Evaluating immune activation and cellular determinants of thrombosis in pre-diabetes

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Submitted in fulfilment of the requirements for the degree:

PhD: Physiology

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As the candidate's supervisor I agree to the submission of this thesis.

Preface

This thesis is in fulfillment of the requirements for Doctor of Philosophy degree Health sciences. Chapter 1 describes the problem statement, the aims and research questions covered in the thesis. Chapter 2 covers the literature review providing an overview of research providing rationale for the current project. Three manuscripts submitted for peer review in accredited journals make up chapters 3, 4 and 5. Chapter 6 is the synthesis and conclusion summarizing the entire project.

This work has not been submitted in any form for any other degree or diploma at another institution. Use of other people's work has been acknowledged accordingly in-text.

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Declaration

I……………Zibusiso Mkandla……………………………. declare that

I. The research reported in this thesis, except where indicated, is my original work.

II. This thesis has not been submitted for any degree or examination at any other university

III. This thesis does not contain other person's data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

IV. This thesis does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a. their words have been re-written but general information.

Publications to international peer-reviewed journals

Under review

1. **Mkandla, Z**., Mutize, T., Dludla, P.V., Nkambule, B.B. 2018. High-fat-diet impairs glucose tolerance and enhances platelet reactivity in C57BL/6 mice. Purinergic signalling, **(submitted for review)**.
2. **Mkandla, Z**., Mutize, T., Mazibuko-Mbeje, S.E., Dludla, P.V., Nkambule, B.B. 2018. Metformin and low-dose aspirin reduce residual platelet activation in pre-diabetic mice. Journal of Diabetes Investigation, **(submitted for review)**.

Published manuscripts

1. **Mkandla, Z**., Mutize, T., Dludla, P.V., Nkambule, B.B. 2019. Impaired Glucose Tolerance is Associated with Enhanced Platelet-Monocyte Aggregation in Short-Term High-Fat Diet-Fed Mice. Nutrients
2. Dludla, P., Nkambule, B., Jack, B, **Mkandla, Z**., Mutize, T., Silvestri, S., Orlando, P., Tinao, L., Louw, J., Mazibuko-Mbeje, S. 2018. Inflammation and oxidative stress in an obese state and the protective effects of gallic acid. Nutrients (Accepted)
3. Mutize, T., **Mkandla, Z**. & Nkambule, B.B. 2018. Global and gene-specific DNA methylation in adult type 2 diabetic individuals: A protocol for a systematic review. Systematic Reviews, 7(1): 1–5.
4. Nkambule, B.B., **Mkandla, Z**., Mutize, T. & Dludla, P.V. 2017. Platelet function and cardiovascular risk in adult HIV-infected patients on HAART: A protocol for a systematic review and meta-analysis. BMJ Open, 7(12): 1–5.

Conference presentations

1. **Mkandla, Z**., Mutize, T., Dludla, P.V., Nkambule, B.B 2017. TLR4 binding ligand mediates the polarization of classical monocytes to M2 monocytes. College of Health Sciences Research Symposium, 5-6 October 2017, University of KwaZulu-Natal, Durban, South Africa.
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Dedication

This thesis is dedicated to Ntandoyenkosi Mkandla and Sandile Mkandla

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Abbreviations

ADP- adenosine diphosphate

AMPK- 5’ AMP-activated protein kinase

AREC- animal research ethics committee

Asp- low-dose aspirin

ASVD- atherosclerotic vascular disease

ATP- adenosine triphosphate

AUC- area under the curve

BD- Becton Dickson

BRU- biomedical research unit

CA- California

CAD- coronary artery disease

CD40L- CD40 ligand

CHS- College of health science

Clo- clopidogrel

COX-1- cyclooxygenase-1

COX-2- cyclooxygenase-2

cPGES- cytosolic prostaglandin E synthase

CST- cytometer set-up and tracking

CVD- cardiovascular disease

DAG- 1,2 diacyl-glycerol

EC- endothelial cell

ECM- extracellular matrix

FIC- fogarty international centre

GM-CSF- granulocyte macrophage colony stimulating factor

GPCR- G-protein coupled receptor

GPIV- glycoprotein-IV

HFD- high fat diet

hsCRP- high sensitivity c-reactive protein

hsIL-6- high sensitivity interleukin-6

HTPR- high on treatment platelet reactivity

IFN-γ- interferon γ

IL-1- interleukin 1

IL-6- interleukin 6

IL-8- interleukin 8

IL-β- interleukin β

IP$_3$- inositol-1, 4, 5-triphosphate

IQR- interquartile range

IR- insulin receptor

JPAD- Japanese primary prevention of atherosclerosis with aspirin for diabetes

LBP- LPS binding protein

LPS- lipopolysaccharide

MCP-1- monocyte chemoattractant protein-1

MeSH- medical subheadings

Met- metformin

MFI- mean fluorescence intensity

MI- myocardial infarct

MIP-1- macrophage inflammatory protein 1

mPGES- microsomal prostaglandin E synthase-1

MPV- mean platelet volume

mRNA- messenger ribonucleic acid

NJ- New Jersey

NO- nitric oxide

NOS- nitric acid synthase

NRF- National Research Foundation

OCS- open canicular system

OGTT- oral glucose tolerance test

PAC-1- procaspase activating compound 1

PAI-1- plasminogen activator inhibitor-1

PAR- protease activated receptor

PBMC- peripheral blood mononuclear cells

PDGF- platelet derived growth factor

PF4- platelet factor 4

PGIS- prostaglandin I synthase

PI3K- phosphoinositide-3 kinase

PKC- protein kinase C

PLA- phospholipase A

PLC- phospholipase C

PLT- freely circulating platelets

PMA- platelet monocyte aggregate

PRI- platelet reactivity index

PSGL-1- P-selectin glycoprotein ligand-1

QC- quality control

RANTES- regulated on activation, normal T-cell expressed and secreted

RBC-PLT- red blood cell bound platelets

RNA- ribonucleic acid

SD- standard deviation

sICAM- soluble intercellular adhesion molecule

sP-selectin- soluble P-selectin

sVCAM- soluble vascular cell adhesion molecule

T2DM- type 2 diabetes mellitus

TF- tissue factor

TF-κB- transcription factor κB

TNF-α- tumour necrosis factor α

TP- thromboxane receptor

TPO- thrombopoietin

TRAP- thrombin receptor activating peptide

TxA2- thromboxane A2

TxS- thromboxane synthase

UKZN- University of KwaZulu-Natal

USA- United States of America

VSMC- vascular smooth muscle cells

vWF- von Willebrand factor

Abstract

Introduction

Platelet dysregulation in pre-diabetes plays a major role in the progression of prothrombotic and pro-inflammatory conditions. Cardiovascular disease (CVD), which may occur at the pre-diabetic stage, are a common cause of morbidity in diabetic individuals. Despite treatment, people living with type 2 diabetes are at an increased risk of developing CVD, which is attributed to increased platelet activation and platelet mediated cellular cross-talks. The aim of the study was to investigate platelet activation and function in prediabetes and to further evaluate the effects of oral glucose lowering and anti-inflammatory therapy on platelet function a pre-diabetic state.

Methodology

Male mice were fed experimental diets, control low-fat diet (D12450J 10 kcal% fat) and a high-fat diet (HFD) (D12492, 60 kcal% fat) (Research Diets, NJ, USA). Platelet activation was determined by measuring the formation of spontaneous platelet-monocyte aggregate (PMA). The high-fat diet (HFD) fed mice were then randomized into 3 treatment groups; metformin (150mg/kg) and low-dose aspirin (3mg/kg) dual therapy; low-dose aspirin (3mg/kg); and clopidogrel (0.25mg/kg). The drugs were administered orally, once a day, every day for 3-weeks. We determined the pre-diabetic status of the mice by measuring their oral glucose tolerance and insulin levels. We further measured the haematological parameters. Platelet function and reactivity were determined by stimulating them with endogenous agonists, adenosine diphosphate (ADP), collagen and arachidonic acid, before and after treatment.

Results

Overall, there were no significant differences in the baseline characteristics such as body weights and insulin levels. However, after three weeks on the experimental diets, the high-fat diet (HFD) fed group exhibited delayed glucose clearance ($p=0.0362$). Baseline levels of platelet-monocyte aggregates were increased in the HFD group, $p=0.0156$. Post-stimulation with 4µM ADP and 20µM ADP, the HFD group expressed elevated levels of activated platelets, $p<0.05$. Metformin and low-dose aspirin-treatment inhibited reversible platelet aggregation, $p=0.0220$. While, residual platelet activation was observed at a concentration of 20µM ADP, $p=0.0535$. In the low-dose aspirin group, there were no significant variations in platelet reactivity following stimulation with ADP, $p>0.05$. Clopidogrel treatment inhibited the platelet response to 20µM ADP ($p=0.0313$).

Conclusion

Despite anti-platelet treatment in pre-diabetes, platelets exhibit a varied response to endogenous agonists. Therapeutic targeting of these pathways may reduce the risk of thrombotic complications in

pre-diabetes. We further highlight the potential synergistic benefit of using dual oral glucose lowering and antiplatelet treatment to minimize the high on-treatment platelet responses observed in pre-diabetes and T2DM.

Chapter 1. Introduction

The metabolic syndrome, a cluster of insulin resistance, hyperinsulinaemia, dyslipidaemia, hypertension and obesity, is a common risk factor for both type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (1,2). In diabetics, the prevalence of masked hypertension may reach 26.5%, which is associated with 31% higher odds of developing CVD in comparison with non-diabetics (3). Most individuals with T2DM are obese, and the global epidemic of obesity has played a major role in the dramatic increase in the incidence and prevalence of noncommunicable diseases in recent years (4). Low-grade chronic inflammation, which is characterised by abnormal adipokine and pro-inflammatory signalling is one of the foremost factors identified in obese and T2DM individuals (5). For the latter, persistent levels of pro-inflammatory proteins can activate and upregulate the expression of tissue factor (TF), which is the initiator of the extrinsic coagulation cascade, on the surface of endothelial cells resulting in increased risk of atherothrombotic conditions (6). Activation of the extrinsic pathway results in the enzymatically active complex (FVIIa) which activates FIX and FX (serine proteases for the coagulation cascade). This ultimately leads to thrombin generation, as well as activation of fibrinogen and fibrin stabilizing factor FXIII in association with fibrin clot formation (7). Currently, there are limited therapies available to protect diabetic individuals at risk of developing the aforementioned complications. However, metformin, which is a commonly used glucose-lowering drug, is known to reduce platelet activation in newly diagnosed subjects with T2DM (8). Some of the mechanisms by which metformin induce its effects include enhancing intracellular antioxidants leading to the reduction of oxidative stress, as well as blocking plasminogen activator inhibitor-1 (PAI-1), thereby promoting fibrinolysis in diabetic individuals (1). Metformin also reduces the levels of FVII, fibrinogen and also shortens fibrinolysis times in non-diabetic individuals with a high hip-to-waist circumference ratio (1,9).

Much evidence points to accelerated thrombogenesis and fibrinolysis in remaining the main underlying factors that contribute to the high risk of atherosclerosis in diabetic individuals (2). However, activated platelets also play a crucial role in the development of hypercoagulable states in T2DM (10,11). Elevated platelet reactivity in T2DM can be influenced by upregulated multiple signalling pathways (12). For example, increased platelet activation in high glucose concentrations can result in enhanced expression of P-selectin and drive fibrinogen binding leading to the formation of platelet-leukocyte aggregates (13). Similarly, activated monocytes have the potential to exacerbate a hypercoagulable state (14). Activated platelets are capable of binding to monocytes to form platelet-monocyte aggregates (PMAs), which are robust markers of exacerbated hypercoagulable state (11). Activated platelets and monocytes express surface TF which initiates the activation of the extrinsic coagulation cascade (15), contributing to the presence of hypercoagulable states in diabetic individuals (16,17). This phenomenon can be explained by the increased levels of TF expression on monocyte surfaces in T2DM (11).

Anti-coagulant drugs including salicylates display a high potential to reduce elevated platelet activation and function, leading to lower risk of cardiovascular events (10,18). However, it has also been noted that commonly used anticoagulant drugs may present limited efficacy in protecting against inflammatory induced complications. Notably, a recent study showed that aspirin monotherapy does not significantly reduce the incidence of CVD in T2DM individuals (19). Thus, in addition to investigating mechanisms involved in pro-inflammatory linked cardiovascular complications in diabetic individuals, novel therapeutics should also be assessed for the protective properties against such complications. To date, limited data is available on the role activated platelets and monocytes play in the development of CVD, hence this phenomenon needs to be evaluated. Therefore, in a series of co-ordinated studies, this project investigated the effects of current T2DM therapy on the thrombotic profile in a pre-diabetic state. Mechanisms involved in disease development and progression, linking inflammation and pre-diabetes induced CVD complications were also assessed. Potential findings may prompt the inclusion of non-steroidal anti-inflammatory drugs to the current therapy which will also aid in reducing the prothrombotic state present in pre-diabetic individuals. It is of note that increasing research is targeting a pre-diabetic state to curb complications associated with diabetes-induced cardiovascular complications.

1.1 Overall aim

To investigate the effects of current T2DM therapy on the thrombotic profile in a pre-diabetic state.

1.2. Objectives

- To investigate platelet activation and function during the development of type 2 diabetes mellitus using a diet-induced model of pre-diabetes.
- To determine the effects of endogenous agonists like arachidonic acid and adenosine diphosphate (ADP) in modulating platelet reactivity and monocyte function in pre-diabetic mice on anti-hyperglycaemic and anti-inflammatory treatment.
- To investigate platelet reactivity in combined therapy of low dose aspirin and metformin-treated pre-diabetic mice.

1.3. Research questions

- How does pre-diabetes affect the reactivity and function of platelets during the development of T2DM?
- What is the polarization status of monocytes in the pre-diabetic state?
- Does aspirin as a monotherapy or as an add-on therapy to metformin inhibit platelet activation and function in pre-diabetic mice?
- Does aspirin as a monotherapy show anti-inflammatory effects in pre-diabetic mice compared to add-on therapy when combined with metformin?

1.3. References

1. Lemkes BA, Hermanides J, Devries JH, Holleman F, Meijers JCM, Hoekstra JBL. Hyperglycemia: A prothrombotic factor? J Thromb Haemost. 2010;8(8):1663–9.

2. Vazzana N, Ranalli P, Cuccurullo C, Davì G. Diabetes mellitus and thrombosis [Internet]. Vol. 129, Thrombosis Research. Elsevier Ltd; 2012 [cited 2015 Feb 25]. p. 371–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22197180

3. Zhao H, Zeng F, Wang X, Wang L. Prevalence, risk factors, and prognostic significance of masked hypertension in diabetic patients. Medicine (Baltimore) [Internet]. 2017;96(43):e8363. Available from: http://insights.ovid.com/crossref?an=00005792-201710270-00049

4. Eckel RH, Kahn SE, Ferrannini E, Goldfine AB, Nathan DM, Schwartz MW, et al. Obesity and type 2 diabetes: What Can be unified and what needs to be individualized? Diabetes Care. 2011;34(6):1424–30.

5. Zimmet P, Magliano D, Matsuzawa Y, Alberti G, Shaw J. The Metabolic Syndrome: A Global Public Health Problem and A New Definition. J Atheroscler Thromb [Internet]. 2005;12(6):295–300. Available from: http://joi.jlc.jst.go.jp/JST.JSTAGE/jat/12.295?from=CrossRef

6. Alzahrani SH, Ajjan RA. Review article: Coagulation and fibrinolysis in diabetes. Diabetes Vasc Dis Res. 2010;7(4):260–73.

7. Hess K, Grant PJ. Inflammation and thrombosis in diabetes. Thromb Haemost. 2011;105(SUPPL. 1):43–54.

8. Formoso G, De Filippis E, Di Fulvio P, Pandolfi A, Bucciarelli T, Ciabattoni G, et al. Decreased in vivo oxidative stress and decreased platelet activation following metformin treatment in newly diagnosed type 2 diabetic subjects. Diabetes Metab Res Rev [Internet]. 2014;32(30):13–23. Available from: http://libweb.anglia.ac.uk/

9. Fontbonne A, Charles M a, Juhan-Vague I, Bard JM, André P, Isnard F, et al. The effect of metformin on the metabolic abnormalities associated with upper-body fat distribution. BIGPRO Study Group. Diabetes Care. 1996;19(9):920–6.

10. Aspirin Therapy in Diabetes. American Diabetes Association. Annals of Internal Medicine. 2004;27(June 1997):1997–8.

11. Davison GM, Nkambule BB, Mkandla Z, Hon GM, Kengne AP, Erasmus RT, et al. Platelet, monocyte and neutrophil activation and glucose tolerance in South African Mixed Ancestry individuals. Sci Rep [Internet]. 2017 Jan 16;7:40329. Available from: http://www.nature.com/articles/srep40329

12. Angiolillo DJ, Bernardo E, Sabat?? M, Jimenez-Quevedo P, Costa MA, Palazuelos J, et al. Impact of Platelet Reactivity on Cardiovascular Outcomes in Patients With Type 2 Diabetes Mellitus and Coronary Artery Disease. J Am Coll Cardiol. 2007;50(16):1541–7.

13. Sudic D, Razmara M, Forslund M, Ji Q, Hjemdahl P, Li N. High glucose levels enhance platelet activation: Involvement of multiple mechanisms. Br J Haematol. 2006;133(3):315–22.

14. Cermak J, Key NS, Bach RR, Balla J, Jacob HS, Vercellotti GM. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. Blood. 1993;82(2):513–20.

15. Shantsila E, Lip GYH. The role of monocytes in thrombotic disorders: Insights from tissue factor, monocyte-platelet aggregates and novel mechanisms. Thromb Haemost. 2009;102(5):916–24.

16. Dorsam RT, Kunapuli SP. Central role of the P2Y 12 receptor in platelet activation. J Clin Invest. 2004;113(3):10–5.

17. Wang Y, Li Z, Wang W. Platelet-Leukocyte Interaction in Atherosclerosis and Atherothrombosis : What We Have Learnt From Human Studies and Animal Models. J Cardiol Ther [Internet]. 2014;1(5):92–7. Available from: http://ghrnet.org/index.php/jct/article/view/710

18. Yeomans ND. Aspirin: Old drug, new uses and challenges. J Gastroenterol Hepatol. 2011;26(3):426–31.

19. Saito Y, Okada S, Ogawa H, Soejima H, Sakuma M, Nakayama M, Doi N, Jinnouchi H, Waki M, Masuda I, Morimoto T. Low-Dose Aspirin for Primary Prevention of Cardiovascular Events in Patients with Type 2 Diabetes Mellitus. Circulation. 2017;135(7):659–70.

Chapter 2. Literature review

2.1. Introduction

Type 2 diabetes (T2DM) is diagnosed based on abnormally elevated fasting plasma glucose concentrations. It continues to be a global pandemic with over 415 million cases reported in 2015, and estimated to rise to 642 million by 2040 (1, 2). The prevalence of diabetes in adults was estimated to be 8.8%, with 75% coming from low and middle-income countries such as those in Southern Africa (2). In 2013, Africa had a diabetes prevalence of 4.9%, and this figure is expected to have an increase of 110% to 41.5 million cases by the year 2035 (3). In addition to a large number of undiagnosed cases, the rate of diabetes prevalence in developing countries has been hugely affected by increasing individuals classified as pre-diabetic (1,2). The state of pre-diabetes can be defined as impaired fasting glucose, impaired glucose tolerance or both (4), with these individuals at higher risk of developing T2DM (5). In 2010, an estimated 57 million people were diagnosed with pre-diabetes. In the United States of America, pre-diabetes contributed to 29.5% of the total diabetes prevalence. More than 470 million people globally are expected to be suffering from this condition by 2030 (5, 6). Pre-diabetes is a high-risk state for diabetes with 5-10% conversion rate and a similar percentage reverting to normoglycaemia. It is associated with insulin resistance and beta β cell dysfunction which occur before noticeable changes in glucose levels (7). T2DM accounts for over 90% of all diabetes cases and is caused by the body's inability to utilise insulin effectively (decreased insulin sensitivity) resulting in increased blood glucose levels (hyperglycaemia) accompanied by subsequent damage to body organs and vasculature (8). Indeed, almost four-fold of diabetic individuals die of cardiovascular complications (9). Therefore, this review discussed the relationship between T2DM and cardiovascular disease (CVD). It focused on the role hyperglycaemia has in the development of pro-inflammatory and ultimately hypercoagulable states associated with CVD. In addition, the review highlights the role of current anti-diabetic and anti-platelet treatment in curbing the susceptibility of vasculature in a diabetic state. To achieve this, a search strategy was developed using medical subheadings (MeSH) keywords type 2 diabetes mellitus, cardiovascular disease, inflammation, anti-diabetic treatment and anti-platelet treatment. MEDLINE (OVID interface, 2000 onwards), EMBASE (OVID interface, 2000 onwards) and Cochrane Central Register of Controlled Trials (Wiley interface, current issue) was used to search for published articles. A comprehensive approach was used to analyse extracted literature, discussing different components of risk factors and detrimental effects associated with inflammation and its role in T2DM induced cardiovascular complications.

2.2. Type 2 diabetes and cardiovascular disease (CVD)

T2DM individuals have a 2-4-fold increased risk of premature atherosclerotic vascular disease (ASVD) when compared to nondiabetic counterparts (10). These include; myocardial infarctions (MI), cerebral vascular disease and peripheral vascular diseases (10). T2DM is a combination of both pro-

inflammatory and hypercoagulable states with alterations in the components of the Virchow's triad (11). These altered components include endothelial damage or dysfunction, abnormal blood stasis, and altered haemostasis, platelets and fibrinolysis (Figure 2.1) (12). Alterations in any two of the Virchow's triad components increases the risk of vascular thrombosis (13).

