cohort. Beyond an understanding of the discussed changes in metabolism, there is suggestion that targeting these processes through novel therapies may have promise [188, 360]. Furthermore, already there has been some interest in the use of existing treatments in tackling these mechanisms. Metformin, known to modulate mitochondrial function, has been proposed as a potential option to reduce mitochondrial respiration. This has been shown in both human platelets and diabetes-rat models. In both cases, reduction in mitochondrial respiration was only evident at doses of metformin greater than those currently used in clinical practice but does suggest its possible use in this context [358, 361].

The bioenergetic changes described are likely driven by changes in substrate and energy sources. In recent years it has been established that platelets appear to have significant metabolic flexibility to maintain energy sources to

meet functional demands. In order to preserve function, key metabolic processes of glycolysis and mitochondrial respiration must rely on different substrates particularly in the absence of glucose, including endogenous glycogen, glutamine and FFA.

The presented data suggest that the reduction in basal glycolysis may in part be driven by a reduction in basal glucose uptake in patients with T1D. Although these were pilot data with relatively low numbers, these differences were still significant. Following stimulation, glucose uptake increased significantly in both patients and healthy controls with the patients with T1D still having a lower absolute glucose uptake than the healthy controls. These changes are likely to be multifactorial, however, it was felt the next step was to establish whether there was impaired glucose transport into the platelets, with the aforementioned GLUT-1 and GLUT-3 of greatest importance. Unfortunately, despite testing a range of concentrations there was no clear evidence of binding of commercially validated antibodies for either GLUT-1 or GLUT-3. Whilst there have been reports of successful use of GLUT-1 fluorophores in red blood cells in cytometric analysis [362], there are limited data for GLUT-1 or GLUT-3 fluorophores in platelets. Alternative options such as western blotting in GLUT-1 and GLUT-3 knockout mice have been used but in the case of the potentially more subtle differences in expression seen between patients with T1D and healthy controls, this is likely to yield far less conclusive evidence nor inform about surface expression [343]. Due to time limitations, this has yet to be explored but would certainly be considered to establish possible defects in GLUT expression driving the changes in basal glucose uptake. Of further note, as previously mentioned, given GLUT-3 mediated

glucose uptake has been shown to be integral for α-granule secretion and the associated platelet activation [341], my data, showing relatively preserved stimulated glucose uptake in patients with T1D may further support this. Furthermore, this may be particularly relevant in the context of the increased P-selectin expression in the same cohort described in earlier chapters, which is also secreted from α-granules upon activation [301].

The ability of platelets to adapt to use different metabolic substrates to meet energy demands is of particular interest in the context of patients with T1D who have altered glucose levels. We have presented evidence to suggest reduced glucose uptake and glycolysis in these patients, thus, an alternative energy source must be driving the increased mitochondrial respiration described in the previous chapter. Existing evidence has already shown the ability of platelets to use FFA as a metabolic substrate [186]. Using a FA oxidation inhibitor to measure the relative contribution of FFA metabolism to basal mitochondrial respiration, the pilot data reported here suggest a trend towards a greater reduction in OCR in patients with T1D. Given the small cohort in whom these data were collected, it would be beneficial to increase the sample size for these data to fully characterise the possible contribution of FA oxidation in driving mitochondrial respiration. In addition, alternative assays such as FA uptake could be considered to further investigate the role of FA oxidation in patients with T1D.

FFA are an area of particular interest in patients with T1D as explored in chapter 1, with characterised changes in lipids and lipid metabolism already established in patients with T1D, particularly in young adults and children [107, 108]. One study demonstrated that in adult patients with T1D, particularly

those with sub-optimal glycaemic control, there were lower overall plasma FFA concentrations [363]. This was further characterised with a higher saturated/unsaturated FFA ratio and increased de novo lipogenesis [363]. In addition, another study demonstrated that inhibition of FA oxidation, albeit not with etomoxir, could be directly linked with attenuated platelet activation and aggregation [364]. Whilst further data in this area are certainly warranted, particularly to aid our understanding of FA oxidation in the context of mitochondrial respiration, there is clear evidence that alterations in FFA and its associated metabolism may be important in patients with T1D.

