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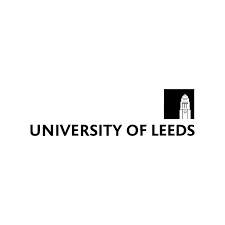
Master of Research in Medicine

SUPERVISED BY PROFESSOR KHALID NASEEM AND PROFESSOR RAMZI AJJAN

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Does diabetes promote metabolic thrombosis

Hawwa Iqbal, University of Leeds



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# 1.Background

Diabetes mellitus is defined as includes a fasting plasma glucose ≥ 7.0mmol/l (126 mg/dL) or oral plasma glucose tolerance ≥ 11.1 mmol/L (200 mg/dL), and HbA1C levels of >48 mmol/mol (WHO, 2011). Currently over 400 million adults and 1 million children currently living with some variation of the disease (IDF, 2019).

Our project focused on the two most prevalent forms: Type 1 (T1DM) and Type 2 diabetes (T2DM). T1DM is an autoimmune mediated condition in which insulin-secreting *β*-cells within the Islets of Langerhans in the pancreas are destroyed by autoimmune reaction (Atkinson et al., 2014), leaving patients in a state of insulin deficiency. T2DM is multifactorial disorder with a range of risk factors, including obesity, age, or being of South Asian, African-Caribbean or black ethnicity (Wilmot and Idris, 2014). ‘Insulin resistance’ is a key pathogenic mechanism; eventually, cells lose the ability to respond to insulin, resulting in reduced glucose uptake by hepatic, adipose and skeletal cells in patients with T2DM (Scheen et al., 1995), with an increase in hepatic glucose production (Kahn et al., 2014), further contributing to hyperglycaemia. High blood glucose levels result in the development of vascular complications, resulting in significant morbidity and mortality in the diabetes population.

### 1.1 Platelets in patients with diabetes

Platelets are metabolically active cells (Seong-Hoon Yun, 2016) that play a critical role in regulating haemostasis. Diabetic platelets show increased activity, resulting in faster rates of aggregation and atherogenesis (Sagel et al., 1975). This increases the risk of thrombus formation thus contributing to vascular complications in diabetes (Vlad G. Zaha 2019). Hyperglycaemia is often cited as the one of the predominant factors in initiating platelet hyperactivity (Sudic et al., 2006). Although the mechanism is poorly understood, it has been noted that under hyperglycaemic conditions, platelets increase their expression of platelet activation markers, including P-Selectin and CD40 (Gresele et al., 2003, Vaidyula et al., 2006, Sudic et al., 2006).

Under healthy conditions, insulin has an anti-aggregatory effect on platelet function by increasing expression of PGI2 receptors on the platelet surface (a potent inhibitor of adhesion) (Randriamboavonjy and Fleming, 2009, Vinik et al., 2001). Platelets taken from T2DM patients have been noted to have a lower expression of insulin receptors and a reduced response to insulin (Udvardy et al., 1985), and showed decreased sensitivity to PGI2 inhibition (T. Akai, 1983). In T1DM-induced mice, an 8-day course of insulin infusion was noted to restore renal and cardiac PGI2 production to normative levels, highlighting the role of insulin resistance specifically in long-term reduction in inhibitor sensitivity (Harrison et al., 1980).

The associations between these factors and platelet hyperactivity have been observed in previous studies, but the outstanding question remains; why do diabetic platelets demonstrate these prothrombotic tendencies? Are platelets born this way, or does the external diabetic milieu drive these changes?

### 1.2 Hypothesis and Study Aims

We hypothesise that the external environment of the platelet, rather than the intrinsic platelet structure, will have a greater effect on platelet activity. The aims of this study are as follows:

1. To develop a plasma swap assay to be used in the diabetic context, that will identify the effects of plasma on platelet function
2. To investigate the relationship between platelets and plasma, and the effect this has on platelet function.
3. To compare the effects between T1DM and T2DM plasma.

This study received ethical approval from the School of Medicine Research Ethics Committee Faculty of Medical Ethics Review committee on 2016, reference number: HSLGLM/12/045.