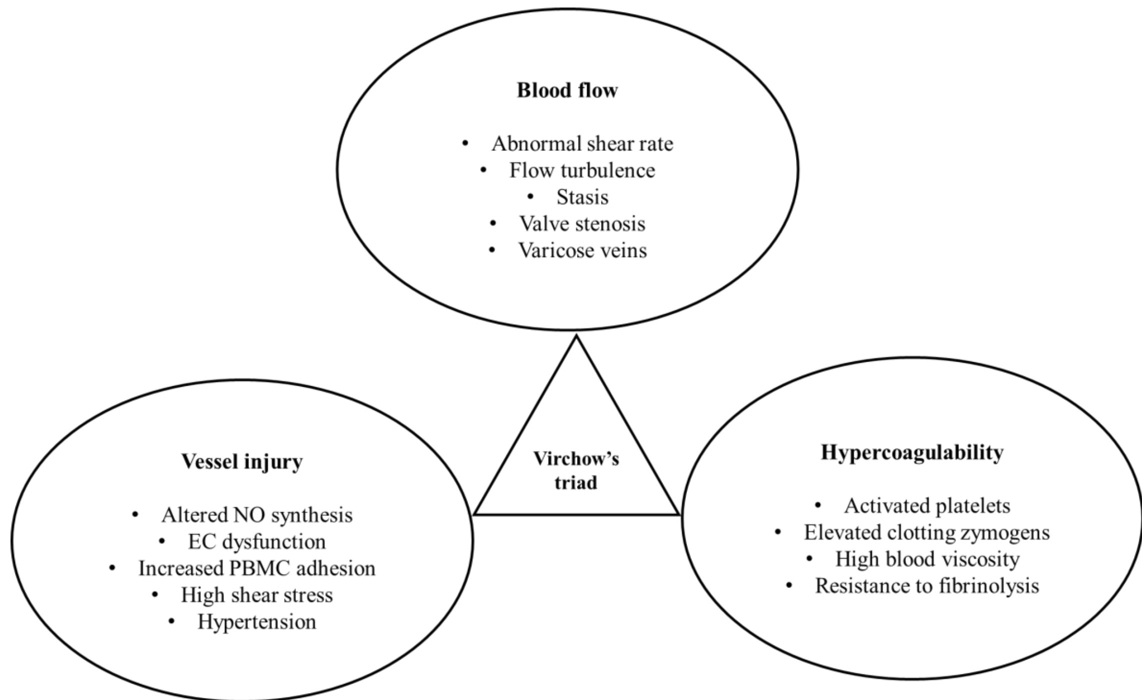

Figure 2.1. Illustration of the components of the Virchow's triad that are altered in type 2 diabetes mellitus (T2DM). Vessel injury: this involves altered nitric oxide (NO) synthesis resulting in increased adhesion of peripheral blood mononuclear cells (PBMCs) to the endothelial surface of blood vessels. Endothelial cell dysfunction also results in altered NO synthesis as well as increased adhesion of PBMCs. Blood flow: stasis, varicose veins and valve stenosis result in alteration of the blood flow through slowing down or blockage caused by fibrin clot formation. Hypercoagulability: T2DM has been associated with increased levels of activated platelets as well as microparticles which contribute to the hypercoagulable state. Increased resistance to fibrinolysis has also been described in T2DM (11).

2.3. Inflammation in obesity and type 2 diabetes

Inflammation is the tissue's response to injury and can be classified into cellular and humoral responses (14, 15). In humoral immune response, antibody-mediated immune response, triggers B-cells to become plasma cells which secrete antibodies. On the other hand, cellular immune response is primarily mediated by T-cells (T-helper and killer T-cells) that can activate other immune cells such as B-cells and natural killer cells (16). Acute inflammation is characterised by increased blood flow and accumulation of fluid, leukocytes and cytokines at the site of injury. It also includes the activation of endothelial cells (EC), tissue macrophages and platelets (15). Chronic inflammation is characterised by specific cellular and humoral immune cell responses at the site of injury and is defined according to the nature of inflammatory cells present (14, 15). Once the tissue injury has been resolved, acute

inflammatory cells are removed by lymphatics or apoptosis (17). Chronic inflammation results in monocyte chemotaxis to the site of inflammation where interferon γ (IFN γ) and monocyte chemoattractant protein-1 (MCP-1) activate macrophages. These macrophages are then retained at the site of inflammation by granulocyte macrophage colony stimulating factor (GM-CSF) and IFN γ where they increase levels of interleukin 1 (IL-1) and tumour necrosis factor (TNF) (14).

Chronic inflammation, which is exacerbated by abdominal obesity, is associated with other metabolic syndrome complications such as insulin resistance (IR), T2DM and CVD (18). In obese individuals, adipocytes secrete adipokines that are involved in the initiation of inflammation (19). Acute hyperglycaemia induces an increase in the production of pro-inflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor (TNF-α) and interleukin 18 (IL-18) (20). Diabetic individuals have increased plasma levels of inflammatory markers, including high-sensitivity C-reactive protein, IL-6, TNF-α, soluble intercellular adhesion molecule (sICAM)-1, soluble vascular cell adhesion molecule (sVCAM)-1, soluble E-selectin, and matrix metalloprotease 2 and 9 (21). In addition, inflammatory conditions can trigger the production of TF in various cells, namely the ECs, vascular smooth muscle cells (VSMCs), monocytes and macrophages, granulocytes and platelets (22).

2.4. Inflammatory mechanisms involved in diabetes-induced cardiovascular complications

2.4.1. Tissue factor is implicated in pro-inflammatory induced injury

Tissue factor is a membrane protein cofactor component of TF-factor VIIa complex enzyme (23, 24). It is associated with microvascular complications which are an indicator of endothelial dysfunction rather than pro-coagulant activity (25), and elevated levels of circulating TF in T2DM (10). The levels of circulating leukocyte and microparticle derived TF in blood have also been associated with the hypercoagulable state (10, 26). Although it remains unclear whether platelets are able to synthesize their own TF messenger RNA (mRNA), it has been previously suggested that TF can be expressed on the surface of platelets as a result of contamination from monocytes (27).

There is still more research to be done to define hypercoagulable states with respect to coagulation, and various factors can initiate a pathological state leading to cardiovascular events. In this case, factor VIIa (activated coagulation factor VII) can also initiate the extrinsic coagulation cascade leading to fibrin deposition and platelet activation (28). When the endothelial surface is disrupted or activated, TF binds to FVIIa to form the complex which is responsible for the downstream activation of FIX and FX to FIXa and FXa, respectively (Figure 2.2). These two factors lead to the formation of prothrombinase complex and thrombin generation (10, 26).

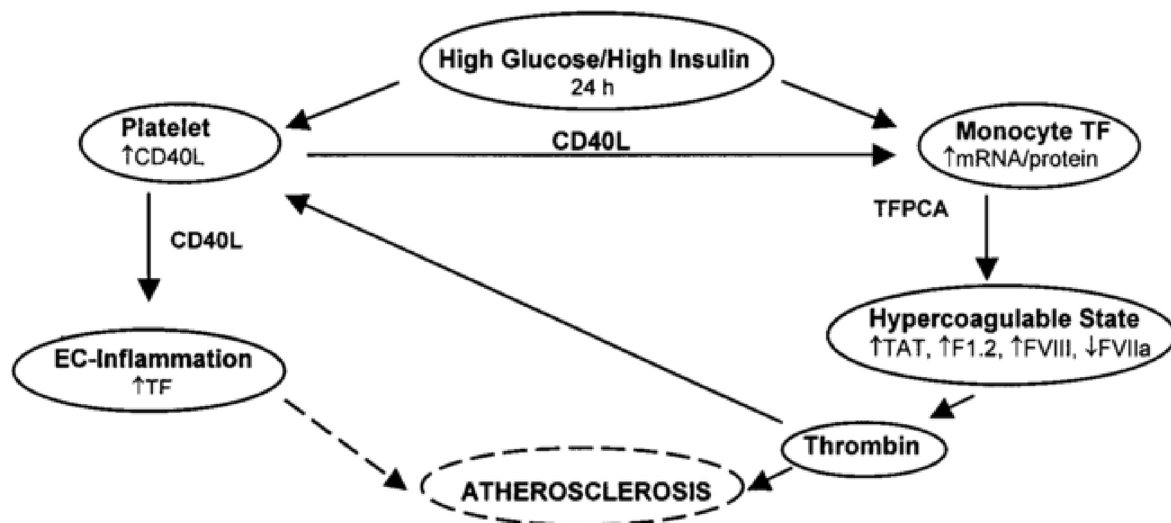

Figure 2.2. Illustration of the effect of hyperglycaemic conditions on platelet CD40L, monocyte tissue factor (TF) mRNA and membrane surface protein. Based on a previous study, exposure to 24-hours of hyperglycaemic (20% glucose solution and glucose levels maintained at 11 mmol/l) conditions results in elevated levels of CD40L (a marker of platelet activation and immune modulation) and monocyte TF mRNA as well as the membrane surface protein. Increase in monocyte mRNA and protein leads to a hypercoagulable state (10).

During T2DM disease progression, it is postulated that monocytes produce TF when they undergo phenotypic change as a result of altered transcription factors (NF-κB) leading to the formation of pro-coagulant cells (27, 29). Lipopolysaccharide (LPS) induces the expression of TF on the surface of monocytes. It binds to LPS binding protein (LBP) to form an LPS/LBP complex which binds to CD14, activating signal transduction pathways and transcription factors to induce TF gene expression (30). Antigen derived T-helper cells, lymphokines, complement-derived anaphylatoxin C5a and antigen-antibody complexes are also able to elicit TF expression on monocyte surface membrane (31). The underlying mechanisms of monocyte and neutrophil function in microvascular thrombosis remain unclear (22, 32). Nonetheless, the presence of leukocytes, monocytes and neutrophils, at the site of inflammation or vascular injury have been reported. Their interaction with platelets may directly contribute to increased fibrin generation by exposing TF, and indirectly by inactivating TF inhibitor (32). The tissue factor pathway inhibitor is a protein associated with the endothelial membrane and its increased levels indicate endothelial damage (33).

2.4.2. Platelet structure and function and its role pro-inflammatory induced endothelial injury

Platelets are very small enucleate cells with a diameter of 2-3µm formed by the fragmentation of much larger megakaryocytes (100µm) in the bone marrow (Figure 2.3) (34, 35).

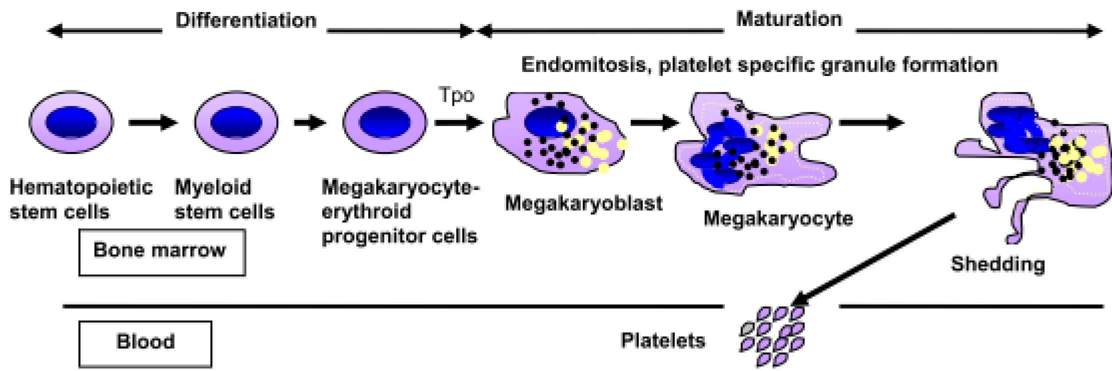

Figure 2.3. Illustration of the differentiation of hematopoietic stem cells to megakaryocyte-erythroid progenitor cells. These cells differentiate into megakaryoblasts through interaction with thrombopoietin (TPO) and subsequent maturation into megakaryocytes. Megakaryocytes in the sinusoidal walls of the bone marrow shed large segments of cytoplasm into the bloodstream where the shear force of circulating blood fragments the segments into individual platelets (36).

Platelets are largely inactive, they circulate close to the endothelium without any firm adhesion due to the anti-adhesive properties of intact endothelial cells. However, vascular damage leads to their activation and adhesion to the endothelial surface (37). Once activated, platelets bind to the exposed extracellular matrix (ECM) proteins such as collagen and von Willebrand factor (vWF) (35, 38). They express a repertoire of cell surface receptors on their phospholipid bilayer membrane, which are responsible for intracellular signalling. This includes CD36 (GPIV, a scavenger receptor), CD63 (receptor for tissue inhibitor of metalloproteinase), CD9 (cell adhesion molecule), G-protein coupled receptors (GPCRs), glycoprotein IIb/IIIa and glucose transporter-3 (GLUT-3) (38). These trigger the release of α-granules that play a role in inflammation, coagulation and wound repair (Figure 2.4). GPCRs trigger the release of adenosine diphosphate (ADP) from dense granules which is an important agonist for platelet activation (38, 39). Low ATP/ADP ratios maintain enhanced glycolysis which arises due to suppressed mitochondrial metabolism, possibly via activation of 5' AMP-activated protein kinase (AMPK), the major regulator of energy metabolism in mammalian cells (40). On the other hand, high ATP/ADP ratios block glycolysis in aerobic non-proliferating cells (41).

Platelet granules contain membrane as well as soluble receptors involved in haemostasis, inflammation and wound healing. Alpha (α) granules are spherical organelles which contain adhesion proteins fibrinogen, fibronectin, vWF, thrombospondin and vitronectin (36, 42). The granule's exocytosis can be evaluated by the membrane expression of its contents such as P-selectin (CD62P), vWF and platelet factor-4 (PF4) (42). Dense granules are unique to platelets for containing mainly bioactive amines such as serotonin, histamine, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) (36, 42). The exocytosis of these granules can also be evaluated by the release of its contents like ADP, ATP and serotonin (42). The exocytosis of both these granules is essential for platelet activation and function.

For example, upon activation of platelets, the P-selectin expressed from the α-granules acts as a point of adherence for leukocytes in the formation of platelet-leukocyte aggregates (43).

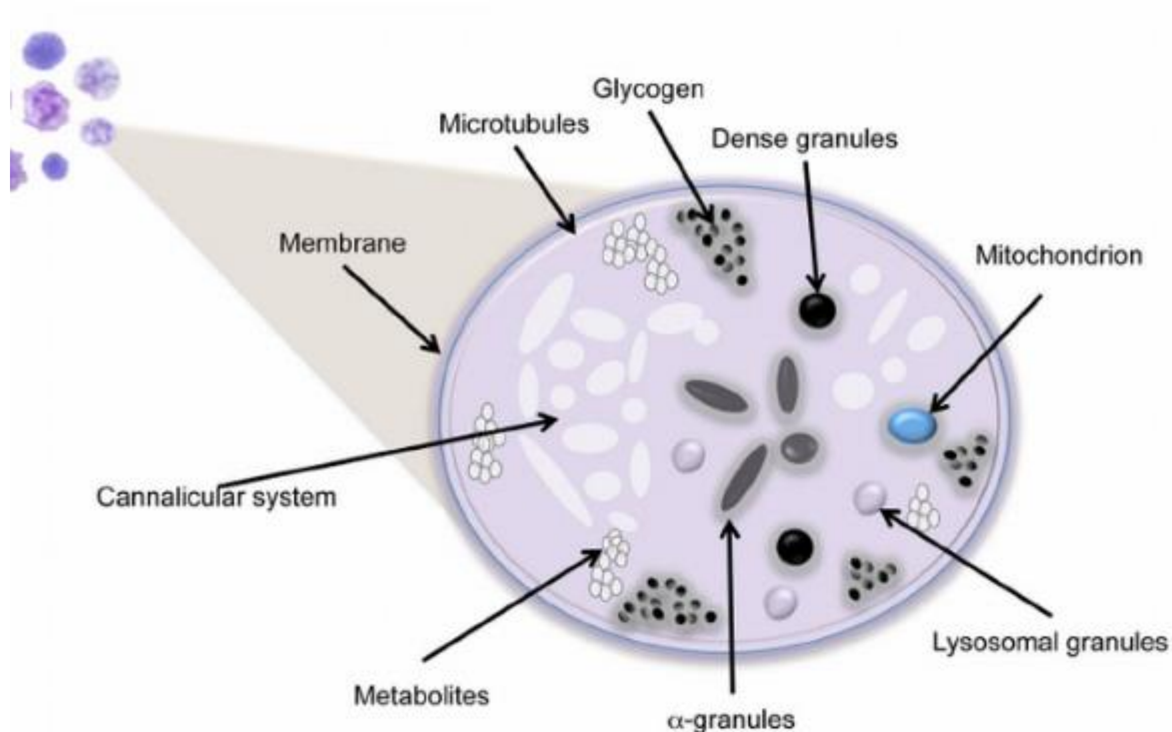

Figure 2.4. Illustration of the structure of a resting platelet. There are four distinct zones; (1) The peripheral zone: which includes the outer membrane. In this zone, there are surface-connected channels known as the open canicular system (OCS) which allows for interaction between the platelet interior and plasma substances. (2) The sol-gel zone houses the platelet cytoskeleton which maintains the discoid shape. (3) The organelle zone contains the alpha (α) and dense granules, lysosomes and mitochondria. (4) The membrane zone contains the dense tubular system where calcium is concentrated and is responsible triggering for contractile events (35, 36).

2.5. Adenosine diphosphate (ADP) receptors involved in platelet activation and function

ADP is released from the dense granules of platelets during degranulation leading to irreversible aggregation (44, 45). Once released, it activates surrounding platelets through the $P2Y_1$ and $P2Y_{12}$ receptors facilitating a positive feedback mechanism (46–49). $P2Y_1$ is responsible for the mobilization of cytoplasmic calcium and mediating platelet shape modification following reversible aggregation. The $P2Y_{12}$ receptor mediates irreversible platelet activation and aggregation without any modification (44). ADP release from the dense granules activates platelets and in-turn results in the release of α-granule components. These components such as macrophage inflammatory protein 1 (MIP-1), normal

T-cell expressed, secreted (RANTES), interleukin-8 (IL-8), interleukin-β (IL-β), CD40 ligand (CD40L) and P-selectin are involved in recruitment and activation of other leukocytes (50).

2.5.1. *P2Y$_1$ receptors*

The P2Y$_1$ is a purinergic receptor and widely expressed in various cell lineages but is mostly expressed by platelets (51, 52). It is coupled with the Gαq proteins thereby triggering calcium release from internal stores in the dense tubular system leading to shape change and reversible aggregation (36, 44, 51). Transgenic P2Y$_1$ deficient mice have been shown to display impaired platelet aggregation and resistance to ADP agonist-induced activation (44, 53), and the inhibition of P2Y$_1$ receptor does not lead to platelet activation and aggregation (51). This is further demonstrated when P2Y$_1$ deficient platelets exhibit no effect on ADP-dependant inhibition of adenylyl cyclase (39).

2.5.2. *P2Y$_{12}$ receptor*

Similar to P2Y$_1$, the P2Y$_{12}$ receptor expression is not limited to platelets but is also present in the brain, glial cells and vascular smooth muscle (54). P2Y$_{12}$ is a Gi-coupled (primarily Gα_{i2}) receptor and plays a role in the activation of the fibrinogen receptor (integrin $\alpha_{IIb}\beta_3$), shear-induced platelet aggregation and thrombus formation (39, 55). Inhibition of this receptor by clopidogrel reduces levels of cyclic adenosine monophosphate (cAMP) suppressing the phosphorylation of vasodilator-stimulated phosphoprotein (VASP-P) by protein kinases (54, 56, 57). It has been shown that platelet reactivity measured in P2Y$_{12}$ reactivity units (PRU) is significantly higher in diabetic individuals when compared to non-diabetic individuals (58). This may suggest the importance of the pathways involved in platelet function and their contribution to the hypercoagulable state in pre-diabetic and T2DM individuals.

2.6. Platelet signalling pathways

Platelet activation and signalling are classified into agonist interactions with their respective receptors and receptor-mediated early platelet activation pathways. The pathways can also be described by intermediate common signalling, integrin intervention inside-out signalling and outside in signalling (59). Platelets interact extracellularly and intracellularly through ligand binding using inside-out signalling. Once ligands bind to the integrins, they pass on information into the cell (outside-in signalling) (60). Some of the pathways that are implicated in the platelet-induced mechanism are briefly described below, namely the cyclooxygenase pathway, calcium signalling and protein kinase C signalling.

2.6.1. *Cyclooxygenase pathway*

Activated platelets release the enzyme cyclooxygenase (COX) which leads to oxidation of arachidonic acid and consequent synthesis of bioactive compounds such as thromboxane A2 (TxA2) (36, 61, 62). Arachidonic acid is metabolised into short-lived intermediates prostaglandin G$_2$ and H$_2$ which result in platelet activation and vasoconstriction (62). Cyclooxygenase 1 and COX 2 are the two main isoforms

which transform arachidonic acid into prostaglandin H_2. This is then transformed into prostanoids, prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostaglandin I_2 (PGI_2) and TxA_2 (63). Prostanoids are lipid mediators generated in response to the action of COX on arachidonic acid; they consist of thromboxanes, prostacyclins and prostaglandins. During low-grade inflammatory conditions such as pre-diabetes and T2DM, the biosynthesis of these prostanoids is elevated (64). Prostaglandin I_2 and TxA_2 are synthesised by COX-1 and COX-2 respectively and play a major role in the physiology and dysregulation of the function of blood vessels (63).

2.6.2. Calcium signalling

Cytosolic calcium plays a crucial role in platelet activation. Its elevation may be induced by intracellular store release of inositol-1,4,5-trisphosphate (IP_3) receptor, or extracellularly by its entry through the plasma membrane by 1,2 diacyl-glycerol (DAG) (59, 65). In T2DM, intracellular calcium concentrations are elevated in resting platelets (66). Elevation of calcium activates the actin-myosin interaction which results in cytoskeleton reorganisation and ultimately platelet shape change (59, 65). Activation of the αIIbβ3 integrin is also attributed to elevated calcium levels, and this results in platelet degranulation, enhanced thromboxane production and aggregation (59, 65, 67). Additional effects of elevated calcium levels include activation of the protein kinase C (PKC), calmodulin and NO synthesis (59).