Beyond the potential use of platelet bioenergetics as an area to target for novel or existing therapeutics, there is emerging evidence to support the synergistic utility of bioenergetics and platelet metabolomics or proteomics. One study closely mapped the platelet metabolome with the parameters of the described Mito stress test [188]. In particular, proton-leak, ATP-linked respiration and basal mitochondrial respiration correlated strongly with the established clusters. Interestingly, these were also the parameters in which we identified the greatest difference between our patients with T1D and the healthy controls. With the growing utility of both proteomics, metabolomics and platelet bioenergetics in the research setting there may be a growing body of evidence to support their use in a clinical setting to support identification of those at greatest risk of developing bioenergetic defects based on their proteome or metabolome alone and ultimately facilitate earlier intervention to reduce cardiovascular morbidity in this cohort.

Limitations to these data are largely around the limited number of patients included. Larger numbers would be needed to fully characterise the relative

impact of glycaemia and also IR. Again, the healthy volunteers were not formally characterised and this may also impact the results from this cohort.

Finally, something that is not fully clear from my data is whether these shifts in bioenergetic profiles and substrate metabolism are directly linked to the functional platelet phenotype described in my earlier chapters. It is therefore crucial for future work to focus not only on increasing our understanding of potential alternative mechanistic pathways contributing to the differences seen in patients with T1D but also to establish whether there are clear associations between the metabolic changes and functional outcomes.

Chapter 7: Summary and General Discussion

The long-standing issue of cardiovascular morbidity and mortality in patients with both T1D and T2D continues to be a concern for those involved either directly or indirectly in the management of these patient groups. The ever-growing worldwide epidemic of obesity only adds to this and indeed the concept of DD, whilst not novel, has only recently gained some traction. The contribution of the platelet research, particularly in patients with T1D and DD has also started to gain momentum in terms of an area of focus amongst the cardiovascular research community. I hope to now summarise my findings and how these contribute to the existing literature, along with a look to the future and what lies beyond the horizon.

7.1 Platelet dysfunction in patients with T1D and DD; hyperreactive, procoagulant and diminished sensitivity to inhibition

The initial focus of this thesis was to further the understanding around platelet hyperreactivity in patients with T1D. Beyond this, exploring the potential effects of glycaemia, both short and medium term as well as insulin resistance is key not only to improve our knowledge around platelet biology but crucially to give us some insight into those patients who may be at greatest risk of platelet driven cardiovascular morbidity long-term.

Most of the early data published with regards to platelet hyperreactivity in diabetes stemmed from research in mouse/rat models or patients with T2D. Even today there remains a relative paucity of equivalent research in patients with T1D, albeit this is steadily improving. Some of the first landmark studies

supporting increased platelet activation in patients with diabetes demonstrated elevated TXA_2 synthesis in patients with diabetes [122]. More recently, attention has turned to the use of flow cytometry to explore different aspects of platelet activation. P-selectin, perhaps the most widely investigated marker of platelet activation, has been shown to be elevated both in patients with T2D [134-136] and in T1D [133]. With regards to the former, further evidence suggests greater long-term hyperglycaemic exposure correlates with higher P-selectin expression with subsequent improved glycaemic control associated with reduced P-selectin expression [134]. Whilst, to our knowledge, no data exist in patients with DD, conditions of hyperinsulinaemia and hyperglycaemia in healthy controls are also associated with increased P-selectin expression [138]. Beyond this, $\alpha_{IIb}\beta_3$, a receptor for fibrinogen-binding, is another marker indicative of platelet activation. This has also been shown at greater levels in activated platelets in patients with T2D than healthy controls [154]. Furthermore, levels were even elevated under basal conditions in a cohort of patients with T2D with sub-optimal glycaemic control (mean HbA1c 83 ± 3 mmol/mol) [155]. Data specifically exploring active $\alpha_{IIb}\beta_3$ expression, in patients with T1D are lacking. Externalisation of PS on the platelet surface provides a haemostatic site for factor Xa binding and can be used as a marker of the procoagulant propensity [302, 303]. At present, I am unaware of any data specifically investigating PS expression in patients with T1D/DD.