# 2. Methodology

### 2.1 Plasma Swap Assay

A ‘plasma swap’ assay is a novel assay designed to isolate the effects of the platelet and blood plasma on platelet hyperactivity. To do this, healthy and diabetic platelets and plasma were isolated. Healthy platelets were transferred into diabetic plasma, and diabetic platelets into healthy plasma, before a series of assays were conducted to assess changes in platelet function. To our knowledge, this is a novel technique that has not been applied in this context prior. Therefore, the preliminary stages of the project required optimisation of the plasma swap protocol, using aggregation and flow cytometry.

### 2.2 Activation and inhibition assays using diabetic plasmas

Following optimisation, it was decided that samples would be made up at plasma concentrations of 20%, with a 60-minute incubation of platelets with plasma. Three reaction conditions were created; healthy platelets with healthy plasma and healthy platelets with T1DM and T2DM plasma respectively. Previous studies have solely focused on comparing either T1DM or T2DM samples with healthy controls. Our unique study design will be using both types of diabetic samples with the same platelet population within the same assay. This eliminates potential confounders and facilitates the direct comparison of the effects of each type of plasma on platelet activity. Diabetic plasma samples were left to thaw before use.

### 2.3 Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software. A two-way ANOVA multiple comparison test was used to analyse the difference in P-Selectin expression between groups in the flow cytometry data. Significance was set at a p-value of 0.05.

# 3. Results

### 3.1 Impact of T1DM and T2DM plasma on platelet activation

Figure 1 – Comparing P-selectin expression in control platelets incubated in either T1DM or T2DM plasma in response to agonists. A) Compares % P-Selectin expression in the sample overall of control platelets incubated in either T1DM or control plasma for 60 minutes (n=4). B) Compares median p-selectin expression of control platelets incubated in either T1DM or control plasma for 60 minutes (n=4). A) Compares % P-Selectin expression in the sample overall of control platelets incubated in either T2DM or control plasma for 60 minutes (n=4). B) Compares median p-selectin expression of control platelets incubated in either T2DM or control (n=4). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001

Having established the basic conditions for the plasma swap assay (data not shown), the effect of patient plasmas was tested. Platelets taken from healthy controls were incubated in control, T1DM and T2DM plasma (thawed from frozen). In all cases the platelets incubated in diabetic plasmas were compared with platelets incubated in healthy controls.

Figures A and C show % P-selectin expression in control platelets incubated in either T1DM (1A) or T2DM (1C) plasma. The difference in % P-selectin expression between plasma swap and control samples was not significant.

Median P-selectin expression (the number of P-Selectin receptors expressed per individual platelet) was also recorded. Figure 1B shows median P-Selectin expression is significantly higher in platelets exposed to T1DM plasma following addition of TRAP (p<0.05), 100ng convulxin (p<0.01) and 250ng convulxin (p<0.0001). Median expression of P-selectin is also elevated in control platelets incubated in T2DM plasma. Under basal conditions P-selectin expression was significantly higher (p<0.05). This effect remained upon addition of agonists showing that individual platelets are expressing more of the adhesive receptor.

### 3.2 Impact of T1DM and T2DM plasma on platelet inhibition

Figure 2 - **Comparing P-selectin expression in control platelets incubated in either T1DM or T2DM plasma in response to increasing concentrations of PGI2 inhibitor.** A) Compares % P-Selectin expression on control platelets in 20% control or T2DM plasma, B) Compared Median P-Selectin expression on control platelets in 20% control or T2DM plasma C) Compares % P-Selectin expression on control platelets in 20% control or T1DM plasma, D) Median P-Selectin expression on control platelets in 20% control or T1DM plasma. All platelet samples were incubated for 60 minutes with plasma alone, followed by a 2-minute incubation of variable concentrations of PGI inhibitor, then stimulated using 250ng convulxin. Control samples (n=3), plasma swap samples (n=2).

In Figure 1, we explored the propensity of platelets to activate in response to agonists.

However, platelet response to agonists is mediated by its response to inhibitors. We therefore compared P-selectin expression in platelets that had been incubated in either T1DM or T2DM plasma for 60 minutes, followed by a 2-minute incubation with the PGI2 inhibitor, compared to a healthy control.

Figures 2A and C show that % P-Selectin expression in platelets incubated in diabetic plasma only decreased in response to the highest concentration of inhibitor (1000nm PGI2). Figure 2B and D corroborate this; in T1DM and T2DM plasma, median P-Selectin expression per platelet was consistently elevated compared to controls, despite increasing concentrations of PGI2. This effect was greater in platelets incubated in T1DM plasma (2D), compared to platelets incubated in T2DM plasma (2B). Statistical analysis could not be conducted as the assay had not been conducted in triplicate.