2.6.3. Protein kinase C

Protein kinase C (PKC) is involved in platelet granule secretion as well as aggregation which can be directly activated by arachidonic acid and DAG (68, 69). Together with increased intracellular calcium, prolonged PKC activation results in irreversible platelet aggregation (69). A study using a PKC inhibitor resulted in inhibited dense granule secretion thereby blocking aggregation and αIIbβ3 activation in platelets (61). In hyperglycaemic conditions, there is increased membrane-bound PKC *in vivo* and DAG levels are chronically elevated resulting in its activation (70, 71).

Endogenous agonists such as ADP, arachidonic acid and collagen may contribute to the development of atherosclerosis. Adenosine diphosphate activates the P2Y pathway, consequently leading to platelet activation, shape change and aggregation (44). Whereas collagen is involved in initiating the intrinsic clotting cascade and has been shown to increase transforming growth factor (TGF-β1) and platelet-derived growth factor (PDGF) (72). Platelet aggregation can be induced by collagen acting on glycoprotein VI/Fc receptor γ chain complex thereby activating enzymes Src and Syk family tyrosine kinases. These prompt signalling of the phosphatidylinositol 3-kinase (PI3K) which leads to activation of the fibrinogen receptor αIIbβ3 and therefore platelet aggregation (73). PI3K regulates cell survival and insulin signalling, and its dysregulation can initiate platelet aggregation (74). Aspirin inhibits the COX-1 and COX-2 pathways thereby attenuating the production of TxA_2 (50), a mediator of platelet activation that can be upregulated in diabetes by the oxidation of arachidonic acid (75, 76).

Arachidonic acid causes an increase in platelet calcium levels in a positive feedback process that promotes its release from intracellular stores and microsomes (68). Current anti-diabetic drugs biguanides, such as metformin, can present anti-atherogenic and cardioprotective effects as shown by their ability to decrease platelet volume (77, 78). However, the mechanism by which this is achieved is not completely understood, with one study citing their ability to lower the mean platelet volume (MPV) in T2DM, as a potential mechanism (79). The use of metformin improves glycaemic control in T2DM individuals and decreases levels of markers of active coagulation such as platelet factor-4, thrombin and d-dimers (80, 81). Anti-platelet drugs such as aspirin and clopidogrel, have since been introduced to T2DM therapy to improve the risk of atherosclerosis by reducing plasma cytokines, C-reactive protein levels and intima-media thickness (82).

2.7. Platelet function in type 2 diabetes

Platelet dysregulation is common in T2DM, as platelets exhibit increased activation, adhesion and aggregation during this state (83). Diabetic individuals already display increased soluble form of P-selectin (sP-selectin) found in plasma, when compared to non-diabetic controls (84). Hyperglycaemic conditions increase levels of ADP and thrombin receptor activating peptide (TRAP) induced platelet P-selectin expression. In human blood incubated with high glucose concentrations, TRAP stimulation also increased fibrinogen binding and platelet-leukocyte aggregates (PLA) (70). Mean platelet volume has been used as a marker for platelet activation and its levels are significantly higher in T2DM that decreased after improved glycaemic control (79). High-fat diet (60% beef lard) induced diabetic C57BL/6J mice have been shown not to exhibit hyperaggregability or hypercoagulability (85). In this study, authors found no differences in P-selectin and CD61 (IIIa portion of the integrin GPIIb/IIIa) between the diabetic and non-diabetic control mice (85). These results highlight the need for more studies to confirm if high fat diet fed mouse is an appropriate model to investigate platelet function and aggregation in obesity and T2DM.

2.8. Glucose-lowering drugs and platelet function

Metformin is a glucose lowering drug that is widely used as the first line of therapy for T2DM individuals. The drug lowers blood glucose levels by improving insulin sensitivity and suppressing hepatic gluconeogenesis (86). The mode of action of metformin is still poorly understood; however, the improvement in insulin sensitivity is mediated by the post-receptor signalling modification in the insulin pathway (87–89). In addition, metformin can reduce platelet activation and oxidative stress by preserving intracellular anti-oxidant function in T2DM (90). This biguanide can also enhance insulin hepatic sensitivity which results in the reduction of gluconeogenesis and glycogenolysis, contributing to lower plasma glucose levels through the modulation of AMPK (89). Metformin induces the upregulation of GLUT1 and GLUT4 transporters in skeletal muscle and adipocytes leading to increased glucose uptake (89). The use of metformin has been tested in a combination therapy with anti-diabetic

add-ons of sulfonylureas and thiazolidinediones like glipizide and pioglitazone, respectively. Pioglitazone greatly reduced procaspase activating compound (PAC-1) binding, P-selectin expression and ADP induced aggregation as compared to glipizide (91). However, glipizide as an add-on therapy to metformin did have a better anti-glycaemic effect that pioglitazone (91). Anti-platelet drugs have also been introduced in addition to the anti-diabetic drugs as dual therapy in order to curb the increased hypercoagulable state associated with diabetics.

Acetylsalicylic acid (ASA) is a non-steroidal anti-inflammatory drug used in low doses as anti-platelet treatment. For instance, aspirin acetylates COX-1 in the serine-530 position preventing arachidonic acid-induced inflammation by binding to the active site of the enzyme (Figure 2.5) (81). The Japanese primary prevention of atherosclerosis with aspirin for diabetes (JPAD) study showed no differences in the risk of CVD between aspirin at 100mg and placebo over a median follow up of 10.3 years (92). The study only investigated primary end-points such as coronary artery events, cerebrovascular events and vascular events (92). Platelets from diabetic individuals remain reactive in spite of anti-platelet treatment such as aspirin and clopidogrel, with 10-40% showing high residual platelet reactivity (57, 93).

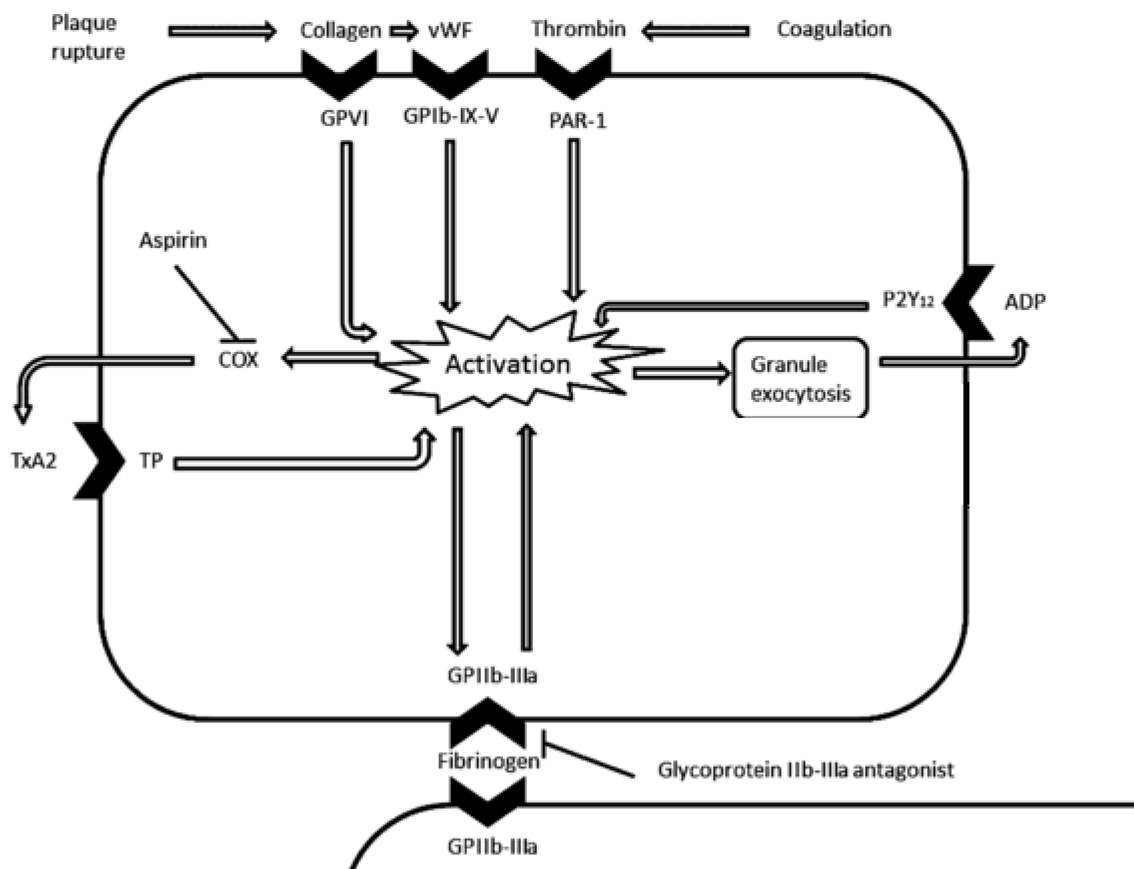

Figure 2.5. Illustration of pathways involved in platelet activation and the cyclooxygenase (COX) inhibitory role of aspirin. Plaque rupture and coagulation released mediators, collagen, von Willebrand factor (vWF) and thrombin bind to their respective receptors on the membrane surface of platelets such as glycoprotein VI (GPVI), glycoprotein Ib-IX-V (GPIb-IX-V), protease activated receptors (PAR) leading to the activation of platelets. This process subsequently leads to the activation of the integrin glycoprotein IIb-IIIa (GPIIb-IIIa), a known a receptor for fibrinogen binding and ultimately platelet aggregation. Activation of platelets also results in granule exocytosis and the increase in ADP agonist. The COX enzyme is also released post platelet activation and increases the synthesis of TxA2 which binds to its receptor thromboxane receptor (TP). Aspirin can inhibit the synthesis of prostanoid TxA_2 and its receptor TP leading to the inhibition of platelet activation. The figure was adapted from a previous publication (94).

Diabetic individuals taking aspirin at 100mg/daily and clopidogrel at 75mg/daily also showed high rates of periprocedural myocardial infarction compared to non-diabetic individuals (58). Similar results of persistently increased platelet reactivity in T2DM despite dual anti-platelet therapy of 85mg aspirin and 150mg clopidogrel daily dose have also been reported (95). Some of the reasons attributed to the lack of response to anti-platelet treatments include insulin resistance, poor glycaemic control and enhanced inflammatory status (58). Insulin is assumed to play an anti-aggregational role in platelet activation through nitric oxide synthase and nitric oxide-dependent mechanisms which are dysregulated in a diabetic state (96). Suggesting more research is necessary to identify therapies that can target diverse mechanism involved in diabetes-induced vascular complications.

2.9. Concluding remarks

Diabetic patients present with multiple risk factors that make them susceptible to cardiovascular complications. Although pathophysiological mechanisms explaining deteriorated vascular function in a diabetic state are multifactorial, inflammation is among the leading features being investigated for its involvement during this process. Chronic low-grade inflammation, which is a major characteristic feature of T2DM, is associated with an increase in immature reticulated large platelets. These platelets are hyperreactive and less responsive to prominent platelet inhibition drugs like aspirin and clopidogrel (57, 58). The use of low dose aspirin was recommended in the early 2000s for diabetic individuals with CVD risk factors (97), based on positive results from studies on hypertension individuals, and for prevention after myocardial infarction (92, 98). However, from the various studies, platelets from diabetic individuals are still hyperreactive regardless of available treatment strategies. Diabetic individuals also experience CVD events despite anti-platelet treatment which prompts further studies into the mechanisms involved in the progression of these events. Most studies have been done on diabetic individuals who may already have developed adverse CVD complications. Studies are needed which will investigate the effects of anti-glycaemic and anti-platelet treatment at early stages in the development of T2DM such as in pre-diabetes.

2.10. References

1. World Health Organization (2006) Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia:Report of a WHO/IDF consultation. Production 1–52 . doi: ISBN 92 4 159493 4

2. Ogurtsova K, da Rocha Fernandes JD, Huang Y, Linnenkamp U, Guariguata L, Cho NH, Cavan D, Shaw JE, Makaroff LE (2017) IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. Diabetes Res Clin Pract 128:40–50 . doi: 10.1016/j.diabres.2017.03.024

3. Peer N, Kengne AP, Motala AA, Mbanya JC (2014) Diabetes in the Africa region: An update. Diabetes Res Clin Pract 103:197–205 . doi: 10.1016/j.diabres.2013.11.006

4. Yip WCY, Sequeira IR, Plank LD, Poppitt SD (2017) Prevalence of pre-diabetes across ethnicities: A review of impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) for classification of dysglycaemia. Nutrients 9:1–18 . doi: 10.3390/nu9111273

5. Aldossari KK, Aldiab A, Al-Zahrani JM, Al-Ghamdi SH, Abdelrazik M, Batais MA, Javad S, Nooruddin S, Razzak HA, El-Metwally A (2018) Prevalence of Prediabetes, Diabetes, and Its Associated Risk Factors among Males in Saudi Arabia: A Population-Based Survey. J Diabetes Res 2018:1–12 . doi: 10.1155/2018/2194604

6. Gossain V V., Aldasouqi S (2010) The challenge of undiagnosed pre-diabetes, diabetes and associated cardiovascular disease. Int J Diabetes Mellit 2:43–46 . doi: 10.1016/j.ijdm.2009.10.004

7. Tabák AG, Herder C, Kivimäki M (2017) Prediabetes : A high-risk state for developing diabetes Progression from prediabetes to diabetes Reversion to normoglycaemia Risk prediction. Pmc 379:1–14 . doi: 10.1016/S0140-6736(12)60283-9.Prediabetes

8. World Health Organization (2016) Global report on diabetes. Decis Support Syst 38:557–573 . doi: 10.1016/j.dss.2003.08.004

9. Leon BM (2015) Diabetes and cardiovascular disease: Epidemiology, biological mechanisms, treatment recommendations and future research. World J Diabetes 6:1246 . doi: 10.4239/wjd.v6.i13.1246

10. Vaidyula VR, Rao AK, Mozzoli M, Homko C, Cheung P, Boden G (2006) Effects of hyperglycemia and hyperinsulinemia on circulating tissue factor procoagulant activity and platelet CD40 ligand. Diabetes 55:202–208 . doi: 10.2337/diabetes.55.1.202

11. Bogdanov VY, Østerud B (2010) Cardiovascular complications of diabetes mellitus: The

Tissue Factor perspective. Thromb Res 125:112–118 . doi: 10.1016/j.thromres.2009.06.033

12. Watson T, Shantsila E, Lip GY (2009) Mechanisms of thrombogenesis in atrial fibrillation: Virchow's triad revisited. Lancet 373:155–166 . doi: 10.1016/S0140-6736(09)60040-4

13. del Zoppo GJ (2008) Virchow's triad: the vascular basis of cerebral injury. Rev Neurol Dis 5 Suppl 1:S12–S21

14. Wright TM (1997) Cytokines in acute and chronic inflammation. Front Biosci 2:A171 . doi: 10.2741/A171

15. Ward PA (2010) Part I. The Inflammatory Response - An Overview - Acute and Chronic Inflammation. Fundam Inflamm I:1–10

16. Nauta J (2011) Statistics in clinical vaccine trials. Stat Clin Vaccine Trials 1–153 . doi: 10.1007/978-3-642-14691-6

17. Serhan CN, Brain SD, Buckley CD, Gilroy DW, Haslett C, O'Neill LAJ, Perretti M, Rossi AG, Wallace JL (2007) Resolution of inflammation: state of the art, definitions and terms. FASEB J 21:325–332 . doi: 10.1096/fj.06-7227rev

18. Hess K, Grant PJ (2011) Inflammation and thrombosis in diabetes. Thromb Haemost 105:43–54 . doi: 10.1160/THS10-11-0739

19. Fernández-Sánchez A, Madrigal-Santillán E, Bautista M, Esquivel-Soto J, Morales-González Á, Esquivel-Chirino C, Durante-Montiel I, Sánchez-Rivera G, Valadez-Vega C, Morales-González J a (2011) Inflammation, oxidative stress, and obesity. Int J Mol Sci 12:3117–3132

20. Esposito K, Nappo F, Marfella R, Giugliano G, Giugliano F, Ciotola M, Quagliaro L, Ceriello A, Giugliano D (2002) Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: Role of oxidative stress. Circulation 106:2067–2072 . doi: 10.1161/01.CIR.0000034509.14906.AE

21. Derosa G, Franzetti IG, Querci F, Carbone A, Ciccarelli L, Piccinni MN, Fogari E, Maffioli P (2013) Variation in inflammatory markers and glycemic parameters after 12 months of exenatide plus metformin treatment compared with metformin alone: A randomized placebo-controlled trial. Pharmacotherapy 33:817–826 . doi: 10.1002/phar.1301

22. Breitenstein A, Tanner FC, Lüscher TF (2010) Tissue Factor and Cardiovascular Disease: Circ J 74:3–12 . doi: 10.1253/circj.CJ-09-0818

23. Butenas S, Orfeo T, Mann KG (2009) Tissue factor in coagulation: Which? where? when? Arterioscler Thromb Vasc Biol 29:1989–1996 . doi: 10.1161/ATVBAHA.108.177402

24. Shantsila E, Lip GYH (2009) The role of monocytes in thrombotic disorders: Insights from tissue factor, monocyte-platelet aggregates and novel mechanisms. Thromb Haemost 102:916–924

25. El-Hagracy R, Kamal G, Sabry I, Saad A, Abou El Ezz N, Nasr H (2010) Tissue Factor, Tissue Factor Pathway Inhibitor and Factor VII Activity in Cardiovascular Complicated Type 2 Diabetes Mellitus. Oman Med J 25:173–178 . doi: 10.5001/omj.2010.52

26. Maroney SA, Ellery PE, Mast AE (2010) Alternatively spliced isoforms of tissue factor pathway inhibitor. Thromb Res 125:S52–S56 . doi: 10.1016/j.thromres.2010.01.038

27. Gerrits A, Koekman C (2010) Platelet tissue factor synthesis in type 2 diabetic patients is resistant to inhibition by insulin. Diabetes 59:1487–1495 . doi: 10.2337/db09-1008.

28. Pendurthi UR, Rao LVM (2008) Factor VIIa Interaction With Tissue Factor and Endothelial Cell Protein C Receptor on Cell Surfaces. Semin Hematol 45:1–7 . doi: 10.1053/j.seminhematol.2008.03.014

29. Metcalf Pate KA, Lyons CE, Dorsey JL, Shirk EN, Queen SE, Adams RJ, Gama L, Morrell CN, Mankowski JL (2013) Platelet activation and platelet-monocyte aggregate formation contribute to decreased platelet count during acute simian immunodeficiency virus infection in pig-tailed macaques. J Infect Dis 208:874–883 . doi: 10.1093/infdis/jit278

30. Guha M, Connell MAO, Pawlinski R, Hollis A, Mcgovern P, Yan S, Stern D, Mackman N (2001) Lipopolysaccharide activation of the MEK-ERK1/2 pathway in human monocytic cells mediates tissue factor and tumor necrosis factor ? expression by inducing Elk-1 phosphorylation and Egr-1 expression. Blood 98:1429–1439 . doi: 10.1182/blood.V98.5.1429

31. Kappelmayer J, Bernabei A, Edmunds LH, Edgington TS, Colman RW (1993) Tissue factor is expressed on monocytes during simulated extracorporeal circulation. Circ Res 72:1075–1081 . doi: 10.1161/01.RES.72.5.1075

32. Darbousset R, Thomas GM, Mezouar S, Frère C, Bonier R, Mackman N, Renné T, Dignat-George F, Dubois C, Panicot-Dubois L (2012) Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation. Blood 120:2133–2143 . doi: 10.1182/blood-2012-06-437772

33. Mitchell CT, Kamineni A, Palmas W, Cushman M (2009) Tissue factor pathway inhibitor, vascular risk factors and subclinical atherosclerosis: The Multi-Ethnic Study of Atherosclerosis. Atherosclerosis 207:277–283 . doi: 10.1016/j.atherosclerosis.2009.04.024

34. Patel SR, Richardson JL, Schulze H, Kahle E, Galjart N, Drabek K, Shivdasani RA, Hartwig

JH, Italiano JE (2005) Differential roles of microtubule assembly and sliding in proplatelet formation by megakaryocytes. Blood 106:4076–4085 . doi: 10.1182/blood-2005-06-2204

35. Zapata JC, Cox D, Salvato MS (2014) The Role of Platelets in the Pathogenesis of Viral Hemorrhagic Fevers. PLoS Negl Trop Dis 8: . doi: 10.1371/journal.pntd.0002858

36. Anjali A. Sharathkumar AS (2008) Platelet Function Disorders Second Edition. World Fed Hemoph 1–28

37. Kaplan ZS, Jackson SP (2011) The Role of Platelets in Atherothrombosis. 51–61

38. Ghoshal K, Bhattacharyya M (2014) Overview of platelet physiology: Its hemostatic and nonhemostatic role in disease pathogenesis. Sci World J 2014: . doi: 10.1155/2014/781857

39. Kim S, Kunapuli SP (2011) P2Y12 receptor in platelet activation. Platelets 22:54–58 . doi: 10.3109/09537104.2010.497231

40. Heidrich F, Schotola H, Popov AF, Sohns C, Schuenemann J, Coskun KO, Lewinski D Von, Hinz J, Bauer M, Mokashi S a, Sossalla S, Schmitto JD (2010) AMPK - Activated Protein Kinase and its Role in Energy Metabolism of the Heart. Curr Cardiol Rev 6:337–342 . doi: 10.2174/157340310793566073

41. Maldonado E, Lemasters J (2015) ATP/ADP Ratio, the Missed Connection between Mitochondria and the Warburg Effect. Pubmed Cent 31:1713–1723 . doi: 10.1109/TMI.2012.2196707.Separate

42. Sharda A, Flaumenhaft R (2018) The life cycle of platelet granules. F1000Research 7:236 . doi: 10.12688/f1000research.13283.1

43. Cerletti C, de Gaetano G, Lorenzet R (2010) Platelet-leukocyte interactions: Multiple links between inflammation, blood coagulation and vascular risk. Mediterr J Hematol Infect Dis 2: . doi: 10.4084/MJHID.2010.023