The data presented in chapters 3, 4 and 5 aimed to explore some of these gaps in existing literature. Compared with healthy controls, our cohort of patients with T1D had elevated levels of all three described markers of

interest. These differences were seen both under basal conditions and following stimulation with platelet agonists, despite the fact that the cohort studied represented low risk patients. Beyond this, I have characterised the effects of various parameters on platelet activation including short and medium-long term glycaemia and insulin resistance. I demonstrated that in patients with T1D, those with elevated HbA1c (a marker of medium-term glycaemic control) and lower TIR (an indicator of short-term glycaemic control) had increased expression of all three of the above markers. Differences in expression were evident under basal conditions, suggesting platelets may circulate in a 'pre-activated' state in those with sub-optimal glycaemic control. Following stimulation, irrespective of the strength of agonist used, P-selectin appeared greater in patients with higher HbA1c and lower TIR. The same was seen with stronger agonist concentrations for fibrinogen-binding. In the presence of dual agonists, PS expression was also highest in those with poorer glycaemic control. Of note, plasma glucose at the time of sampling did not correlate with increased platelet activation and therefore the observed effects are related to short- and medium-term glycaemic control. Some of the patients with generally good glycaemic control, with lower HbA1c and higher TIR may have very transient high glucose excursions, e.g., post-prandially, yet reassuringly these did not appear to influence platelet activation significantly in the same way as persistently high glucose levels. Approaches towards managing young adult patients with T1D in clinical practice already have a strong focus on reducing HbA1c and more recently focussing on improving TIR to meet the widely agreed target of $\geq$70%. However, it appears that factors other than glycaemic control may also play a role. My data show that patients with the highest insulin resistance, measured through the lowest eGDR, had

increased levels of all three markers. When using the eGDR of <6mg/kg/min, shown to correlate with significantly increased cardiovascular mortality [235], these differences were most apparent. Again, differences were seen both under basal conditions and following stimulation.

In addition to hyperreactivity, existing data around platelet sensitivity to inhibition in diabetes is somewhat outdated and has historically relied upon platelet aggregation, which is a crude test and fails to detect subtle, yet clinically important, differences [326, 327, 365]. My data have explored novel insights into the effects of PGI_2 on all of the above-described markers in patients with T1D/DD. Collectively, these data showed evidence of diminished sensitivity to PGI_2. CD62P showed a generally lower response to PGI_2 compared with PAC1 and AnnV-binding in both cohorts, though following stimulation with a lower concentration of agonist stimulation, there was a smaller reduction in CD62P expression seen in patients with T1D than the healthy controls. With the higher dose of $PGI_{2,}$ the reduction in expression of AnnV-binding as well as PAC1 expression appeared reduced in patients with T1D compared with healthy controls, supporting the concept of poorer sensitivity to PGI_2. Glycaemic exposure may again be implicated, with patients who had higher HbA1c and lower TIR having a more limited response to PGI_2 particularly when assessing PAC1 and AnnV expression. In addition to the role of glycaemia, my data showed that patients with the lowest eGDR (<6 mg/kg/min), and thus greatest insulin resistance, also appeared to have reduced sensitivity to PGI_2 when compared to patients considered not to have insulin resistance (eGDR $\geq$8 mg/kg/min).

To summarise, my data therefore suggest that in young adult patients with T1D, there is a functional phenotype of hyperreactive platelets with a diminished response to the inhibitor PGI$_2$ compared with healthy controls. Those with sub-optimal glycaemic control and DD appear to have the greatest change in platelet function and thus may be at greatest risk of cardiovascular disease. This suggests a shift in clinical management may be warranted with earlier recognition of insulin resistance in patients with T1D. Furthermore, benefit may be gained through a proactive approach to minimising this risk through better education and the more widespread use of adjunctive therapies such as metformin in patients with T1D. Whilst undeniably multifactorial, given the relatively young age and lack of pre-existing macrovascular disease in the patient cohort included in this study, it suggests that T1D alone can drive the described platelet dysfunction.