# 4. Discussion

The question of ‘nature vs nurture’ has been long debated in the field of platelet biology. The concept of a ‘plasma swap’ assay is crucial in exploring this question as it highlights the influence of the external plasma on platelet function, as well as showing the extent to which platelet activity is independent to its environment. Unfortunately, due to the early termination of the project we were unable to explore the effects of healthy plasma on diabetic platelets, but this distinction has clear clinical implications in developing precise therapeutic targets that should either act on controlling the external platelet environment or work intracellularly within the platelet itself.

### 4.1 Activation Plasma Swap Assay

Figures 1A and C show that there is no difference in overall % P-selectin expression in control platelets incubated in T1DM and T2DM plasma is and healthy control samples. However, Figures 1B and D show an elevated median P-selectin expression per platelet in response to agonists and crucially, under basal conditions (significant in platelets incubated in T2DM plasma (p<0.05)). To our knowledge, this is a novel finding, and one that implicates the extrinsic plasma in inducing intrinsic changes to platelet function, resulting in increased expression of activation markers. This suggests that there is something unique to diabetic plasma that acts to ‘prime’ platelets at the basal level; so that when platelets are exposed to agonists, the activation response is much greater. Furthermore, P-selectin translocation to platelet surface is agonist dependent; therefore under basal conditions, the elevation in median P-selectin expression highlights that there are unidentified factors in both T1DM and T2DM plasma that is mimicking agonist interaction, resulting in low-level background activation.

Given the similar findings between T1D and T2D plasma, the data suggest a direct role for hyperglycaemia rather than insulin resistance. Previous studies have noted in whole blood samples taken from a healthy subject, there was a dose-dependent increase in platelet and endothelial P-Selectin expression in response to increasing glucose concentrations (Keating et al., 2003, Burger et al., 2017). However, it is likely that other factors (rather than hyperglycaemia alone) are also responsible for platelet hyperactivity. Elevated levels of insulin seen in T2DM plasma has been associated with increased P-selectin expression, (Shanik et al., 2008), a feature characteristically absent in T1DM plasma (American Diabetes, 2009). From the literature, we know that hyperinsulinaemia is associated with the decreased activity of fibrinolytic PAI-1 and an increased release of vWF from platelet *α-*granules following exposure to agonists (Vinik et al., 2001). In T1DM platelets incubated with 10µU/ml insulin, there was a significant increase in fibrinogen binding (p<0.05) suggesting an insulin-associated drop in fibrinolytic activity, although there was no change in P-selectin expression (Hu et al., 2002). However, in patients with T2DM, an infusion of post-prandial 0.2ml/kg insulin was associated with an 23% increase in P-selectin expression (n=18) (Spectre et al., 2012). These studies suggest that there is some relationship between insulin levels and platelet P-selectin expression, although this can differ between T1DM and T2DM subjects.

### 4.4 Inhibition Plasma Swap Assay

Platelet hyperactivity could also be caused by an increased resistance to inhibition. Natural platelet inhibitors prostacyclin (PGI2) and nitric oxide (NO) are responsible for preventing platelet-platelet adhesion and platelet-endothelium binding (Vinik et al., 2001). In our study, we found that control platelets that had been incubated in both T1DM and T2DM plasma showed an increased resistance to PGI2 compared to controls (Figure 8). Unfortunately, given the early termination of the project due to COVID-19, only a n=2 was achieved, preventing the use of statistical analysis to calculate the significance of these findings. However, the results seem to highlight that platelets demonstrate a stronger resistance to higher concentrations of PGI2 following incubation with diabetic plasma samples, compared to healthy controls, as highlighted by the maintained expression of activation marker P-selectin.

# 5. Conclusions and Future Work

To conclude, this study has shown that exposure to T1DM and T2DM plasma alone is enough to significantly increase platelet hyperactivity in response to agonists, as well as conferring resistance to PGI2 inhibition. We hypothesise that hyperglycaemia could be sensitising platelets to agonists. Further work could explore a dose response to glucose added to healthy control platelets in healthy plasma and recording P-Selectin expression.

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