44. Oury C, Toth-Zsamboki E, Vermylen J, Hoylaerts MF (2006) The platelet ATP and ADP receptors. Curr Pharm Des 12:859–875 . doi: 10.2174/138161206776056029

45. Kapur R, Semple JW (2017) Platelet functions beyond hemostasis. Mol Cell Biol Platelet Form Implic Heal Dis 7:221–237 . doi: 10.1007/978-3-319-39562-3_10

46. Krinsky NI, Sladdin DG, Levine PH, Taub IA, Simic MG (1981) Modification of platelet function by radical species produced during irradiation of oxygenated water. Thromb Haemost 45:116–120

47. Grove EL (2012) Antiplatelet effect of aspirin in patients with coronary artery disease. Dan

Med J 59:B4506

48. Cunningham MR, Nisar SP, Mundell SJ (2013) Molecular mechanisms of platelet P2Y $_{12}$ receptor regulation. Biochem Soc Trans 41:225–230 . doi: 10.1042/BST20120295

49. Nkambule BB, Davison G, Ipp H (2015) The value of flow cytometry in the measurement of platelet activation and aggregation in human immunodeficiency virus infection. Platelets 26:250–257 . doi: 10.3109/09537104.2014.909021

50. Schrottmaier WC, Kral JB, Badrnya S, Assinger A (2015) Aspirin and P2Y12 Inhibitors in platelet-mediated activation of neutrophils and monocytes. Thromb Haemost 114:478–489 . doi: 10.1160/TH14-11-0943

51. Gachet C (2012) P2Y12 receptors in platelets and other hematopoietic and non-hematopoietic cells. Purinergic Signal 8:609–619

52. Abbracchio MP, Burnstock G, Boeynaems J, Eric A, Boyer JL, Kennedy C, Knight GE, Gachet C, Jacobson KA, Weisman GA (2012) International Union of Pharmacology LVIII: Update on the P2Y G Protein-Coupled Nucleotide Receptors: From Molecular Mechanisms and Pathophysiology to Therapy. Am Soc Pharmacol Exp Ther 58:281–341 . doi: 10.1124/pr.58.3.3.International

53. Léon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P, Dierich A, LeMeur M, Cazenave JP, Gachet C (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y1receptor-null mice. J Clin Invest 104:1731–1737 . doi: 10.1172/JCI8399

54. Hechler B, Gachet C (2011) P2 receptors and platelet function. Purinergic Signal 7:293–303 . doi: 10.1007/s11302-011-9247-6

55. Dorsam RT, Kunapuli SP (2004) Central role of the P2Y 12 receptor in platelet activation. J Clin Invest 113:10–15 . doi: 10.1172/JCI200420986.The

56. Geisler T, Mueller K, Aichele S, Bigalke B, Stellos K, Htun P, Ninci E, Fateh-Moghadam S, May AE, Gawaz M (2010) Impact of inflammatory state and metabolic control on responsiveness to dual antiplatelet therapy in type 2 diabetics after PCI: Prognostic relevance of residual platelet aggregability in diabetics undergoing coronary interventions. Clin Res Cardiol 99:743–752 . doi: 10.1007/s00392-010-0179-x

57. Kakouros N, Rade JJ, Kourliouros A, Resar JR (2011) Platelet function in patients with diabetes mellitus: From a theoretical to a practical perspective. Int J Endocrinol 2011:1–14 . doi: 10.1155/2011/742719

58. Mangiacapra F, Patti G, Peace A, Gatto L, Vizzi V, Ricottini E, D'Ambrosio A, Muller O,

Barbato E, Di Sciascio G (2010) Comparison of platelet reactivity and periprocedural outcomes in patients with versus without diabetes mellitus and treated with clopidogrel and percutaneous coronary intervention. Am J Cardiol 106:619–623 . doi: 10.1016/j.amjcard.2010.04.015

59. Li Z, Delaney MK, O'Brien K a., Du X (2010) Signaling during platelet adhesion and activation. Arterioscler Thromb Vasc Biol 30:2341–2349 . doi: 10.1161/ATVBAHA.110.207522

60. Jin J, Quinton TM, Zhang J, Rittenhouse SE, Kunapuli SP (2002) Adenosine diphosphate (ADP)-induced thromboxane A2generation in human platelets requires coordinated signaling through integrin αIIbβ3and ADP receptors. Blood 99:193–198 . doi: 10.1182/blood.V99.1.193

61. Yeung J, Apopa PL, Vesci J, Kenyon V, Rai G, Jadhav A, Simeonov A, Holman TR, Maloney DJ, Boutaud O, Holinstat M (2012) Protein kinase C regulation of 12-lipoxygenase-mediated human platelet activation. Mol Pharmacol Fast Forw 3:420–430

62. Khan N, Farooq AD, Sadek B (2015) Investigation of cyclooxygenase and signaling pathways involved in human platelet aggregation mediated by synergistic interaction of various agonists. Drug Des Devel Ther 9:3497–3509 . doi: 10.2147/DDDT.S84335

63. Korbecki J, Baranowska-Bosiacka I, Gutowska I, Chlubek D (2014) Cyclooxygenase pathways. Acta Biochim Pol 61:639–649

64. Smyth EM, Grosser T, Wang M, Yu Y, FitzGerald GA (2009) Prostanoids in health and disease. J Lipid Res 50:S423–S428 . doi: 10.1194/jlr.R800094-JLR200

65. Varga-Szabo D, Braun A, Nieswandt B (2009) Calcium signaling in platelets. J Thromb Haemost 7:1057–1066 . doi: 10.1111/j.1538-7836.2009.03455.x

66. El Haouari M, Rosado JA (2008) Platelet signalling abnormalities in patients with type 2 diabetes mellitus: a review. Blood Cells Mol Dis 41:119–23 . doi: 10.1016/j.bcmd.2008.02.010

67. Damman P, Woudstra P, Kuijt WJ, De Winter RJ, James SK (2012) P2Y12 platelet inhibition in clinical practice. J Thromb Thrombolysis 33:143–153

68. Signorello MG, Segantin A, Leoncini G (2009) The arachidonic acid effect on platelet nitric oxide level. Biochim Biophys Acta - Mol Cell Biol Lipids 1791:1084–1092 . doi: 10.1016/j.bbalip.2009.07.003

69. Wu CC, Wu SY, Liao CY, Teng CM, Wu YC, Kuo SC (2010) The roles and mechanisms of PAR4 and P2Y 12/phosphatidylinositol 3-kinase pathway in maintaining thrombin-induced platelet aggregation. Br J Pharmacol 161:643–658 . doi: 10.1111/j.1476-5381.2010.00921.x

70. Sudic D, Razmara M, Forslund M, Ji Q, Hjemdahl P, Li N (2006) High glucose levels enhance platelet activation: Involvement of multiple mechanisms. Br J Haematol 133:315–322 . doi: 10.1111/j.1365-2141.2006.06012.x

71. Geraldes P, King GL (2011) Activation of Protein Kinase C Isoforms & Its Impact on Diabetic Complications. Circ Res 106:1319–1331 . doi: 10.1161/CIRCRESAHA.110.217117.Activation

72. Harrison S, Vavken P, Kevy S, Jacobson M, Zurakowski D, Murray MM (2011) Platelet activation by collagen provides sustained release of anabolic cytokines. Am J Sports Med 39:729–734 . doi: 10.1177/0363546511401576

73. Weng Z, Li D, Zhang L, Chen J, Ruan C, Chen G, Gartner TK, Liu J (2010) PTEN regulates collagen-induced platelet activation. Blood 116:2579–2581 . doi: 10.1182/blood-2010-03-277236

74. Manna P, Jain SK (2015) Phosphatidylinositol-3,4,5-Triphosphate and Cellular Signaling: Implications for Obesity and Diabetes. Cell Physiol Biochem 35:1253–1275 . doi: 10.1159/000373949

75. Evangelista V, De Berardis G, Totani L, Avanzini F, Giorda CB, Brero L, Levantesi G, Marelli G, Pupillo M, Iacuitti G, Pozzoli G, Di Summa P, Nada E, De Simone G, Dell'elba G, Amore C, Manarini S, Pecce R, Maione A, Tognoni G, Nicolucci A (2007) Persistent platelet activation in patients with type 2 diabetes treated with low doses of aspirin. J Thromb Haemost 5:2197–2203 . doi: 10.1111/j.1538-7836.2007.02728.x

76. Bambace NM, Holmes CE (2011) The platelet contribution to cancer progression. J Thromb Haemost 9:237–249 . doi: 10.1111/j.1538-7836.2010.04131.x

77. Dolasik I, Sener SY, Celebi K, Aydin ZM, Korkmaz U, Canturk Z (2013) The effect of metformin on mean platelet volume in diabetic patients. Platelets 24:118–121 . doi: 10.3109/09537104.2012.674165

78. Verdoia M, Schaffer A, Barbieri L, Cassetti E, Nardin M, Bellomo G, Marino P, Sinigaglia F, De Luca G (2014) Diabetes, glucose control and mean platelet volume: A single-centre cohort study. Diabetes Res Clin Pract 104:288–294 . doi: 10.1016/j.diabres.2013.12.020

79. Demirtunc R, Duman D, Basar M, Bilgi M, Teomete M, Garip T (2009) The relationship between glycemic control and platelet activity in type 2 diabetes mellitus. J Diabetes Complications 23:89–94 . doi: 10.1016/j.jdiacomp.2008.01.006

80. Lemkes BA, Hermanides J, Devries JH, Holleman F, Meijers JCM, Hoekstra JBL (2010)

Hyperglycemia: A prothrombotic factor? J Thromb Haemost 8:1663–1669 . doi: 10.1111/j.1538-7836.2010.03910.x

81. Gonçalves LH, Silva MVF, Duarte RCF, Dusse LMS, Fernandes AP, Bosco AA, Gomes KB, Carvalho M das G (2014) Acetylsalicylic acid therapy: Influence of metformin use and other variables on urinary 11-dehydrothromboxane B2 levels. Clin Chim Acta 429:76–78 . doi: 10.1016/j.cca.2013.11.028

82. Hartge MM, Unger T, Kintscher U (2007) The endothelium and vascular inflammation in diabetes. Diabetes Vasc Dis Res 4:84–88 . doi: 10.3132/dvdr.2007.025

83. Kim JH, Bae HY, Kim SY (2013) Clinical marker of platelet hyperreactivity in diabetes mellitus. Diabetes Metab J 37:423–428 . doi: 10.4093/dmj.2013.37.6.423

84. Soma P, Swanepoel AC, du Plooy JN, Mqoco T, Pretorius E (2016) Flow cytometric analysis of platelets type 2 diabetes mellitus reveals 'angry' platelets. Cardiovasc Diabetol 15:1–7 . doi: 10.1186/s12933-016-0373-x

85. Henry M, Davidson L, Cohen Z, McDonagh PF, Nolan PE, Ritter LS (2009) Whole blood aggregation, coagulation, and markers of platelet activation in diet-induced diabetic C57BL/6J mice. Diabetes Res Clin Pract 84:11–18 . doi: 10.1016/j.diabres.2009.01.011

86. He L, Sabet A, Djedjos S, Miller R, Sun X, Mehboob A, Radovick S, Wondisford FE (2010) Metformin and Insulin Suppress Hepatic Gluconeogenesis by Inhibiting cAMP Signaling Through Phosphorylation of CREB Binding Protein (CBP). Cell 137:635–646 . doi: 10.1016/j.cell.2009.03.016.Metformin

87. Hawley SA, Gadalla AE, Olsen GS, Grahame Hardie D (2002) The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. Diabetes 51:2420–2425 . doi: 10.2337/diabetes.51.8.2420

88. Shaw RJ, Lamia K a, Vasquez D, Koo S, Depinho RA, Montminy M, Cantley LC (2005) The Kinase LKB1 Mediates Glucose Homeostasis in Liver and Therapeutic Effects of Metformin. Science (80-) 310:1642–1646 . doi: 10.1126/science.1120781.The

89. Bösenberg LH, van Zyl DG (2008) The mechanism of action of oral antidiabetic drugs: A review of recent literature. J Endocrinol Metab Diabetes South Africa 13:80–88 . doi: 10.1080/22201009.2008.10872177

90. Formoso G, De Filippis E, Di Fulvio P, Pandolfi A, Bucciarelli T, Ciabattoni G, Nicolucci A, Davi G, Consoli A (2008) Decreased in vivo oxidative stress and decreased platelet activation following metformin treatment in newly diagnosed type 2 diabetic subjects. Diabetes Metab

Res Rev 24:13231–237 . doi: 10.1002/dmrr

91. Xiao CC, Ren A, Yang J, Ye SD, Xing XN, Li SM, Chen C, Chen RP (2015) Effects of pioglitazone and glipizide on platelet function in patients with type 2 diabetes. Eur Rev Med Pharmacol Sci 19:963–970

92. Saito Y, Okada S, Ogawa H, Soejima H, Sakuma M, Nakayama M, Doi N, Jinnouchi H, Waki M, Masuda I, Morimoto T (2017) Low-Dose Aspirin for Primary Prevention of Cardiovascular Events in Patients with Type 2 Diabetes Mellitus. Circulation 135:659–670 . doi: 10.1161/CIRCULATIONAHA.116.025760

93. Calkin AC, Drew BG, Ono A, Duffy SJ, Gordon M V., Schoenwaelder SM, Sviridov D, Cooper ME, Kingwell BA, Jackson SP (2009) Reconstituted high-density lipoprotein attenuates platelet function in individuals with type 2 diabetes mellitus by promoting cholesterol efflux. Circulation 120:2095–2104 . doi: 10.1161/CIRCULATIONAHA.109.870709

94. Linden MD (2013) Using platelet function testing to guide antiplatelet therapy. Drug Dev Res 74:517–525 . doi: 10.1002/ddr.21110

95. Angiolillo DJ, Shoemaker SB, Desai B, Yuan H, Charlton RK, Bernardo E, Zenni MM, Guzman LA, Bass TA, Costa MA (2007) Randomized comparison of a high clopidogrel maintenance dose in patients with diabetes mellitus and coronary artery disease: Results of the optimizing antiplatelet therapy in diabetes mellitus (OPTIMUS) study. Circulation 115:708–716 . doi: 10.1161/CIRCULATIONAHA.106.667741

96. Suslova TE, Sitozhevskii A V., Ogurkova ON, Kravchenko ES, Kologrivova I V., Anfinogenova Y, Karpov RS (2015) Platelet hemostasis in patients with metabolic syndrome and type 2 diabetes mellitus: cGMP- and NO-dependent mechanisms in the insulin-mediated platelet aggregation. Front Physiol 5:1–8 . doi: 10.3389/fphys.2014.00501

97. American Diabetes Association (2004) Aspirin Therapy in Diabetes. Ann Intern Med 27:1997–1998 . doi: 10.2337/diacare.27.2007.S72

98. Collaboration AT (2002) Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. Bmj 324:71–86 . doi: 10.1136/bmj.324.7329.71

Chapter 3. Research article one

Increased platelet-monocyte aggregates in diet-induced pre-diabetes.

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3. Abstract

Introduction

The formation of platelet-monocyte aggregates (PMA) is a physiological feature which mediates the pro-inflammatory role of platelets. Elevated PMAs in pre-diabetes may result in the development of atherosclerosis. The aim of this study was to evaluate PMA formation in a pre-diabetes using a high-fat diet mouse model, providing evidence of a link between inflammation and the hypercoagulable state in pre-diabetes.

Methods

Whole blood was collected from two animal groups on low fat (control group) and high-fat diet (pre-diabetic group). The glucose tolerance was determined using the oral glucose tolerance test and insulin measurement was done using the enzyme linked immunoassay (ELISA) technique. Baseline haematological parameters were also measured using the haemocytometer. PMAs were determined using whole blood flow cytometry. To determine the level of PMA formation, blood from mice was stained with an antibody cocktail containing CD45-BV500, CD14-PE and CD41-FITC antibodies.

Results

The mice had comparable body weights and insulin levels, however, the high-fat diet fed mice demonstrated delayed glucose clearance, indicating that these mice were pre-diabetic. Baseline PMA levels were elevated in the pre-diabetic group when compared to the controls. In addition, stimulation with the adenosine diphosphate (ADP) agonist also significantly increased the level of platelet-monocyte aggregates in the non-diabetic control mice however these remained comparable in the pre-diabetic mice. Baseline levels of monocytes, measured using the CD14 monocyte marker, were significantly decreased in the pre-diabetes.

Conclusions

In pre-diabetes, platelets readily interact with circulating monocytes and form PMAs which are early markers for atherosclerosis. For the present study, the decrease in CD14 monocyte marker, indicated a shift in the monocyte phenotype to the pro-inflammatory form CD14^{++}, further providing a possible link of the pro-thrombotic and pro-inflammatory role of platelets in pre-diabetes.

Keywords: flow cytometry; platelet-monocyte aggregates; pre-diabetes; pro-inflammation

3.1. Introduction

Chronic platelet activation has been associated with a sustained pro-inflammatory response and an increased risk of cardiovascular complications (1). Monocytes, via the surface membrane P-selectin glycoprotein ligand-1 (PSGL-1), bind to P-selectin expressed on the activated endothelial surface

mediated interaction (2). This represents the early events in the pathophysiological mechanisms leading to atherosclerosis in type 2 diabetes mellitus (T2DM) (3). In a similar manner, activated platelets are able to bind circulating peripheral blood leucocytes via P-selectin and PSGL-1 interactions, forming platelet-leukocyte aggregates (PLAs) (4,5). The binding of P-selectin to its counter-receptor PGSL1 induces leukocyte tethering and firm adhesion of monocytes to the endothelium (6,7). As a result of the receptor-ligand binding, leukocytes are activated through the translocation of nuclear factor κB (NF-κB), an important transcriptional factor that is responsible for pro-inflammatory molecule synthesis (7).

Activation of the NF-κB pathway, in addition to increasing superoxide anion production, promotes accelerated levels of monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor-α (TNF-α), interleukin-8 (IL-8), IL-1β and cyclooxygenase-2 (COX-2) (5). Elevated expression of these pro-inflammatory markers is concomitant with the formation of monocyte aggregates (PMA) (8). Previous studies have shown that platelets preferentially bind to monocytes, thus PMAs are regarded as stable markers of platelet activation (3,5,9). These interactions provide a link between the inflammatory and thrombotic responses involved in conditions such as T2DM where elevated levels of PMAs have been reported (10–12). The aim of this study was to evaluate PMA formation in a pre-diabetic mouse model, providing evidence of a link in the inflammatory status of diabetes with hypercoagulable status. Investigating a pre-diabetic state could identify essential pathophysiological mechanisms that are involved in early-stage development of T2DM.

3.2. Methodology

3.2.1. Study design

Five-week-old C57BL/6 male mice were housed, individually in a cage, at the University of KwaZulu-Natal (UKZN) biomedical research unit (BRU). Animals were handled according to the principles of laboratory Animal Care of the National Society of Medical Research and the National Institutes of Animal Care and Use of Laboratory Animals of the National Academy of Sciences (National Institute of Health publication 80-23, revised 1978). Ethical clearance was granted by the UKZN animal research ethics committee (AREC), ethics registration number AREC/086/016. Animals were allowed to acclimatize for a week before being fed on the experimental diets, control low-fat diet (n=8) (D12450J 10 kcal% fat) and a high-fat diet (HFD) (n=22) (D12492, 60 kcal% fat) (Research Diets, NJ, USA). All mice had free access to water and food *ad libitium*. The animals were regularly monitored while the cages were cleaned once a week, to ensure a clean environment for the mice.

3.2.2. Oral glucose tolerance tests

Oral glucose tolerance tests were performed as previously described (13). In brief, after an 8 hour fast, 2g/kg of glucose was administered using oral gavage technique. Blood glucose levels were then measured at different time intervals (30, 60, 120 minutes) using the Accu-Chek active blood glucometer (Roche, Basel, Switzerland).

3.2.3. Blood collection for haematology characteristics and flow cytometry analysis

After 3 weeks on an HFD or control diet, 200µl of venous blood was collected using the tail bleeding method and the mice were terminated using inhalation of halothen. Venous blood was collected into 3.2% citrate coated microtainer tubes (Sigma Aldrich, St Louis, Missouri, USA).

3.2.4. Measurement of haematological parameters

The Beckman Coulter Ac T diff™ analyser (Beckman Coulter, Brea, CA, USA.) was used to measure the baseline haematological parameters such as red blood cell count, white cell count, platelet count, haematocrit, mean cell volume and plateletcrit as per manufacturer's protocol.

3.2.5. Instrument set-up and optimization

The BD FACSCanto II flow cytometer (BD Bioscience, NJ, USA) was used and the cytometer set-up and tracking (CST) beads (BD Bioscience, NJ, USA) were used to perform internal quality control (QC) as per manufacturer's protocol. To compute and compensate for spectral overlap the BD ™ Compbead compensation particles (BD Bioscience, NJ, USA) were used. In addition, SPHERO ™ 6-peak Rainbow calibration particles (BD Bioscience, NJ, USA) were used daily as QC for the median fluorescence intensity (MFI).

3.2.6. Measurement of baseline platelet-monocyte aggregates (PMA)

The measurement of baseline (unstimulated) PMA levels was performed within 30 min blood collection. Briefly, 25µl of the blood was stained with 2.5µl (ratio 1:10) of the anti-mouse monoclonal antibody cocktail containing CD14-PE (clone: rmC5-3) (monocyte marker), CD41-FITC (clone: MWReg30) (platelet marker) and CD45-BV510 (clone: 30-F11) (leukocyte marker) for 10 minutes in the dark at room temperature. These samples were fixed using 25µl of thrombofix (Beckman Coulter, Brea, CA, USA) prior to red blood cell lysis. The samples were then lysed with 350µl FACSLyse lysis buffer (BD Bioscience, NJ, USA) for 15minutes in the dark at room temperature. This was then analysed on the BD FACSCanto II flow cytometer (BD Bioscience, NJ, USA).