7.2 Bioenergetic and metabolism changes in patients with T1D and DD

Whilst increasing our understanding of the functional phenotypes seen in platelets from patients with T1D and DD improves our understanding of cardiovascular morbidity and mortality in these patient cohorts and highlighting patients most at risk, next it is crucial to explore why these changes occur in the first place. To do this, my data explored possible changes in metabolism and the bioenergetic signatures of platelets from patients with T1D.

As already described, platelets have been shown to possess a degree of metabolic flexibility, seamlessly switching between substrates dependent on availability in order to preserve important mitochondrial functions of glycolysis and oxidative phosphorylation [186]. Data in patients with T1D are lacking and there are only limited data in animal models of T2D. Mitochondrial dysfunction and alterations in mitochondrial respiration have been linked to a number of disease states including T2D, with evidence of shifts in ATP-linked respiration and altered mitochondrial membrane potential [191, 197]. Data regarding glycolysis in patients with T1D are non-existent but reduced platelet glycolysis has been shown in mouse models [189]. Furthermore, glucose metabolism has been shown as a crucial component to maintaining these key mitochondrial functions and has also been strongly linked with platelet activation thus linking bioenergetics and functional outcomes. Glucose uptake was first shown to be reduced in platelets from patients with T1D in the 1980s [366] but there has only been minimal further data contributing to this area in the four decades since. GLUT transporters are the key facilitators of glucose uptake, with GLUT-1 and GLUT-3 the most predominant in platelets, however, there are no studies investigating GLUTs in platelets from patients with T1D.

My data, predominantly presented in chapter 6, first explored differences in basal glycolysis and basal mitochondrial respiration in patients with T1D. There appeared a reduction in basal glycolysis in the patients with T1D and conversely an increase in basal mitochondrial respiration. There were no overt correlations demonstrated between glycaemia and the bioenergetic functions, though a trend towards lower basal glycolysis was evident in those with insulin resistance. However, numbers were limited and this remains an area for future research.

In terms of meeting energy demands, there appeared to be no clear differences between patients with T1D and healthy controls in glycolytic capacity and reserve. Thus, platelets from the patient group appear to correct for the glycolysis deficit seen under basal conditions. In contrast, my data identify a number of differential responses to stress in terms of respiratory function. There was an increase in non-mitochondrial respiration in individuals with T1D, which has been linked with other disease states and in particular in the presence of ROS [359]. Additionally, the data suggest comparatively increased proton leak in the T1D cohort, consistent with mitochondrial membrane impairment due to mitochondrial dysfunction [188]. Elevated ATP-linked respiration was also demonstrated in the patients with T1D and linked to increased oxidative stress. There were, however, no clear differences in maximal respiratory capacity. Overall, changes in platelet bioenergetic signatures in patients with T1D appear to be most profound under basal conditions with somewhat preserved function in response to energy demand facilitating platelet functions. Further to this, glycolysis and respiration were also measured in response to platelet activation with agonists. Whilst there were comparative increases in both glycolysis and respiration across the two groups (patients with T1D and healthy controls), in response to thrombin there was a slightly greater increase in glycolysis in the patient cohort.

Having established these changes in bioenergetic functions, I next examined glucose uptake by platelets, which was reduced T1D individuals compared with healthy controls. Under basal conditions these differences were more pronounced and may, in part, be driving the reduction in basal glycolysis already described. Following stimulation with a platelet agonist, there was a significant increase in platelet glucose uptake in both cohorts, though the

overall glucose uptake in patients remained lower than that seen in healthy controls. Yet, when accounting for the lower basal glucose uptake in patients, this difference was not apparent. I next tried to establish whether these changes may be influenced by the relative expression of important GLUT transporters, GLUT-1 (largely linked to basal glucose uptake) and GLUT-3 (largely accounting for glucose uptake following platelet activation). Unfortunately, the commercial antibodies obtained did not yield results and this remains an area requiring further investigation.