3.2.7. Measurement of PMA post stimulation with ADP

To investigate the role of agonist-activated platelets in the formation of PMAs in hyperglycemic conditions, ADP was used to stimulate platelets and PMA levels were determined by flow cytometry. Briefly, 25µl of the citrated blood was incubated with 10µl (20µM) ADP for 15 minutes and then fixed with 25µl of thrombofix (Beckman Coulter, Brea, CA, USA). The sample was then stained with 2.5µl (ratio 1:10) of anti-mouse monoclonal antibody cocktail containing CD14-PE PE (clone: rmC5-3), CD41-FITC (clone: MWReg30) and CD45-BV510 (clone: 30-F11) (BD Bioscience, NJ, USA) and incubated for 10 minutes, at room temperature in the dark. The analysis was then done on the BD FACSCanto II flow cytometer (BD Bioscience, NJ, USA).

3.2.8. Gating strategy

The pan-leukocyte marker (CD45) was used to identify leukocyte populations. The specific monocyte specific marker (CD14) was used to identify monocytes. In addition, CD41 was used to identify platelets bound monocytes and enumerate PMAs (Figure 3.1).

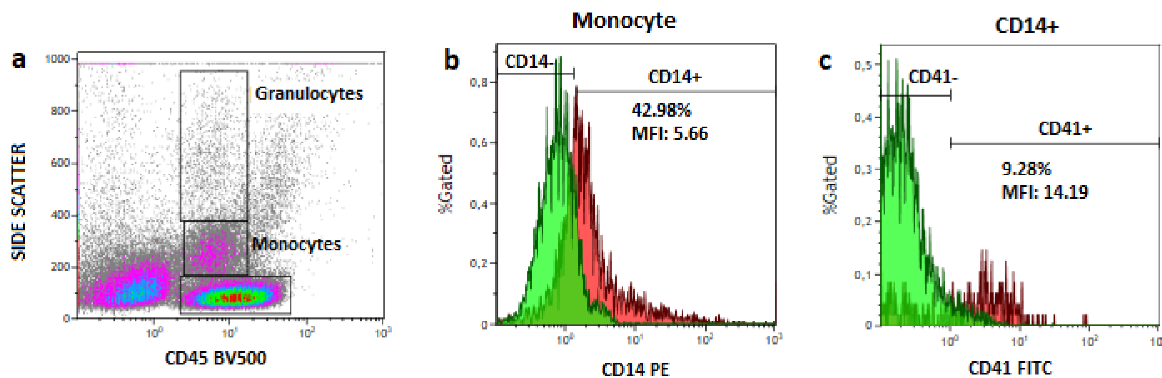

Figure 3.1. Gating strategy for the analysis for platelet-monocyte aggregates (PMAs). (a) The pan-leukocyte marker CD45 that was used to identify leukocytes. The primary gate on the monocyte was determined using the side scatter properties which are higher compared to other leukocytes. (b) An unstained sample was used to set the negative population for autofluorescence and to set the gate to determine the quantitative (%CD14+) and qualitative level of monocytes (CD14+ MFI). (c) To determine the level of PMAs formed, the level of platelet-bound monocytes was measured based on the (CD14+) monocyte population in (b). The quantitative (%CD41+) and qualitative (CD41+ MFI) expressions were determined based on the unstained sample as a negative control. PMA: platelet-monocyte aggregate; HFD: high-fat diet; MFI: median fluorescence intensity.

3.2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA). Non-parametric and parametric data were analysed using the Mann-Whitney test and unpaired t-test respectively. Non-parametric data were reported as the median IQR. Parametric data were reported as the mean ± standard deviation (SD). Statistical significance was defined as a p-value <0.05.

3.3. Results

3.3.1. Baseline characteristics

Baseline characteristics of the control and experimental group are shown in Table 3.1. There were no significant differences in the baseline characteristics such as body weights or insulin levels, well as the white cell count, platelet count and monocyte count between the control group (n=8) and HFD group (n=22). However, these markers such as red blood cell count (p=0.0178), haematocrit (p=0.0433) and mean cell volume (p=0.0025) showed a significant difference when the HFD group was compared to the controls (Table 3.1). The oral glucose tolerance test (OGTT) showed that the HFD group had a higher postprandial blood glucose levels compared to the control group. This was

also illustrated by the higher area under the curve (AUC) observed in the HFD group indicating delayed blood glucose clearance in the HFD pre-diabetic group (p= 0.0091) (table 3.1). There were no differences in the insulin levels (0.2667) and body weights between the treatment groups (p=0.1749).

Table 3.1. Baseline characteristics.

Parameter	Control (n=8)	High-fat diet (n=22)	p-value
Weight (g)	24.33[23.11- 26.83]	23.54[22.83- 24.49]	0.1749
Insulin (µIU/ml)	16.16[15.43- 22.71]	19.69[17.04- 24.51]	0.2667
OGTT (AUC)	852.0[717.0 - 918.0]	1068[1003 - 1095]	**0.0091**
White cell count (10^3/µl)	5.35[3.68- 9.00]	7.50[4.80- 8.40]	0.4699
Red blood cell count (10^6/µl)	7.17[7.04- 7.69]	6.910[5.53- 7.17]	**0.0178**
Haemoglobin (g/dL)	25.85[20.00- 29.23]	22.40[16.75- 25.00]	0.6683
Haematocrit (%)	31.10[30.05- 33.30]	29.00[23.00- 31.40]	**0.0433**
Mean cell volume (fL)	43.00[43.00- 43.75]	42.00[41.00- 44.00]	**0.0025**
Platelet count (10^3/µL)	782.9± 206.4	697.2± 151.1	0.5789
Mean platelet volume (fL)	5.30[5.03- 5.50]	5.20[5.10- 5.40]	0.6957
Neutrophil count (%)	7.75[7.00- 9.48]	8.000[6.90- 9.30]	0.9640
Lymphocyte count (%)	89.85[88.15- 90.73]	89.20[87.80- 90.50]	0.5271
Monocyte count (%)	1.96± 0.24	2.05± 0.56	0.6840
Basophil (%)	0.25[0.13- 0.50]	0.2000[0.10- 0.80]	0.7997

Data presented as mean ±SD and median (IQR); $p<0.05$ represented in boldface.

3.3.2. *Baseline levels of monocytes and platelet-monocyte aggregates*

The levels of monocytes were determined by measuring the levels of CD14 expression from each sample. The HFD 25.93±12.17 showed lower quantitative levels of monocyte (%CD14) compared to the control group 42.98±16.34, p=0.0093. In contrast, the qualitative median fluorescence intensity (MFI) was elevated in the HFD group 14.18±18.80 compared to the control group 5.66±0.51, p=0.0078 (Table 3.2, Figure 3.2).

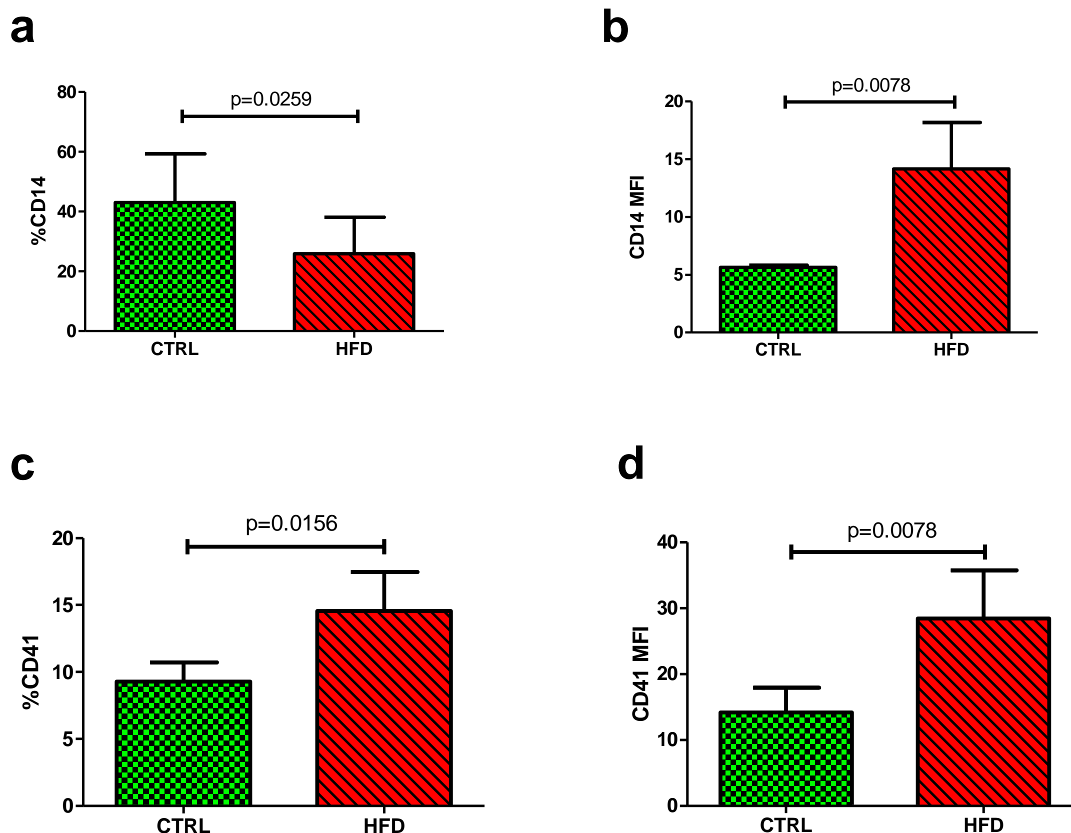

Figure 3.2. Monocyte and platelet-monocyte aggregate levels between the control group (n=8) and the high-fat diet (HFD) group (n=22). (a) Monocyte levels (%CD14) were significantly lower in the HFD group compared to the control group at baseline, p=0.0259. (b) The qualitative measurement (CD14 MFI) however was increased in the HFD compared to the control group, p=0.0078. PMAs were determined by the level of platelet-bound monocytes. (c) The HFD group had higher levels of PMA compared to the control group at baseline measurement, p=0.0156. (d) Similarly, the qualitative measurement (CD41 MFI) was increased in the HFD compared to the control group, p=0.0078. PMA: platelet-monocyte aggregate; HFD: high-fat diet; MFI: median fluorescence intensity.

Baseline levels of platelet-monocyte aggregates (%CD41) were increased in the HFD group 14.55±13.66 compared to the control group 9.28±4.05, p=0.0156. The qualitative analysis measured using the median fluorescence intensity (MFI), also showed increased levels in the HFD group 28.45±34.13 compare to the control group 14.19±10.64, p=0.0078 (Table 3.2, Figure 3.2).

Table 3.2. Baseline platelet monocyte aggregate (PMA) levels.

	Control (n=8)	High-fat diet (n=22)	p-value
%CD14	42.98±16.34	25.93±12.17	**0.0093**
CD14 MFI	5.66±0.51	14.18±18.80	**0.0078**
%CD41	9.28±4.045	14.55±13.66	**0.0156**
CD41 MFI	14.19±10.64	28.45±34.13	**0.0078**

Data presented as mean ±SD; p<0.05 represented in boldface; MFI: Median fluorescence intensity.

3.3.3. Increased levels of PMAs post stimulation with ADP in the control group

Post-stimulation with ADP showed a significant decrease in %CD14 in the control group 17.99[8.46-20.31] compared to the unstimulated levels 63.16[61.10-63.80], p=0.0074 (Table 3.3). There was no significant differences post stimulation with ADP in qualitative measurements (CD14 MFI). PMA levels (%CD41) were significantly increased post stimulation with ADP 25.97[20.02-33.22] compared to unstimulated levels 12.55[12.49-16.34], p=0.0438 (Table 3.3).

Table 3.3. PMA levels post stimulation with ADP.

Control diet	**Unstimulated**	**Post-ADP**	**p-value**
%CD14	63.16[61.10-63.80]	17.99[8.46-20.31]	**0.0074**
CD14 MFI	6.13[5.98-6.55]	18.00[12.53-64.61]	0.2596
%CD41	12.55[12.49-16.34]	25.97[20.02-33.22]	**0.0438**
CD41 MFI	25.84[22.45-31.69]	34.22[24.06-41.80]	0.2854
High-fat diet	**Unstimulated**	**Post-ADP**	**p-value**
%CD14	13.52[4.590-16.08]	14.37[8.430-18.98]	**0.0405**
CD14 MFI	16.53[11.28-45.47]	20.32[14.66-73.78]	0.3125
%CD41	29.96±11.40	28.94±10.79	0.4375

CD41 MFI	67.22±28.19	40.08±14.95	0.0938

Data presented as mean ±SD; p<0.05 represented in boldface; MFI: Median fluorescence intensity; ADP: adenosine diphosphate.

3.3.4. Increased levels of PMAs post stimulation with 20µM ADP in HFD group

The HFD group showed a significant decrease in the %CD14 post stimulation with ADP 14.37[8.430-18.98] compared to unstimulated levels 13.52[4.590-16.08], p=0.0405. Interestingly there was no significant difference between the unstimulated PMA levels 29.96±11.40 and post-ADP stimulation 28.94±10.79, p=0.4375 (Table 3.3).

3.4. Discussion

The aim of this study was to evaluate PMA formation in an HFD induced pre-diabetic mouse model. The C57BL/6 mice used were the ideal model for this study as they develop glucose intolerance when fed on an HFD (13) This study also aimed at elucidating a link between inflammation and the hypercoagulable state observed in diabetes. Although animals presented with nonsignificant differences in basic parameters such as body weight and insulin levels, markers indicating platelet activation enhanced. This result may suggest that pathogenesis of pre-diabetes leading to the development of T2DM may initiate even before individuals present significant changes in body weight or insulin levels. Overall, this study was able to demonstrate that in pre-diabetes, activated platelets readily interact with monocytes forming PMAs which have been described as early markers for atherosclerosis in T2DM (14). The formation of these aggregates at this early stage of pre-diabetes may indicate the hyperreactive nature of platelets which characterize the diabetic condition. We were able to demonstrate that activated platelets were capable of forming these interactions by flow cytometry measurements of platelet-bound monocytes. This is in agreement with previous studies which demonstrated that activated platelets interact with monocytes via P-selectin and its counter-receptor PSGL-1 expressed on the surface of monocytes (3,4,15). To the best of our knowledge, our study is the first to assess PMAs using a diet-induced pre-diabetes animal model, which could significantly enhance our current understanding on the susceptibility of the vasculature to abnormally enhanced platelet activation.

The animal experimental model showed that pre-diabetic mice exhibited elevated levels of PMAs, indicating increased interactions between platelets and monocytes than in non-diabetic states. The qualitative increase of the PMAs also reiterates the increased levels of platelet-monocyte interactions in the pre-diabetes which may promote thrombosis. A previous study showed that increased PMA levels in individuals who already had coronary artery disease (15). In any case, individuals with myocardial infarctions exhibit high levels of PMAs (16,17). It has been suggested that increased levels of PMA as well as platelet-neutrophil aggregates in T2DM further highlighting the importance of the platelet-monocyte interactions in the progression of the pro-thrombotic state (12).

Consistently, in response to ADP stimulation, the pre-diabetic group exhibited an increase in the formation of PMAs indicating the hyperreactive nature of platelets in this disease state. ADP is a platelet agonist which activates the P2Y12 pathway resulting to its the translocation of P-selectin from the alpha granules to the cell surface (16,18,19). Activated platelets form interactions with monocytes, this is mediated by the binding of P-selectin to its receptor PSGL-1 on the surface of these cells (3). Platelet-bound monocytes are activated and differentiated into pro-inflammatory monocytes. This is accompanied by an increase in the expression of CD11b, a marker of monocyte activation (3,16,20). To that effect, our study showed a decrease in CD14 monocyte marker, indicating a shift in the monocyte phenotype to the pro-inflammatory form $CD14^{++}$ which can be explained by the higher qualitative analysis (CD14 MFI).

The hyperreactive nature of platelets in pre-diabetes may be associated with increased PMA formation, the early marker of atherosclerosis that is known to promote a pro-inflammatory state (13,21). A drawback of this study may be that the levels of pro-inflammatory markers produced as a result of PMA formation were not determined. Further studies evaluating the monocyte pro-inflammatory response as a result of platelet binding interactions in pre-diabetes will provide insight into the role of these in the transmigration of monocytes into metabolic tissue.

3.5. Acknowledgements

We would like to acknowledge the Biomedical research unit and the Department of Human Physiology, College of Health Sciences (CHS), University of KwaZulu-Natal for providing access to the flow cytometry analysis facility and Biomedical research unit for the animal housing facilities.

3.6. Author contributions

ZM: contributed in the conceptualisation and drafting of the article, analysis and interpretation of data and final approval of the version to be published.

TM: revision and final approval of the article.

PVD: revision and final approval of the article.

BBN: contributed to the conceptualisation analysis and interpretation of data and final approval of the version to be published.

3.7. Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animals were handled according to the principles of laboratory Animal Care of the National Society of Medical Research and the National Institutes of Animal Care and Use of Laboratory Animals of the National Academy of Sciences (National Institute of Health publication 80-23, revised

1978). Ethical clearance was granted by the UKZN animal research ethics committee (AREC), ethics registration number AREC/086/016

3.8. References

1. Samad F, Ruf W. Review Article In flammation , obesity , and thrombosis. 2016;122(20):3415–23.

2. Yngen M, Östenson CG, Hu H, Li N, Hjemdahl P, Wallén NH. Enhanced P-selectin expression and increased soluble CD40 Ligand in patients with Type 1 diabetes mellitus and microangiopathy: Evidence for platelet hyperactivity and chronic inflammation. Diabetologia. 2004;47(3):537–40.

3. Bournazos S, Rennie J, Hart SP, Fox KAA, Dransfield I. Monocyte functional responsiveness after PSGL-1-mediated platelet adhesion is dependent on platelet activation status. Arterioscler Thromb Vasc Biol. 2008;28(8):1491–8.

4. Liang H, Duan Z, Li D, Li D, Wang Z, Ren L, et al. Higher levels of circulating monocyte-platelet aggregates are correlated with viremia and increased sCD163 levels in HIV-1 infection. Cell Mol Immunol [Internet]. 2015;12(4):435–43. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4496539&tool=pmcentrez&rendertype=abstract

5. Hui H, Fuller KA, Erber WN, Linden MD. Imaging flow cytometry in the assessment of leukocyte-platelet aggregates. Methods [Internet]. 2017;112:46–54. Available from: http://dx.doi.org/10.1016/j.ymeth.2016.10.002

6. Dorsam RT, Kunapuli SP. Central role of the P2Y 12 receptor in platelet activation. J Clin Invest. 2004;113(3):10–5.

7. Cerletti C, Tamburrelli C, Izzi B, Gianfagna F, Gaetano G De. Platelet-leukocyte interactions in thrombosis. Thromb Res [Internet]. 2012;129(3):263–6. Available from: http://dx.doi.org/10.1016/j.thromres.2011.10.010

8. Wrigley BJ, Shantsila E, Tapp LD, Lip GYH. Increased Formation of monocyte-platelet aggregates in ischemic heart failure. Circ Hear Fail. 2013;6(1):127–35.

9. Michelson AD, Barnard MR, Krueger LA, Valeri CR, Furman MI. Circulating Monocyte-Platelet Aggregates Are a More Sensitive Marker of In Vivo Platelet Activation Than Platelet Surface P-Selectin: Studies in Baboons, Human Coronary Intervention, and Human Acute Myocardial Infarction. Circulation [Internet]. 2001;104(13):1533–7. Available from: http://circ.ahajournals.org/cgi/doi/10.1161/hc3801.095588

10. van Gils JM, Zwaginga JJ, Hordijk PL. Molecular and functional interactions among monocytes, platelets, and endothelial cells and their relevance for cardiovascular diseases. J Leukoc Biol. 2009;85(2):195–204.

11. Li Z, Yang F, Dunn S, Gross AK, Smyth SS. Platelets as immune mediators: Their role in host defense responses and sepsis [Internet]. Vol. 127, Thrombosis Research. Elsevier B.V.; 2011. p. 184–8. Available from: http://dx.doi.org/10.1016/j.thromres.2010.10.010

12. Davison GM, Nkambule BB, Mkandla Z, Hon GM, Kengne AP, Erasmus RT, et al. Platelet, monocyte and neutrophil activation and glucose tolerance in South African Mixed Ancestry individuals. Sci Rep [Internet]. 2017 Jan 16;7:40329. Available from: http://www.nature.com/articles/srep40329

13. Andrikopoulos S, Blair AR, Deluca N, Fam BC, Proietto J. Evaluating the glucose tolerance test in mice. 2008;1323–32.

14. Patkó Z, Császár A, Acsády G, Ôry I, Takács ÉVA, Fûrész J. Elevation of monocyte – platelet aggregates is an early marker of type 2 diabetes. 2012;4(2):181–5.

15. Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. J Am Coll Cardiol [Internet]. 1998;31(2):352–8. Available from: http://dx.doi.org/10.1016/S0735-1097(97)00510-X

16. Projahn D, Koenen RR. Platelets: key players in vascular inflammation. J Leukoc Biol [Internet]. 2012;92(6):1167–75. Available from: http://www.jleukbio.org/cgi/doi/10.1189/jlb.0312151

17. Furman MI, Barnard MR, Krueger LA, Fox ML, Shilale EA, Lessard DM, et al. Circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction. J Am Coll Cardiol. 2001;38(4):1002–6.

18. Sudic D, Razmara M, Forslund M, Ji Q, Hjemdahl P, Li N. High glucose levels enhance platelet activation: Involvement of multiple mechanisms. Br J Haematol. 2006;133(3):315–22.

19. von Hentig N, Förster A-K, Kuczka K, Klinkhardt U, Klauke S, Gute P, et al. Platelet-leucocyte adhesion markers before and after the initiation of antiretroviral therapy with HIV protease inhibitors. J Antimicrob Chemother [Internet]. 2008;62(5):1118–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18753189

20. Barnard MR, Linden MD, Frelinger AL, Li Y, Fox ML, Furman MI, et al. Effects of platelet binding on whole blood flow cytometry assays of monocyte and neutrophil procoagulant activity. J Thromb Haemost. 2005;3(11):2563–70.

21. Rutten B, Tersteeg C, Vrijenhoek JEP, Van Holten TC, Elsenberg EHAM, Mak-Nienhuis EM, et al. Increased platelet reactivity is associated with circulating platelet-monocyte complexes and macrophages in human atherosclerotic plaques. PLoS One. 2014;9(8):1–8.

3.9. Bridging chapter

The formation of platelet monocyte aggregates provides a robust measurement of platelet activation. It gives a basis for further analysis on the functionality of the activated platelets in the following chapter. After determining increased platelet activation levels in pre-diabetes, we further elucidated on the role of platelet hyperactivity in pre-diabetes by measured platelet reactivity and function in response to stimulation of the P2Y, cyclooxygenase and glycoprotein VI mediated signalling pathways.

Chapter 4. Research article two

High-fat-diet impairs glucose tolerance and enhances platelet reactivity in C57BL/6 mice

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Total word count: 4953 words

4. Abstract

Background

Platelet hyperactivity is associated with microvascular and macrovascular complications in type 2 diabetics. Although such complications have been identified in pre-diabetes, the role of platelet activity in a pre-diabetic state is poorly understood. This study aimed at assessing the levels of platelet activation and function in a pre-diabetic mouse model to improve our understanding of mechanisms involved in the development of diabetes induced vascular damage.

Methods

A 60% high-fat diet (HFD) was used to induce pre-diabetes in C57BL/6 mice. Body weights, glucose tolerance, insulin levels and haematological parameters were measured after 3 weeks on the diet. In order to investigate platelet reactivity in HFD fed mice, their whole blood samples were stimulated with varying concentrations of adenosine diphosphate (ADP) and then stained with the platelet markers including glycoprotein IIb/IIIa (CD41), P-selectin (CD62P), and glycoprotein IV (CD36).

Results

Results showed that despite no significant differences in body weights, glucose tolerance was impaired in these pre-diabetic mice. There were no significant differences in the haematological parameters measured between the two study groups. Post-stimulation with different agonists concentrations resulted in elevated %CD62P expression in the HFD group.

Conclusions

The results show that despite not gaining weight, mice fed an HFD diet displayed impaired glucose tolerance and platelet reactivity. In addition to inferring that a pre-diabetic state may occur independent of weight gain in individuals, it also shows the high risk of such individuals in this state to develop cardiovascular complications.

Keywords: Pre-diabetes; Platelet reactivity; Platelet function; Flow cytometry

4.1. Introduction

Type 2 diabetes mellitus (T2DM) is characterised by insulin resistance, hyperglycaemia, hyperinsulinemia and an increased incidence of microvascular and macrovascular complications (1,2). On the other hand, pre-diabetes is defined as a state of impaired glucose tolerance that is associated with at higher risk of developing T2DM (3,4). Previous studies have focused on the pathophysiological mechanisms in a pre-diabetic state, in efforts to identify interventions to curb diabetic complications that may lead to the development of cardiovascular disease (CVD). In pre-diabetic individuals, elevated levels of activated peripheral blood platelets have been associated with myocardial infarcts of coronary

artery disease (5,6). Although precise mechanisms involved in this process are not fully known, disturbances in several platelet signalling pathways such as adenosine diphosphate receptor (P2Y12) may initiate these irregularities (5,7). It is also reported that activated platelets play a crucial role in initiating inflammation, coagulation and subsequent thrombus formation through the actions of a cell adhesion molecule, P-selectin (8,9). Elevated levels of P-selectin have already been linked with an increased risk of coronary artery disease in individuals living with T2DM (5,10), suggesting that increased platelet activation plays a major role in the development of CVD.

Platelets also express glycoprotein IV (CD36), a major platelet glycoprotein that is involved in thrombus formation (11,12). In resting platelets, CD36 is localised on the alpha granule membrane and the open canalicular system (13). Whereas on activated platelets, CD36 is densely expressed on pseudopods and the plasma membrane (13). The soluble form of CD36, sCD36, has been described as a novel biomarker of T2DM (14). Several other studies have shown that sCD36 is associated with the development of obesity, insulin resistance, increased risk of T2DM and atherosclerosis (14–16).

Although elevated levels of both P-selectin and CD36 are linked with the aggravation of inflammation, comprehensive understanding of enhanced platelet-mediated responses in a pre-diabetic state remain unclear. However, it is currently understood that an elevated platelet reactivity index (PRI) may be associated with an increased incidence of long-term cardiovascular events (5,17). In this study, we aimed at assessing the role of platelet activation and function in a pre-diabetic state through the use of endogenous agonists which stimulate the purinergic receptor ($P2Y_{12}$), phospholipase C (PLC) and cyclooxygenase (COX) platelet signalling pathways.

4.2. Methodology

4.2.1. Study design and animal care

Male C57BL/6 mice (age: 5 weeks) were purchased from the University of KwaZulu-Natal (UKZN) biomedical research unit (BRU) and handled according to the principles of laboratory animal Care of the National Society of Medical Research and the National Institutes of Animal Care and Use of Laboratory Animals of the National Academy of Sciences (National Institute of Health publication 80-23, revised 1978). Ethical clearance was granted by the UKZN animal research ethics committee (AREC), ethics registration number AREC/086/016.

After the 1-week acclimatization period, the mice (n=18) were randomized into two diet groups and kept on the respective diets for a duration of 3 weeks. Mice in the experimental group (n=13) were fed a high-fat diet (HFD), which contained 60% kilocalories derived from fats (Research diet no. D12492; Research Diets, New Brunswick, NJ, USA). The control group (n=5) received a low-fat control diet containing 10% kilocalories fat (Research diet no. D12450J; Research Diets, New Brunswick, NJ, USA). Mice were housed in a controlled environment with a twelve-hour light/dark cycle (lights were

switched on at 6:00 AM and switched off at 6:00 PM) and a temperature range of 23-25ºC (relative humidity: ~50%). The mice also had unlimited access to water. Furthermore, they were regularly monitored while the cages were cleaned once a week, to ensure a clean environment.

4.2.2. Oral glucose tolerance tests

After 3-weeks on HFD, all mice fasted for 8 hours before their fasting blood glucose levels were measured as previously described (18). Briefly, using oral gavage method, 2g/kg of glucose was administered, and blood glucose levels were measured at varying time intervals (0 minutes (baseline), 30 minutes, 60 minutes, and 120 minutes), using the Accu-Check active blood glucometer (Roche, Basel, Switzerland).

4.2.3. Blood collection

After 3 weeks on the experimental diets (at 9 weeks of age), 200µl of venous blood was collected from the lateral tail vein. This was collected into 3.2% sodium citrate coated microtainer tubes (Sigma Aldrich, St Louis, Missouri, USA).

4.2.4. Measurement of baseline haematological parameters

The baseline haematological parameters which included the platelet counts and mean platelet volume were measured using the Beckman Coulter AcT diff™ analyser (Beckman Coulter, Brea, CA, USA), following manufacturer's instructions.

4.2.5. Platelet flow cytometry and platelet function analysis

Flow cytometry analysis was performed within 30 minutes of blood collection. Internal quality control (QC) was performed using the Becton Dickson (BD) cytometer set-up and tracking (CST) beads (BD Bioscience, NJ, USA), as per manufacturer's instructions. The BD ™ Compbead compensation particles were used to calculate and compensate for spectral overlap between the fluorescent channels. In addition, daily monitoring of potential instrumental drifts was performed using the SPHERO ™ 6-peak Rainbow calibration particles (BD Bioscience, NJ, USA) and used as QC for all median fluorescence intensity (MFI) measurements.

4.2.6. Baseline measurement of markers of platelet activation

The baseline levels of circulating activated platelets were determined as previously described (19). Briefly, 25µl of whole blood was stained with 2.5µl of a titrated antibody cocktail containing glycoprotein IIb/IIIa, CD41-FITC (used as a platelet marker); P-selectin, CD62P-BV421 (used as a platelet activation marker) and glycoprotein IV, and CD36-PE (used as a platelet aggregation marker) (BD Bioscience, NJ, USA). The samples were then incubated at room temperature for 10 minutes and immediately analysed on the BD FACS Canto II flow cytometer (BD Bioscience, NJ, USA).

4.2.7. *Platelet function analysis: Agonist-induced platelet activation*

Two varying concentrations of ADP (4µM for reversible and 20µM for irreversible platelet activation), collagen (0.19mg/mL) and arachidonic acid (500µg/mL) (irreversible single wave platelet activation) (Bio-data co-operation, Pennsylvania, USA) were used as previously described (19). Briefly, 25µl of whole blood was stimulated with 10µl of agonist in a staggered manner and incubated at room temperature for 15 minutes. The samples were then stained with 2.5µl of the antibody cocktail containing CD41-FITC (clone MWReg30), CD62P-BV421 (clone RB40.34), and CD36-PE (clone CRF D-2712) (BD Bioscience, New Jersey, USA) and incubated for 10 minutes in the dark, at room temperature. The lysis of red blood cells was omitted as this has been shown to induce platelet activation by releasing ADP as described by Helms et. al (20). Thus, 350µl of phosphate buffer saline was added before the sample analysed on the BD FACS Canto II flow cytometer (BD Bioscience, NJ, USA). The percentage expression (quantitative) and MFI (qualitative) of both markers (CD62P and CD36) were also measured. The PRI for each activation marker was calculated, using the baseline CD62P MFI and CD36 MFI according to the following equation.

$$\text{Platelet reactivity Index} = \left(\frac{\text{MFI post Agonist stimulation -MFI at Baseline}}{\text{MFI post Agonist Simulation}} \text{ x } 100\right).$$

4.2.8 *Gating strategy*

The platelet marker CD41 was used to gate on unbound, freely circulating platelets (PLT) with lower side scatter properties. The red-blood cell bound platelets (RBC-PLT) were also gated using CD41 and higher side scatter properties. An unstained control was used to gate out autofluorescence. The level of level of activated, freely circulating platelets was determined by the membrane surface expression of activation marker CD62P (P-selectin) (Figure 4.1)

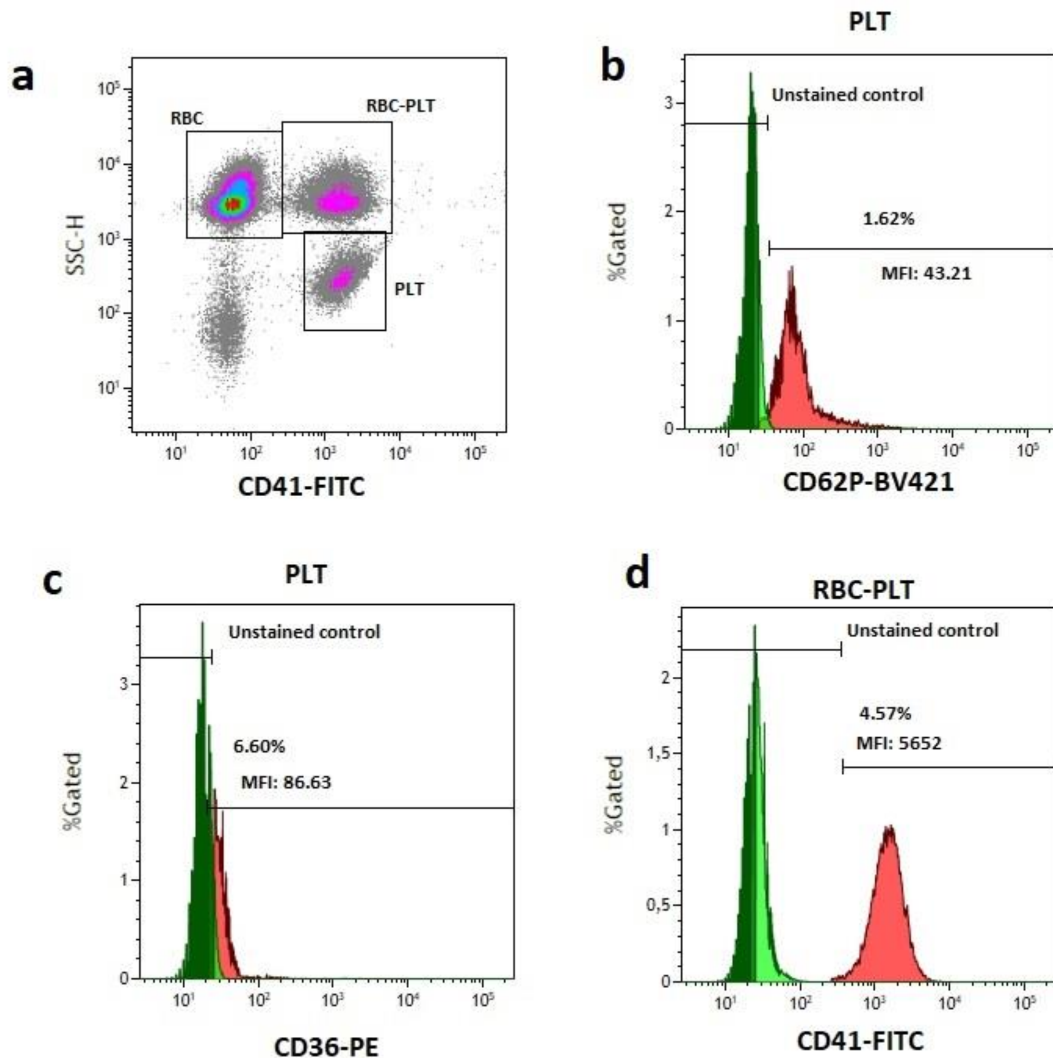

Figure 4.1. Platelet gating strategy. (a) The primary gating strategy was based on the platelet marker (CD41) used to distinguish platelet populations. The side scatter properties were used to distinguish unbound platelets (PLT) from red blood cell-bound (RBC-PLT) platelets. (b) The quantitative (%CD62P) and qualitative (CD62P MFI) expression of CD62P was determined. (e) Quantitative (%CD36) and qualitative (CD36 MFI) levels of CD36 expression on platelets was determined. (d) Quantitative (%CD41) and qualitative (CD41 MFI) red blood cell-bound platelet population was determined based on the RBC-PLT primary gate in (a). *RBC: Red blood cell; RBC-PLT: Red blood cell-bound platelets; PLT: Platelet; MFI: Median fluorescence intensity.*

4.2.9 Statistical analysis

All statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA). Parametric data were analysed using the repeated measures ANOVA and Bonferroni's Multiple Comparison Test post hoc test. Parametric data were reported as the mean ±

standard deviation (SD), and non-parametric data reported as the median ± interquartile range (IQR).

4.3. Results

4.3.1. Establishment of a pre-diabetic model

There were no body weight differences between the HFD group and control diet mice (Table 4.1, Figure 4.2). Notably, the basal levels of %CD62P and CD62P MFI were comparable between the two groups (Table 4.1).

Table 4.1. Baseline parameters

	Control group (n=5)	HFD (n=13)	p-value
Body weight (g)	26.60± 3.44	27.07± 1.49	0.9648
White cell count x 10^3/µL	7.2±3.49	8.471±2.84	0.5168
Red blood cell count x 10^6/µL	7.33±0.3	6.24±1.21	**0.0109**
Platelet count x 10^3/µL	923.3± 147.8	771.8± 147.8	**0.0475**
Mean platelet volume (fL)	5.58± 0.42	5.39± 0.21	0.2673
Plateletcrit (PCT)	0.49[0.42-0.59]	0.40[0.37-0.44]	**0.0475**
Platelet distribution width (PDW)	0.055[0.053-0.059]	0.054[0.050-0.055]	0.2673
%CD62P	6.43[3.88-9.86]	4.98[3.89-5.99]	0.5569
CD62P MFI	95.93[73.03-111.5]	87.19[51.62-98.22]	0.2782
%CD36	2.06±1.50	1.69±0.82	0.2127
CD36 MFI	43.90±1.95	41.60±1.98	**0.0475**

Significant values (p<0.05) are shown in bold. HFD: High-fat diet group; MFI: Median fluorescence intensity

Overall, the oral glucose tolerance showed that the HFD group had a higher postprandial blood glucose levels compared to the control group. This was also illustrated by the higher area under the curve (AUC) observed in the HFD group (Figure 4.2). Notably, the HFD group showed significant levels of impaired glucose clearance at 30 minutes postprandial blood glucose levels (p=0.0233). There was also a decrease in the plateletcrit in the HFD group as compared to the control group, p=0.0475. While the baseline GPIV (CD36) MFI was reduced in the HFD group (p=0.0475) (Table 4.1).

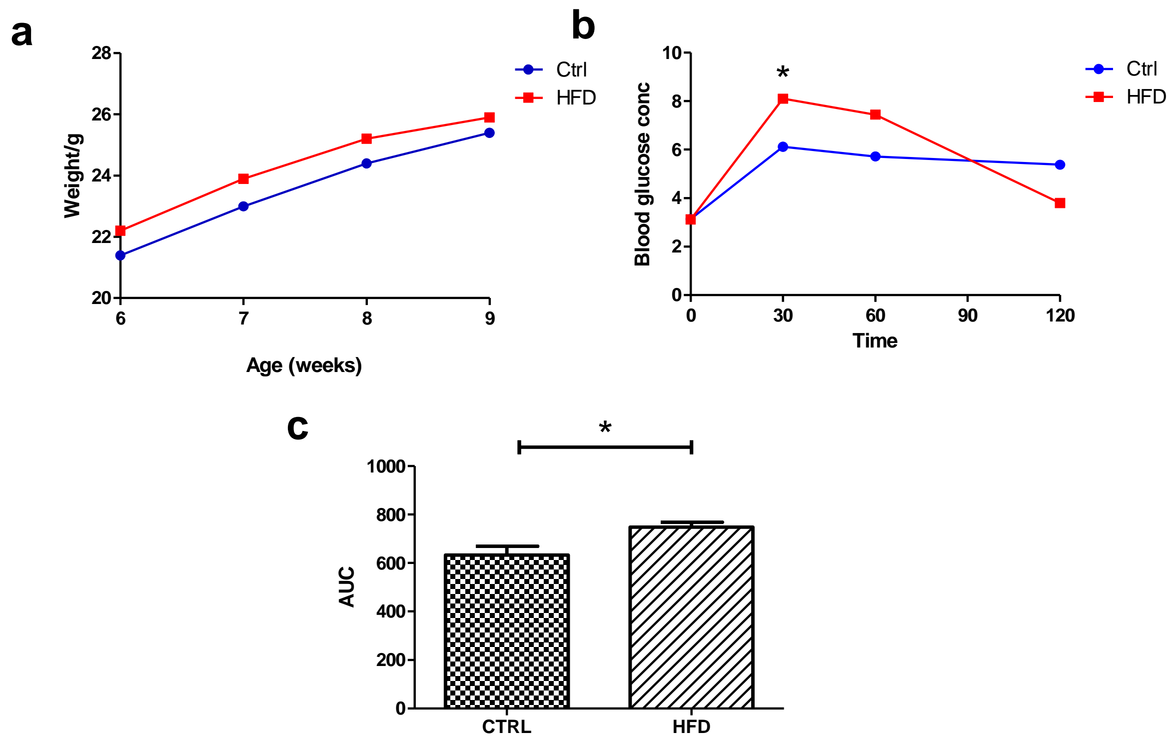

Figure 4.2. Animal weight and glucose control. (a) The C57BL/6 mice showed a steady increase in weight with no significant difference between the 2 study groups. (b) The oral glucose tolerance test (OGTT) showed significant poor glycaemic control for the HFD group at 30minutes compared to the control group, p= 0.0233. (c) Overall, the area under the curve demonstrated increased poor glycaemic in the HFD compared to the control group, p= 0.0362. CTRL: Control diet group; HFD: High-fat diet group; AUC: area under the curve.

For haematological parameters, the HFD group had lower red blood cell count compared to the control diet group, p=0.0109. Similarly, the HFD group had a lower platelet count compared to the control diet group, p=0.0475. The platelet count showed a positive correlation with the red blood cell count (RBC), r=0. 5504, p=0. 0414. There was a significant correlation between the red blood cell-bound platelet (RBC-PLT) and freely circulating platelets (PLT) post stimulation with arachidonic acid in the high-fat diet group (r=0.6233; p=0.0013) (Figure 4.4). No other significant correlations were observed with the other parameters measured, including mean platelet volume, plateletcrit and platelet distribution width (Figure 4.3 and 4.4).