With the already established metabolic flexibility in platelets, I next investigated whether in lieu of glucose, platelets from patients with T1D may be utilising FFA. The ability of platelets to use FFA as substrates has already been proven, typically through the use of FA oxidation inhibitors. Whilst my data are preliminary, they suggest that in the presence of one such FA oxidation inhibitor, a greater reduction in respiration is seen in patients with T1D compared with healthy controls. Overall, my data suggest that FA, in the setting of reduced glucose uptake, may be driving the alterations in bioenergetic functions described in the patients with T1D.

Again, within this young cohort without pre-existing macrovascular complications, these differences highlight the urgent need for more proactive cardiovascular risk stratification in patients with T1D. Whilst these data are insufficient to clearly determine the impact of IR on these parameters, the early suggestion of links between more profound alterations in mitochondrial respiration and higher IR once again support the need for integration of accurate IR measures in clinical practice.

7.3 Limitations

To summarise the main limitations detailed in the relevant chapters, it must first be acknowledged that particularly with respect to the bioenergetic data, numbers are small and difficult to draw definitive conclusions. Furthermore, the comparison healthy volunteer group are not formally characterised and it would be prudent to ensure availability of detailed medical history including relevant medications, smoking status and alcohol consumption. Whilst all experiments were conducted myself to reduce inconsistency, stock reagents and fluorophores were replaced over the course of the study and thus there may be variations that cannot be accounted for. Equally, whilst the same Cytoflex S machine was used, lasers had to be re-aligned and engineer work took place during the study. Again, I tried to mitigate for this through compensation experiments conducted after engineer work but this could never be fully accounted for.

As touched upon, there is an awareness that from these data alone we cannot establish true causality and again, larger and more longitudinal data would be required to further determine this association.

7.4 Future directions

Whilst the data presented in this thesis have made some inroads into the relative paucity of evidence around platelet function, bioenergetics and metabolic processes in patients with T1D and DD, there still remains a number of unanswered questions arising from the study that merit further research.

Beyond this, there are novel concepts and treatments that may lend themselves to answering some of these questions and ultimately contribute to changing the clinical landscape for managing cardiovascular complications in patients with T1D and DD.

7.4.1 Further characterisation of alterations in platelet metabolism and functional outcomes in T1D

Initially, I could use the techniques described to examine the potential impact of smoking status, alcohol intake or even possible interfering medications on both functional outcomes and platelet metabolism. Beyond this there may also be a role for investigating the effects of potential insulin sensitising interventions such as adjunctive therapies e.g., metformin or even a regular defined exercise regime. As previously mentioned, it would also be important to link the alterations seen in platelet metabolism and the established bioenergetic signature shifts with the functional outcomes described. A larger cohort with paired data of the two aspects would facilitate a better understanding of the role of metabolic changes in the functional platelet phenotype in patients with T1D and whether this may be a potential area to develop interventions.

Whilst my data are the first to explore bioenergetic signatures and the use of glucose and FA as energy sources in platelets, further data are needed to fully characterise and understand these changes. First, it would be prudent to establish whether it is predominantly GLUT transporter defects driving changes in glucose handling through either further flow cytometry attempts [362], or through techniques such as western blotting, though this may fail to

detect subtle differences. As briefly explored, further characterisation of the impact of FA oxidation inhibitors, and thus the role of FA in respiration, are also required. Crucially, there is a need for experiments also linking the changes in FA metabolism to the functional phenotypes demonstrated from my data. One recent study has explored this link and demonstrated the role of FA oxidation in both agonist-stimulated platelet activation and thrombus formation [364]. Interestingly the inhibitor used in my data, etomoxir, failed to be linked to these changes and thus it may be prudent to use alternative FA oxidation inhibitors such as those in the described study [364].