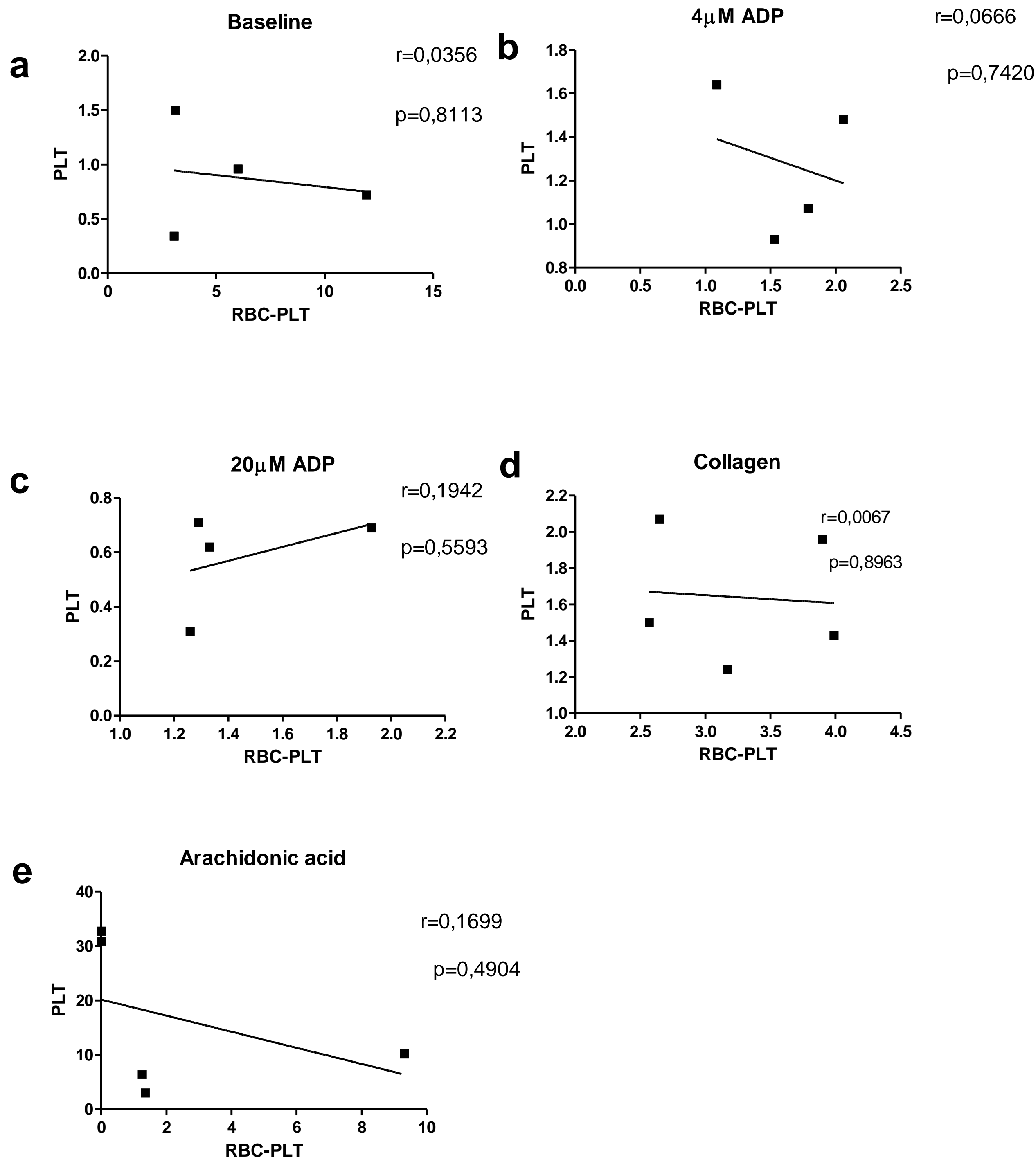

Figure 4.3. Correlations between red blood cell-bound platelet and freely circulating platelets in the control diet group. (a) Correlation between RBC-PLT and PLT at baseline was statistically insignificant (r=0.0356; p=0.8113). (b) Post-stimulation with 4μM ADP, the correlation was also insignificant (r=0.0666; p=0.7420). (c) 20μM ADP stimulation did not elicit any significant correlation between the RBC-PLT and PLT populations (r=0.1942; p=0.5593). (d) The stimulation of platelets with collagen also provided no significant correlation between the two populations (r=0.0067; p=0.8963). (e) There was also no significant correlation between the populations post

stimulation with arachidonic acid (r=0.1699; p=0.4904). ADP: adenosine diphosphate; RBC-PLT: red blood cell-bound platelets; PLT: freely circulating unbound platelets.

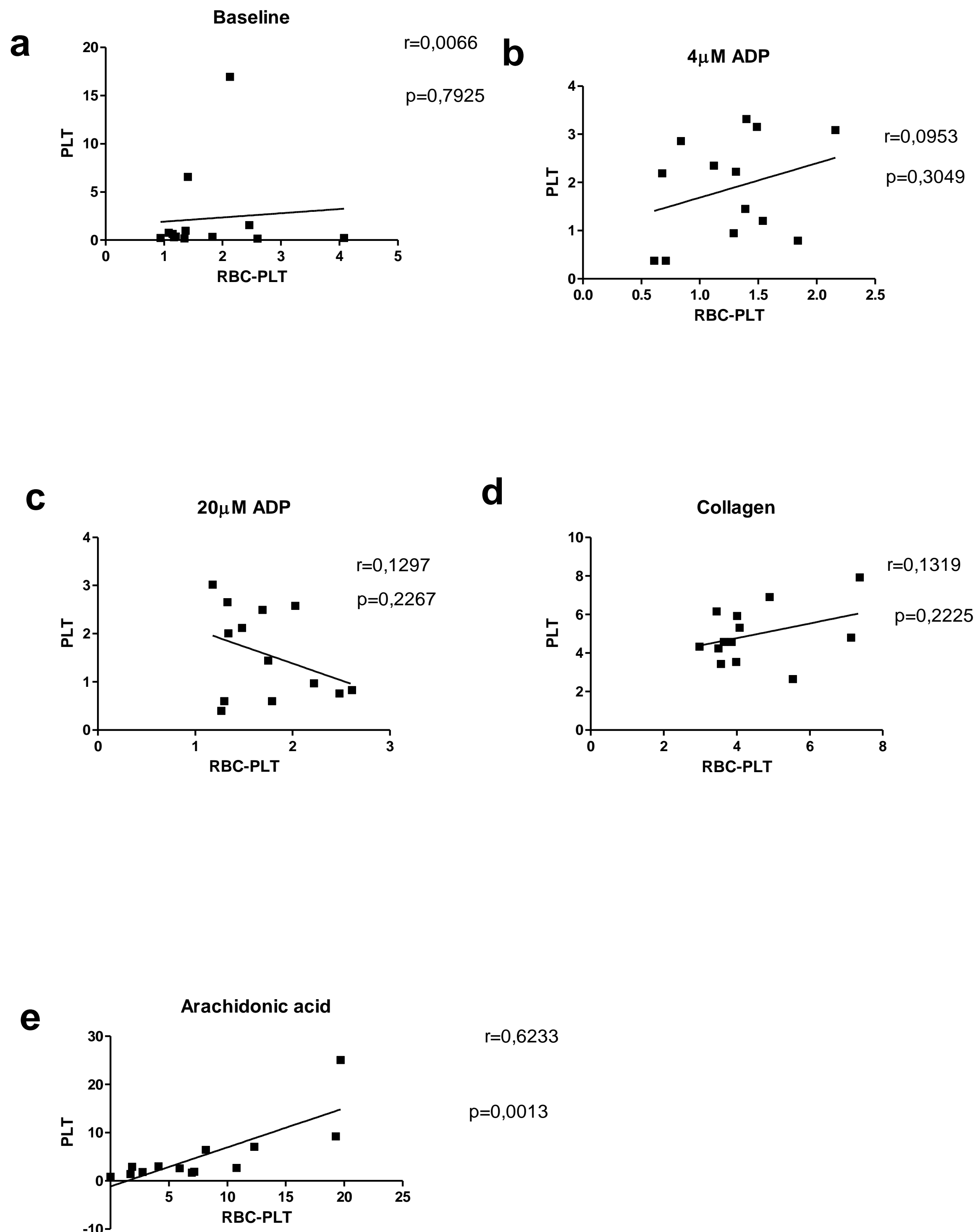

Figure 4.4. Correlations between red blood cell-bound platelet and freely circulating platelets in the high-fat diet group. (a) In this group baseline correlation between the red blood cell-bound platelets and freely circulating platelets was non-significant (r=0.0066; p=0.7925). (b) Stimulation of platelets with 4μM ADP, the correlation between the two populations was also non-significant (r=0.0953; p=0.3049). Similarly, post stimulation with

20μM ADP resulted in non-significant correlation between the populations (r=0.1297; p=0.2267). (d) Collagen platelet stimulation also did not elicit any significant correlation (r=0.1319; p=0.2225). (e) There was a significant positive correlation between the red blood cell-bound platelets and the freely circulating platelets post stimulation with Arachidonic acid (r=0.6233; p=0.0013). ADP: adenosine diphosphate; RBC-PLT: red blood cell-bound platelets; PLT: freely circulating unbound platelets.

Quantitative analysis of the RBC-PLT population demonstrated significantly lower levels in the HFD group at baseline (p=0.0078), however, it was markedly increased post stimulation with arachidonic acid (p=0.0482) (Table 4.2).

Table 4.2. Quantitative (%CD41) comparison of red blood cell-bound platelets between control and high-fat diet

Agonist	**Control diet**	**HFD**	**p-value**
Baseline	4,565[3,075-10,45]	1,370[1,160-2,295]	**0,0078**
4μM ADP	1,530[0,7350-1,925]	1,310 [0,7750-1,515]	0,6934
20μMADP	1,310 [1,268-1,780]	1,690 [1,315- 2,125]	0,2572
Collagen	3,170 [2,610- 3,945]	3,990 [3,535- 5,220]	0,0681
Arachidonic acid	1,260 [0,0000- 5,335]	6,970 [2,305- 11,56]	**0,0482**

Significant values (p<0.05) are shown in bold. HFD: High-fat diet group

Qualitative analysis of this population also showed significantly lower levels in the HFD group (p=0.0273) and higher levels in the same group post stimulation with 4μM ADP (p=0.0487) (Table 4.3).

Table 4.3. Qualitative (CD41 MFI) comparison of red blood cell-bound platelets between control and high-fat diet

Agonist	**Control diet**	**HFD**	**p-value**
Baseline	5652 [5225-6341]	4933 [4755-5330]	**0,0273**
4μM ADP	4944 [4752-4998]	5162 [4980-5543]	**0,0487**
20μM ADP	4488 [4377-5081]	4884 [4555-5249]	0,1570
Collagen	5385 [4854-5535]	5164 [4992-5342]	0,6221
Arachidonic acid	4630 [0,0000-6020]	6423 [4575-7422]	0,1384

Significant values (p<0.05) are shown in bold. HFD: High-fat diet group; MFI: Median fluorescence intensity

4.3.2. Platelet activation post stimulation with ADP

To evaluate the ability of platelets to undergo dose-dependent reversible and irreversible activation, platelets were stimulated using two ADP concentrations, 4µM and 20µM. The HFD group showed a comparable response after stimulation with 4µM ADP 2.22[0.79-3.09] and 20µM ADP 1.44[0.60-2.58], p=0.0956. However, post stimulation with 4µM ADP and 20µM ADP, the HFD group expressed elevated %CD62P compared to the control group 0.35[0.22-0.76], p =0.0020 and p=0.0052 respectively (Figure 4.5). In addition, the HFD group had a higher CD36 PRI compared to the control group, p=0.0362.

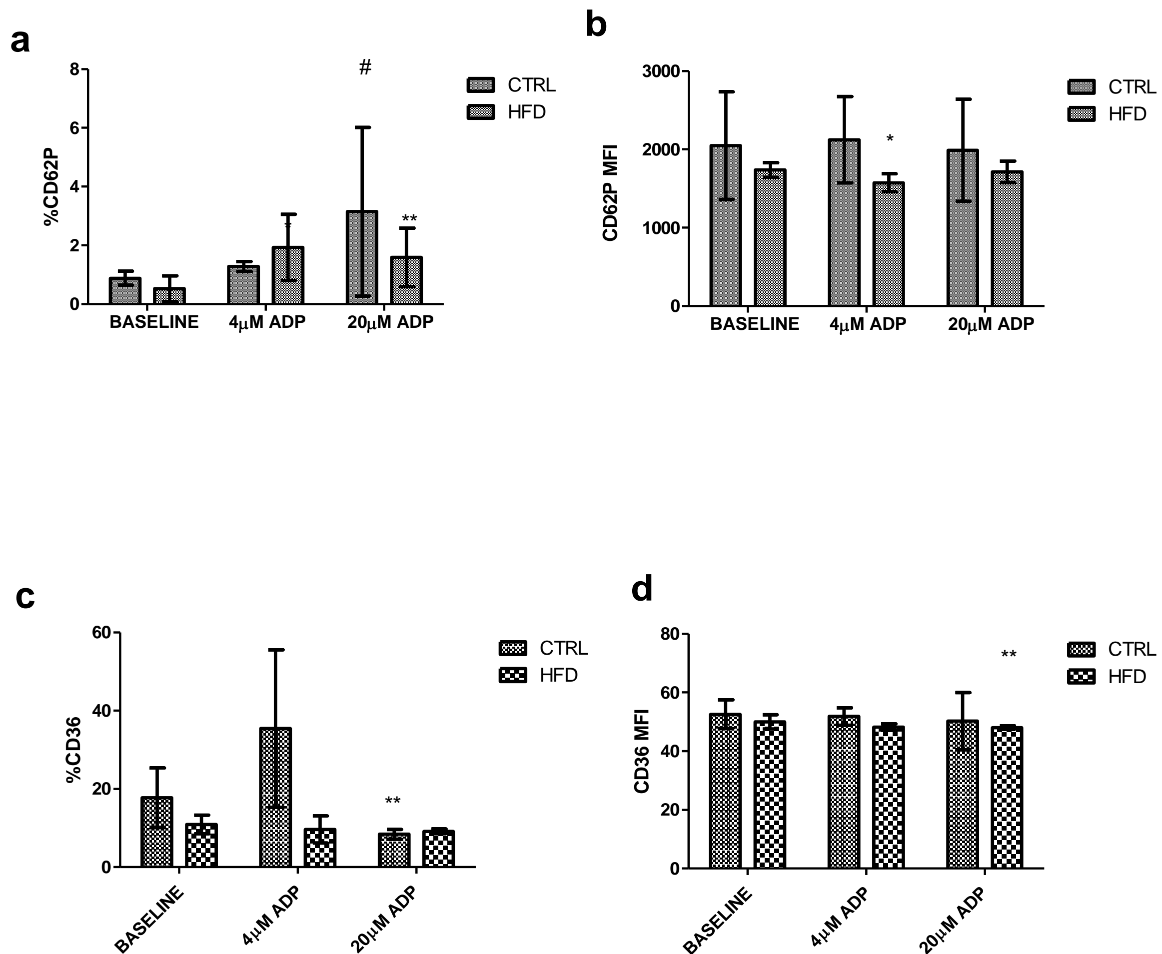

Figure 4.5. Biphasic platelet activation post stimulation with 4µM and 20µM ADP. The levels of platelet CD62P and CD36 at baseline and post stimulation with varying concentrations of ADP. (a) Stimulation with 4µM (p=0.0020) and 20µM (p=0.0052) increased levels of %CD62P in HFD group. (b) The MFI levels in the HFD group were significantly decreased post stimulation with 4µM ADP but remained comparable across all other groups. (c) Stimulation with 20µM ADP resulted in a decrease in the %CD36 expression in the control group, p=0.0286. (d) While stimulation with the 20µM of ADP,

significantly decreased the CD36 MFI in the HFD group. *#: p<0.05 4µM ADP vs 20µM ADP; *: p<0.05 Baseline vs 4µM ADP; **: p<0.05 Baseline vs 20µM ADP; Ctrl: control group; HFD: High fat diet group; MFI: Median fluorescence intensity.*

4.3.3. Increased levels of platelet activation (%CD62P) and reactivity in response to endogenous agonists in HFD group

In order to determine the level of platelet activation post stimulation with endogenous agonists collagen and arachidonic acid, we measured the percentage expression of CD62P and the corresponding MFI.

Table 4.4. Platelet reactivity post stimulation with endogenous agonists

Agonist	%CD62P			CD62 MFI		
	Control group	HFD	p-value	Control group	HFD	p-value
Collagen	1,05 [0,31 - 2,84]	14,61 [6,041-20,21	**0,0475**	0,39 [0,10-0,51]	-0,05 [-0,10-0,03]	**0,0007**
Arachidonic acid	19,30 [14,32-28,59] *	6,82 [2,96-16,12]	0,4617	-0,01 [-0,34-0,45]	0,07 [-0,04-0,23] *	0,0519
	%CD36			CD36 MFI		
	Control group	HFD	p-value	Control group	HFD	p-value
Collagen	-0,30[-0,41--0,17]	0,39[0,15-0,55]	0,2263	-0,04[-0,12--0,01]	0,02[-0,06-0,04]	0,2157
Arachidonic acid	4,25[3,43-4,69] *	7,78[6,02-9,01] *	0,3512	4,73[1,22-12,64]	1,14[0,89-1,64] *	**0,0088**

Significant values (p<0.05) are shown in bold and with an asterisk (*). HFD: High-fat diet group; MFI: Median fluorescence intensity

At post-stimulation with collagen, the %CD62P expression was higher in the HFD group 4.58[3.89-6.04] compared to the control group 1.50[1.34–2.02], p=0.0195. Similarly, the CD62P MFI was elevated in the HFD group compared to the control diet group, p=0.0016. Arachidonic acid stimulation also resulted in higher %CD62P expression in the control group 10.20[4.69-31.82] compared to the HFD group 2.70[1.79-6.77], p=0.0301 (Table 4.5).

Table 4.5: CD62P expression post stimulation with collagen and arachidonic acid

	CTRL	HFD	p-value
Collagen			
%CD62P	1,50 [1,34 - 2,02]	4,58[3,89- 6,04]	**0,0016**
CD62P MFI	2640 [2383 - 2677]	1643 [1586 - 1709]	**0,0016**
Arachidonic acid			
%CD62P	10,20 [4,69- 31,82]	2,70[1,79- 6,77]	**0,0301**
CD62P MFI	1916 [1611 - 2437]	1793 [1710 - 2093]	0,7674

Significant values (p<0.05) are shown in bold; CTRL: Control diet group; HFD: High-fat diet group; MFI: Median fluorescence intensity.

4.3.4. Increased CD36 expression post stimulation with agonists in HFD group

The expression of CD36 was determined both quantitatively (%CD36) and qualitatively (CD36 MFI) post stimulation with collagen and arachidonic acid. The HFD group had lower CD36 MFI expression compared to the control group, p=0.0193 (Table 4.6, Figure 4.6).

Table 4.6: CD36 expression post stimulation with collagen and arachidonic acid

	CTRL	HFD	p-value
Collagen			
%CD36	11,92 [10,67-13,18]	12,69 [11,58-16,84]	0,2369
CD36 MFI	48,99 [48,62-50,25]	49,51 [48,97-50,16]	0,4902
Arachidonic acid			
%CD36	91,44 [78,99-97,64]	95,11 [90,46-96,54]	0,3562
CD36 MFI	201,9 [91,63-612,2]	109,1 [95,45-127,2]	**0,0193**

Significant values (p<0.05) are shown in bold; CTRL: Control diet group; HFD: High-fat diet group; MFI: Median fluorescence intensity

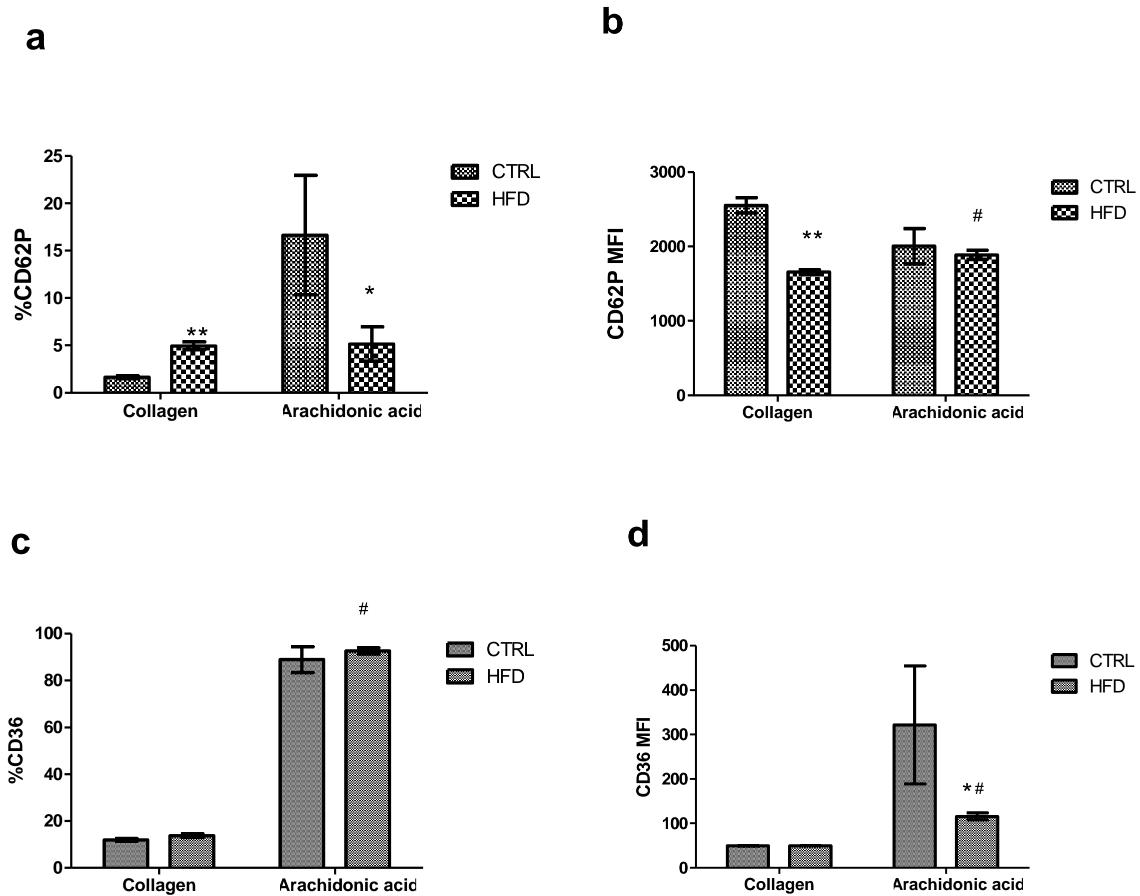

Figure 4.6. Platelet activation and function post stimulation with collagen and arachidonic acid. (a) Increased platelet %CD62P compared to the control (Ctrl) post stimulation with collagen (p=0.0016) and decreased post arachidonic acid stimulation (p=0.0301). (b) The CD62P MFI was decreased in the HFD group compared to the control group post stimulation with collagen (p=0.0016). Post-stimulation with arachidonic acid had increased CD62P MFI compared to collagen post stimulation in the HFD group (p=0.0046). (c) There was higher platelet %CD36 expression post stimulation with arachidonic acid compared to collagen stimulation (p=0.0002). (d) Increased platelet CD36 MFI post stimulation with arachidonic acid was higher than collagen, in the HFD group (p=0.0002). The HFD group had lower CD36 MFI compared to the control group, p=0.0193. **Ctrl vs HFD (Arachidonic acid); **Ctrl vs HFD (Collagen); # p<0.05 Collagen vs Arachidonic acid (HFD).*

4.4. Discussion

The aim of this study was to investigate the role of platelet activation and function in an HFD-induced pre-diabetic mouse model. Previous research shows that male C57BL/6 mouse strain can develop obesity, impaired glucose tolerance and insulin resistance during high-fat feeding (21). In our study, 3-weeks of high-fat feeding in mice led to the development of impaired postprandial glucose control, a common feature of pre-diabetes (22). Although mice did not gain significant weight, such

characteristics have been identified in non-obese individuals with T2DM, where it was shown that these individuals may develop insulin resistance more rapidly as compared with obese counterparts (23). Nonetheless, haematological indices measured, which included RBC, platelet count and the plateletcrit, were all decreased in the HFD fed group when compared to the controls. These findings suggest that pre-diabetic mice might have developed thrombocytopenia. Interestingly, it has been shown that a diabetic state can increase the severity of thrombocytopenia in dengue virus-infected individuals in the first three days of hospitalisation (24).