7.4.2 Platelet proteomics

Beyond detailing the alterations in function, bioenergetics and substrate metabolism, exploring the underlying changes in the platelet proteome, as detailed in earlier chapters, may help to establish the characteristics of those at greatest risk of platelet dysfunction which in turn indicates those most at risk of developing cardiovascular morbidity [188, 200]. Furthering our understanding in this area may also identify potential biomarkers or targets to actively mitigate this risk. As this is a relatively novel concept, most existing data for platelet proteomics are from healthy controls, in whom it has been shown there is relatively little interindividual variation [367]. This in itself makes studying the platelet proteome from patients with chronic diseases even more important and indeed reliable. I have already collected additional pilot data from a subgroup of the DEVELOP patients, with a view to explore platelet proteomic analysis in patients with T1D. Whilst this area is still growing, associations have already been established between an alterations in the

platelet proteome and cardiovascular disease [368]. One of the earliest studies demonstrated differential regulation of 6 proteins between those with no pre-existing cardiovascular disease and those with either established angina or NSTEMI [369]. Further to this, patients presenting with NSTEMI have also been shown to have identifiable variations in platelet proteomics compared with patients with stable coronary artery disease, with the GPIIb/IIIa signalling pathway particularly implicated [205]. Whilst these studies did not focus on patients with T1D, data show the potential utility in patients with cardiovascular disease. Proteomics may open the door for novel biomarkers and identifying those at greatest risk of cardiovascular complications. The utility of this has been shown in detection of early stage lung and pancreatic cancers and such concepts would be easily transferrable to cardiovascular disease [370]. Furthermore, and perhaps more crucially, platelet proteomics may allow detection of new, key proteins that are differentially expressed either in T1D or those patients at greatest risk of cardiovascular complications. These may prove targets for the development of new therapies to minimise risk and in term improve long-term outcomes.

7.4.3 Biomarkers and therapeutics

Whilst new techniques in platelet proteomics may allow identification of new targets, novel direct therapies would undeniably be many more years into the future. At present, a number of newer treatment options have already been in development. Current focus on direct anti-platelet therapies and alternative regimes were discussed in chapter 1. Minimising risk-profiles through optimising glycaemic control and reducing insulin resistance remain key aspects of the indirect management of cardiovascular risk in patients with

diabetes, there are a number of future therapies or approaches that may also be of interest.

Oxidative stress has been already established as one of the key contributors to the development of cardiovascular disease in patients with diabetes thus studies to combat its effects are important. As described earlier, FA oxidation inhibitors allow us to explore the impact of FA oxidation on platelet function. Crucially, as has been postulated, if a link between the described metabolic flexibility can be made with platelet activation and function, such inhibitors may be important options to explore as therapeutic interventions to reduce cardiovascular risk [364]. Indeed, there has already been some progress in this area, with some data demonstrating potential benefit with these inhibitors in angina and atherosclerosis [371]. The use of such inhibitors has also been linked to suppression of NLRP3 inflammasome, discussed in chapter 1, which has been shown to be upregulated in diabetes (T2D and diabetic mouse models) [372]. This has implications for reducing IL-1β synthesis and minimising the associated inflammation described in chapter 1.

Finally, several miRNAs as detailed in chapter 1, have been implicated in platelet related pathways in patients with diabetes. This includes miR-223, miR-26b and miR-140 which have been shown to be downregulated in conditions of hyperglycaemia and are all linked with elevated platelet activation[212, 373]. It is hoped that with further research some of these may prove novel targets. Some progress has already been made with evidence to support the silencing of miR-155 linked to foam cell formation and atherosclerosis, resulting in a fall in the formation of atherosclerotic plaques [289].
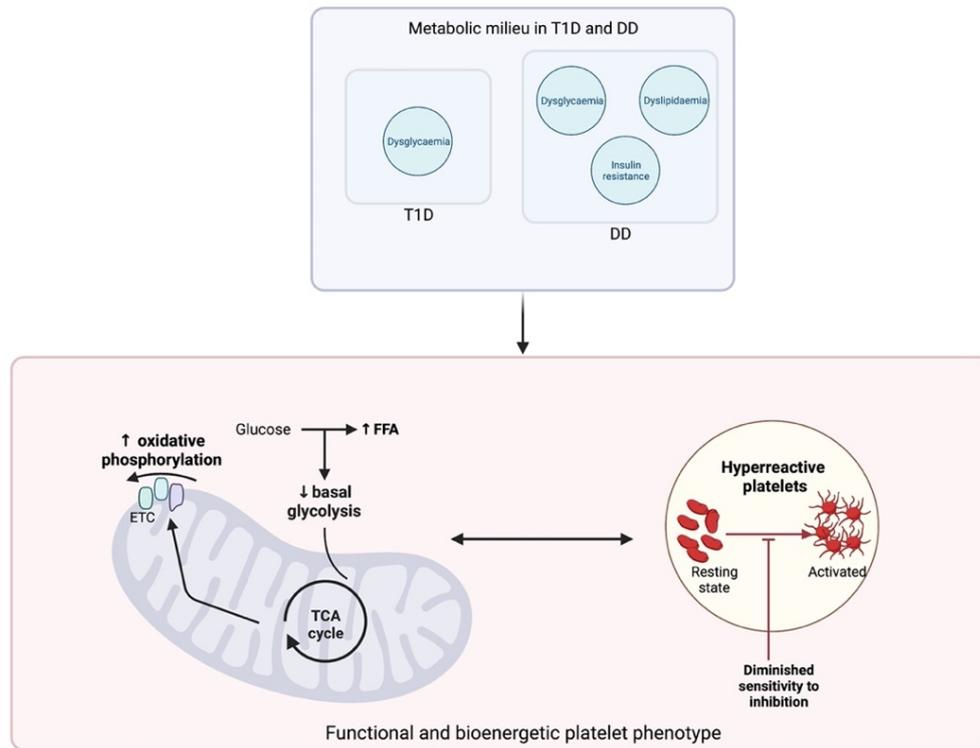
Figure 7-1: Impact of metabolic milieu of type 1 diabetes (T1D) and double diabetes (DD) on the functional and bioenergetic phenotypes of platelets.

The metabolic environment seen in patients with T1D encompasses a number of abnormalities including hyperglycaemia, hypoglycaemia and glycaemic variability along with further changes in a subgroup of these individuals with DD who additionally have dyslipidaemia and insulin resistance. These changes appear to result in a functional shift in platelets towards hyperreactivity and diminished sensitivity to inhibition, resulting in a pro-thrombotic phenotype. These functional alterations may be partly fuelled by, and further contribute to, a bioenergetic shift with changes in glucose metabolism and free fatty acid (FFA) oxidation resulting in altered glycolysis and mitochondrial respiration. The net effect is the development of a prothrombotic environment that contributes to increased risk of vascular events in this population of patients. TCA: tricarboxylic acid cycle (also known as Krebs cycle). ETC: electron transport chain. FFA: Free fatty acids.

7.5 Conclusion

Research in the field of platelet function in patients with T1D and even more so in patients with DD remains comparatively lacking to T2D. We have shown that even in young adults with no macrovascular disease, there is evidence of platelet hyperreactivity with a propensity towards procoagulant subpopulations. This is further driven by sub-optimal glycaemic control and co-existing insulin resistance, or DD. To compound the issue further, platelets from this cohort appear to have a diminished sensitivity to inhibition. This functional phenotype may be driven in part by alterations in bioenergetic signatures with alterations in energy sources with a possible increased use of FFA over glucose compared with healthy populations.

Whilst data for existing anti-platelet therapies and approaches have predominantly stemmed from patients without diabetes, focussed evidence and specific guidelines are becoming more available. Furthermore, the ever-developing arsenal of tools for cardiovascular risk stratification, individualisation of care and an increasingly proactive, preventative approach offer hope for the future of these important patient cohorts. I hope my data will contribute to the evidence required to optimise anti-thrombotic management in T1D, both for prevention and treatment, through appropriate phenotyping and the use of "precision medicine".

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List of Abbreviations

ACD- acid-citrate-dextrose

ACS- acute coronary syndrome

ADAM- a disintegrin and metalloproteinase

ADP- adenosine diphosphate

AGE- advanced glycation end-product

AnnV- Annexin-V

ATP- adenosine triphosphate

BMI- body mass index

$CaCl_2$- calcium chloride

CaCS- calcium containing Modified Tyrode's buffer

CAD- coronary artery disease

cAMP- cyclic adenosine monophosphate

CD40L- CD40-ligand

CGM- continuous glucose monitor

COX- cyclo-oxygenase

CoV- coefficient of variation

CRP- C-reactive protein

CRP-XL- Collagen related peptide cross-linked

CXCL- chemokine C-X-C motif

CXN- convulxin

DAG- diacylglycerol

DAMP- damage-associated molecular pattern

DAPT- dual anti-platelet therapy

DCCT- Diabetes Control and Complications Trial

DD- Double Diabetes

DEVELOP- Double diabetes and adverse clinical outcome: identification of mechanistic pathways

DM- Diabetes Mellitus

DMARD- disease modifying anti-rheumatic drug

DMEM- Dulbecco's Modified Eagle Medium

ECAR- extracellular acidification rate

EDC- epidemiology data center

EDTA- ethylenediaminetetraacetic acid

eGDR- estimated glucose disposal rate

eNOS- endothelial nitric oxide synthase

ETC- electron transport chain

FCCP- carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone

FFA- free fatty acids

FSC- forward scatter

GLUT- glucose transporter

GP-glycoprotein

GPIIb/IIIa- Glycoprotein IIb/IIIa

GPRP- Glycine-Proline-Arginine-Proline

GV- glycaemic variability

HbA1c- glycated haemoglobin

HDL- high-density lipoprotein

HFpEF- heart failure with preserved ejection fraction

HUVEC- human umbilical cord endothelial cells

IL- interleukin

IR- insulin resistance

IRS- insulin receptor substrate

LDL- low-density lipoprotein

LPL- lipoprotein lipase

LTA- light-transmission aggregometry

MFI- median or mean fluorescence intensity

MI- myocardial infarction

miRNA- micro ribonucleic acid

MODY- maturity onset diabetes of the young

NF-$\kappa\beta$- nuclear factor kappa β

NHS- National Health Service

NLRP3- NOD-like receptor protein 3

NO- Nitric oxide

NSTEMI- non-ST elevation myocardial infarction

OCR- oxygen consumption rate

OxLDL- Oxidised low-density lipoprotein

PAI-1- Plasminogen Activator Inhibitor-1

PAMP- pathogen-associated molecular pattern

PBMC- peripheral blood mononuclear cell

PBS- phosphate-buffered saline

PDGF- platelet-derived growth factor

PDH- pyruvate dehydrogenase

PFA- paraformaldehyde

PGI_2- Prostacyclin

PKC- protein kinase C

PPP- platelet poor plasma

PRP- platelet-rich plasma

PS- phosphatidylserine

PSGL- P-selectin glycoprotein ligand

PTGS2- prostaglandin-endoperoxide synthase 2

RAGE- receptor for advanced glycation end-products

RCT- randomised-controlled trial

RNA- ribonucleic acid

ROS- Reactive oxygen species

SELP- selectin-P gene

SSC- side scatter

SD-standard deviation

SFLLRN- thrombin-reepctor activating peptide

STEMI- ST-elevation myocardial infarction

T1D- Type 1 Diabetes Mellitus

T2D- Type 2 Diabetes Mellitus

TAR- time above range

TBR- time below range

TIR- time in range

TLR- toll-like receptor

TNF- tumour-necrosis factor

TXA_2- thromboxane

UKPDS- UK Prospective Diabetes Study

VASP- vasodilator-stimulated phosphoprotein

VLDL- very low-density lipoprotein

vWF- von Willebrand Factor

2-NBDG- 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose

2-DG- 2-deoxyglucose