It is currently known that platelets are essential for haemostasis, especially in preserving healthy vascular endothelium (25). However, together with the low RBCs, decreased platelet count in a diabetic state may indicate dysfunctional coagulation cascade (25). Our analysis of the red blood cell-bound platelets demonstrated no relationship at baseline but was increased when stimulated with arachidonic acid in the HFD fed group. Interestingly, despite the differences in basal platelet counts, in our study, the baseline levels of activated platelets were comparable between the pre-diabetic and experimental control group. A similar finding was reported by Dogru and colleagues, where they showed no significant differences in basal levels of sP-selectin levels between pre-diabetic and non-diabetic individuals (26). The use of P-selectin and ADP to study platelet activation in mice may provide evidence of the hyperreactivity of platelets from pre-diabetes despite normal basal activation levels.

In the current study, a biphasic response was observed post-ADP stimulation in the pre-diabetic group. This effect suggests that sub-threshold concentration of ADP was able to elicit maximum platelet response which may indicate high platelet susceptibility to submaximal stimulation in the pre-diabetic state. Briefly, ADP is known to be released from platelet dense granules in response to inside-out PLC signalling, this subsequently leads to platelet shape change and aggregation (27,28). During this process, the integrin $\alpha_{IIb}\beta_3$ undergoes conformational changes resulting in a high-affinity state for attachment to fibrinogen resulting in platelet aggregation (29). The G_q-coupled protein $P2Y_1$ is an ADP receptor, which activates the release of calcium from the dense tubular system (DTS) leading to platelet activation, reversible aggregation and shape change. A previous study identified increased levels of intracellular cytosolic Ca^{2+} following ADP and thrombin stimulation in mice lacking $P2Y_1$ (30). It is also reported that hyperglycaemia can alter Ca^{2+} mobilisation and ultimately promote its reactivity with aggregation agents [31, 32]. In diabetic individuals, platelets can exhibit reduced membrane fluidity as a result of modifications in lipid components or glycation of membrane proteins, as well as an increase in intracellular Ca^{2+} mobilisation (33). The glycation of membrane proteins may then cause changes in protein structure and conformation enhancing the expression of platelet receptors such as P-selectin (31). In our study, we activated the $P2Y_{12}$ receptors with the ADP agonist and hence we can hypothesise that intracellular Ca^{2+} levels were increased. As a result, we observed elevated platelet activation levels.

In our study, platelet reactivity was also increased in pre-diabetic mice post stimulation with arachidonic acid. Stimulation with this agonist resulted in a higher PRI in pre-diabetic mice, with an overall increased expression of both P-selectin and CD36 when compared to the controls. Arachidonic acid is known to induce platelet aggregation and release of thromboxane A_2 (TXA_2), which is associated with detrimental effects through the generation of oxidative stress and activation of the PLC pathway (34). Thus, peroxidation of arachidonic acid forming active isoprostanes may provide an important connection between impaired glucose tolerance and platelet activation. Oxidative stress induced by the chronic hyperglycaemia in pre-diabetes enhances peroxidation of arachidonic acid and the production of prostaglandins and thromboxanes (35–37). Interestingly, as a previous study showed a negative correlation between arachidonic levels in blood and glycated haemoglobin (38). This may suggest that poor glucose control such that observed in a pre-diabetic state may influence platelet activation through thromboxane mediated signalling (38). However, further studies are needed to study this phenomenon in detail.

Furthermore, we studied another major platelet glycoprotein, CD36, and its involvement in thrombus formation in our model. CD36 is involved in platelet aggregation, adhesion to collagen and angiogenesis which contribute to the progression of atherosclerosis in T2DM (39,40). The present study demonstrated an inverse association between the qualitative and quantitative measurements of CD36. Currently, it is known that increased expression of CD36 correlates with markers of inflammation, which include high sensitivity C-reactive protein (hsCRP) and high sensitivity interleukin-6 (hsIL-6) which are independent predictors of CVD (41). Diabetic individuals display increased levels of sCD36 compared to non-diabetic individuals, which have been linked with insulin resistance and obesity-driven low-grade inflammation (14). Contrary to these findings, no significant changes in the expression of CD36 concomitant to reduced platelet activation have been observed in diabetic individuals following 3 months of improved glucose control (39). This may suggest that improved metabolic control alone may not be enough to normalise the levels of platelet function in people with T2DM. However, such findings have to be confirmed in other models of pre-diabetes.

Nonetheless, our findings demonstrate that platelets from pre-diabetic mice were more reactive compared to those from the control group. This suggests that thrombotic complications such as platelet hyper-reactivity that are associated with hyperglycaemia and insulin resistance may develop as earlier as the pre-diabetic state. Our results further suggest that multiple signalling pathways in addition to the purinergic receptors may mediate platelet activation and function. However, before such hypothesis can be accepted, investigations to evaluate the involvement of platelet-leukocyte aggregates and platelet microparticles, which are a major contributor of circulating soluble glycoprotein IV and early biomarkers of T2DM are necessary (42). Future studies can also investigate the intracellular mechanisms involved in leukocyte-platelet interactions in a pre-diabetic state as this may provide further insight into the pathophysiology involved in platelet function in a pre-diabetic state.

4.5. Conclusion

Platelet hyperreactivity is a common feature of pre-diabetes and T2DM (43), which consequently increases the risk of developing CVD (7). Platelet activation involves a convergence of several activation pathways. Our study showed that in a pre-diabetic state, platelets are hyper-reactive to arachidonic acid compared to ADP and collagen. This may suggest that the COX-1 regulated signalling pathway is highly activated in pre-diabetics and early anti-platelet treatment interventions targeting this pathway in pre-diabetic individuals may lower the risk of thrombotic complications in overt diabetes.

4.7. Declarations

4.7.1. Ethics approval and consent to participate

All institutional and national guidelines for the care and use of laboratory animals were followed and approved. Ethical clearance was granted by the UKZN animal research ethics committee (AREC), ethics registration number AREC/086/016.

4.7.2. Consent of publication

Not applicable

4.7.3. Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

4.7.4. Competing interests

The authors declare no competing financial interests

4.7.5. Funding

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4.7.6. Author contributions

ZM: contributed in the conceptualisation and drafting of the article, analysis and interpretation of data and final approval of the version to be published.

TM: revision and final approval of the article.

PVD: revision and final approval of the article.

BBN: contributed to the conceptualisation analysis and interpretation of data and final approval of the version to be published.

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4.8. References

1. Reaven GM. Role of insulin resistance in human disease. Diabetes. 1988;37(12):1595–607.

2. Duckworth W, Abraira C, Moritz T, Reda D, Emanuele N, Reaven PD, et al. Glucose control and vascular complications in veterans with type 2 diabetes. NEnglJMed. 2009;360(1533–4406 (Electronic)):129–39.

3. World Health Organization. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia:Report of a WHO/IDF consultation. Production. 2006;1–52.

4. Bansal N. Prediabetes diagnosis and treatment: A review. World J Diabetes [Internet]. 2015;6(2):296. Available from: http://www.wjgnet.com/1948-9358/full/v6/i2/296.htm

5. Ferreiro JL, Gómez-Hospital JA, Angiolillo DJ. Platelet abnormalities in diabetes mellitus. Diab Vasc Dis Res. 2010;7(4):251–9.

6. Davison GM, Nkambule BB, Mkandla Z, Hon GM, Kengne AP, Erasmus RT, et al. Platelet, monocyte and neutrophil activation and glucose tolerance in South African Mixed Ancestry individuals. Sci Rep [Internet]. 2017 Jan 16;7:40329. Available from: http://www.nature.com/articles/srep40329

7. El Haouari M, Rosado JA. Platelet signalling abnormalities in patients with type 2 diabetes mellitus: a review. Blood Cells Mol Dis [Internet]. 2008;41(1):119–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18387322

8. Stokes KY, Granger DN. Platelets: a critical link between inflammation and microvascular dysfunction. J Physiol. 2012;590(Pt 5):1023–34.

9. Haugaard AK, Lund TT, Birch C, Rönsholt F, Trøseid M, Ullum H, et al. Discrepant coagulation profile in HIV infection: elevated D-dimer but impaired platelet aggregation and clot initiation. AIDS. 2013;

10. Angiolillo DJ, Bernardo E, Sabat?? M, Jimenez-Quevedo P, Costa MA, Palazuelos J, et al. Impact of Platelet Reactivity on Cardiovascular Outcomes in Patients With Type 2 Diabetes

Mellitus and Coronary Artery Disease. J Am Coll Cardiol. 2007;50(16):1541–7.

11. Podrez EA, Byzova T V, Febbraio M, Salomon RG, Ma Y, Valiyaveettil M, et al. Platelet CD36 links hyperlipidemia , oxidant stress and a prothrombotic phenotype. 2007;13(9).

12. Febbraio M, Hajjar DP, Silverstein RL. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis,inflammation, and lipid metabolism. J Clin Invest. 2001;108(6):785–91.

13. Berger G, Caen JP, Berndt MC, Cramer EM. Ultrastructural demonstration of CD36 in the alpha-granule membrane of human platelets and megakaryocytes. Blood. 1993;82(10):3034–44.

14. Handberg A, Lopez-Bermejo A, Bassols J, Vendrell J, Ricart W, Fernandez-Real JM. Circulating soluble CD36 is associated with glucose metabolism and interleukin-6 in glucose-intolerant men. Diabetes Vasc Dis Res [Internet]. 2009;6(1):15–20. Available from: http://journals.sagepub.com/doi/10.3132/dvdr.2009.003

15. Gachet C. P2Y12 receptors in platelets and other hematopoietic and non-hematopoietic cells. Vol. 8, Purinergic Signalling. 2012. p. 609–19.

16. Burnstock G, Novak I. Purinergic signalling and diabetes. Vol. 9, Purinergic Signalling. 2013. p. 307–24.

17. Chu SG, Becker RC, Berger PB, Bhatt DL, Eikelboom JW, Konkle B, et al. Mean platelet volume as a predictor of cardiovascular risk: A systematic review and meta-analysis. J Thromb Haemost. 2010;8(1):148–56.

18. Andrikopoulos S, Blair AR, Deluca N, Fam BC, Proietto J. Evaluating the glucose tolerance test in mice. Am J Physiol Endocrinol Metab 2008;1323–32.

19. Nkambule BB, Davison G, Ipp H. The value of flow cytometry in the measurement of platelet activation and aggregation in human immunodeficiency virus infection. Platelets. 2015;26(3):250–7.

20. Helms CC, Marvel M, Zhao W, Stahle M, Vest R, Kato GJ, et al. Mechanisms of hemolysis-associated platelet activation. J Thromb Haemost. 2013;11(12):2148–54.

21. Henry M, Davidson L, Cohen Z, McDonagh PF, Nolan PE, Ritter LS. Whole blood aggregation, coagulation, and markers of platelet activation in diet-induced diabetic C57BL/6J mice. Diabetes Res Clin Pract. 2009;84(1):11–8.

22. Ford ES, Zhao G, Li C. Pre-Diabetes and the Risk for Cardiovascular Disease. A Systematic Review of the Evidence. J Am Coll Cardiol [Internet]. 2010;55(13):1310–7. Available from: http://dx.doi.org/10.1016/j.jacc.2009.10.060

23. Vaag A, Lund SS. Non-obese patients with type 2 diabetes and prediabetic subjects: distinct phenotypes requiring special diabetes treatment and (or) prevention? Appl Physiol Nutr Metab [Internet]. 2007;32(5):912–20. Available from: http://www.nrcresearchpress.com/doi/abs/10.1139/H07-100

24. Chen CY, Lee MY, Lin K Der, Hsu WH, Lee YJ, Hsiao PJ, et al. Diabetes mellitus increases severity of thrombocytopenia in dengue-infected patients. Int J Mol Sci. 2015;16(2):3820–30.

25. Vinik AI, Erbas T, Park TS, Nolan R, Pittenger GL. Platelet dysfunction in type 2 diabetes. Diabetes Care [Internet]. 2001;24(8):1476–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11473089

26. Dogru T, Tasci I, Sonmez A, Genc H, Gok M, Yilmaz MI, et al. The plasma levels of soluble P-selectin in subjects with prediabetes. Int J Clin Pract. 2006;60(9):1048–52.

27. Léon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y1receptor-null mice. J Clin Invest. 1999;104(12):1731–7.

28. Jurk K, Kehrel BE. Platelets: Physiology and biochemistry. Semin Thromb Hemost. 2005;31(4):381–92.

29. Oury C, Toth-Zsamboki E, Vermylen J, Hoylaerts MF. The platelet ATP and ADP receptors. Curr Pharm Des. 2006;12(7):859–75.

30. Fabre JE, Nguyen M, Latour A, Keifer JA, Audoly LP, Coffman TM, et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. Nat Med [Internet]. 1999;5(10):1199–202. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10502826

31. Natarajan A, Zaman AG, Marshall SM. Platelet hyperactivity in type 2 diabetes: role of antiplatelet agents. Diabetes Vasc Dis Res [Internet]. 2008;5(2):138–44. Available from: http://journals.sagepub.com/doi/10.3132/dvdr.2008.023

32. Santilli F, Vazzana N, Liani R, Guagnano MT, Davì G. Platelet activation in obesity and metabolic syndrome [Internet]. Vol. 13, Obesity Reviews. 2012 [cited 2015 Feb 25]. p. 27–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21917110

33. Colwell JA, Nesto RW. The platelet in diabetes: focus on prevention of ischemic events. Diabetes Care. 2003;26(7):2181–8.

34. Signorello MG, Segantin A, Leoncini G. The arachidonic acid effect on platelet nitric oxide level. Biochim Biophys Acta - Mol Cell Biol Lipids [Internet]. 2009;1791(11):1084–92.

Available from: http://dx.doi.org/10.1016/j.bbalip.2009.07.003

35. Helmersson J, Vessby B, Larsson A, Basu S. Association of Type 2 Diabetes with Cyclooxygenase-Mediated Inflammation and Oxidative Stress in an Elderly Population. Circulation. 2004;109(14):1729–34.

36. Ferroni P, Basili S, Falco a, Davì G. Platelet activation in type 2 diabetes mellitus. J Thromb Haemost. 2004;2(8):1282–91.

37. Angiolillo DJ, Bernardo E, Ramírez C, Costa M a., Sabaté M, Jimenez-Quevedo P, et al. Insulin Therapy Is Associated With Platelet Dysfunction in Patients With Type 2 Diabetes Mellitus on Dual Oral Antiplatelet Treatment. J Am Coll Cardiol. 2006;48(2):298–304.

38. Jones DB, Carter RD, Haitas B, Mann JI. Low phospholipid arachidonic acid values in diabetic platelets. Br Med J (Clin Res Ed). 1983;286(6360):173–5.

39. Eibl N, Krugluger W, Streit G, Schrattbauer K, Hopmeier P, Schernthaner G. Improved metabolic control decreases platelet activation markers in patients with type-2 diabetes. Eur J Clin Invest. 2004;34(3):205–9.

40. Saboor M, Ayub Q, Ilyas S, Moinuddin. Platelet receptors: An instrumental of platelet physiology. Pakistan J Med Sci. 2013;29(3).

41. Sun Y, Scavini M, Orlando, A R, Murata G, Servilla K, Tzamaloukas A, et al. Increased CD36 Expression Signals Monocyte Activation Among Patients With Type 2 Diabetes. Diabetes Care. 2010;33(9):2065–7.

42. Alkhatatbeh MJ, Enjeti AK, Acharya S, Thorne RF, Lincz LF. The origin of circulating CD36 in type 2 diabetes. Nutr Diabetes [Internet]. 2013;3:e59-7. Available from: http://dx.doi.org/10.1038/nutd.2013.1

43. Kakouros N, Rade JJ, Kourliouros A, Resar JR. Platelet function in patients with diabetes mellitus: From a theoretical to a practical perspective. Int J Endocrinol. 2011;2011.

4.9. Bridging chapter

In pre-diabetes, platelets exhibited hyperreactivity to stimulation of the purinergic receptor, glycoprotein VI and cyclooxygenase mediated signalling pathways. The hyperreactivity of platelets in this early stage of pre-diabetes may be the major driver of atherosclerotic conditions and cardiovascular disease at later stages in the progression into overt type 2 diabetes. The occurrence of cardiovascular disease in treated type 2 diabetes has been reported and persistent platelet reactivity may be a major role player in this phenomenon. The following study investigated the effect of combinational use of metformin and low-dose dual therapy in inhibiting platelet hyperreactivity to protect against diabetes-induced vascular complications. Treatment with low-dose aspirin and clopidogrel agents was used as a comparative therapy.

Chapter 5. Research article three

Dual metformin and low-dose aspirin therapy reduces residual platelet activation in pre-diabetic mice

Short title: Metformin and low-dose aspirin therapy

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5. Abstract

Introduction

Aspirin is a common drug used in the prevention of cardiovascular disease (CVD), whereas metformin is used to improve glucose homeostasis in type 2 diabetic patients. There is currently limited evidence on the effects and benefits of combined of metformin and low-dose aspirin therapy in reducing diabetes-associated cardiovascular complications. This study investigated the platelet function in the combined metformin and low-dose aspirin therapy, in comparison we further evaluated clopidogrel a commonly used anti-platelet drug.

Methods

Pre-diabetic mice were randomized into different treatment groups; combination therapy of metformin (150mg/kg) and low-dose aspirin (3mg/kg) (n=5), low-dose aspirin (3mg/kg) (n=5) or clopidogrel (0.25mg/kg) (n=5) monotherapy. Baseline measurements were taken before treatment and 4 weeks after treatment, the body weights and insulin levels were again measured. Platelet function was assessed using varying concentrations of ADP (4µM and 20µM), collagen (0.19mg/mL) and arachidonic acid (500µg/mL).

Results

The animal body weights were comparable across the groups ($p>0.05$). The dual-treatment with metformin and low-dose aspirin inhibited platelet activation post stimulation with 4µM ADP ($p=0.0220$). Interestingly, the low-dose aspirin-treated group showed no significant difference in the residual levels of platelet activation post stimulation with both ADP concentrations ($p>0.05$). Clopidogrel treatment inhibited the response to collagen stimulation ($p=0.0270$).

Discussion

Our study showed varied platelet response to agonist-induced activation in the pre-diabetic group despite treatment. This highlights the involvement of different signalling pathways in platelet activation and the potential benefit of dual-therapy in pre-diabetes. The synergistic use of metformin and low-dose aspirin led to greater inhibition of platelet activation compared to low-dose aspirin monotherapy treatment.

Keywords: Pre-diabetes; platelet reactivity; dual-therapy

5.1. Introduction

People living with type 2 diabetes mellitus (T2DM) are at a higher risk of developing cardiovascular disease compared to nondiabetic individuals (1). There is no specific treatment currently available to protect diabetic individuals at risk of cardiovascular complications (2,3). However, conventional therapies that target both T2DM and cardiovascular disease are used to prolong the lives of diabetic patients. In fact, low-dose aspirin is used in the primary prevention of cardiovascular complications and subclinical atherosclerosis in patients with T2DM, who may be at risk of developing vascular events (4). While the use of low-dose aspirin is recommended in the prevention of cardiovascular events (4,5), cyclooxygenase (COX) and $P2Y_{12}$ receptor inhibitors such as clopidogrel are the commonly used anti-platelet drugs to prevent such complications (6,7). Alarmingly, a sub-optimal response to low-dose aspirin and clopidogrel treatments has been reported in T2DM patients with coronary artery disease (8–10). Residual platelet activation persists in type 2 diabetics who are on low-dose aspirin (11).

Interestingly, the use of a combination of metformin and low-dose aspirin can ameliorate diabetes-associated complications, involving lipogenesis and hepatic glucose production, leading to improved insulin sensitivity (12,13). Metformin acts on the liver and the gut to lower glucose production and enhance glucose utilization (14). Furthermore, metformin lowers the cardiovascular mortality rate in diabetic patients (15). The use of metformin in combination with low-aspirin reduces the levels of urinary thromboxane A2 metabolite (11-dehydrothromboxane B_2) which is directly associated with the levels of sCD40L, a platelet activation marker (13,16).

Clopidogrel acts on blood platelets by inhibiting the $P2Y_{12}$ receptor, thus preventing the binding of adenosine diphosphate (ADP) and consequently inhibit platelet activation (17–19). This process inhibits the function of platelet-monocyte aggregates (PMAs), which are associated with cardiovascular complications in T2DM associated (17–19). The therapeutic benefits of clopidogrel in people living with T2DM include a reduction in the relative risk of developing myocardial infarcts and strokes (20).

Although the benefits of dual metformin and low-dose aspirin therapy include, reduced in platelet hyperreactivity and chronic inflammation (16), high on-treatment platelet reactivity (HTPR) has been associated with thrombotic complications in T2DM patients on combined aspirin and clopidogrel treatment (21). There is minimal data available on combinational effects of metformin and low dose aspirin in protecting against diabetes-induced cardiovascular complications. In this study, we assessed platelet activation and reactivity in pre-diabetes following combined metformin and low-dose aspirin treatment, as well as clopidogrel monotherapy.

5.2. Methodology

5.2.1. Animal husbandry

A total of 20 C57BL/6 male mice were purchased from the University of KwaZulu-Natal (UKZN) biomedical research unit (BRU). The mice were housed in a controlled environment with a twelve-hour light/dark cycle (lights were switched on at 6:00 AM and switched off at 6:00 PM) and a temperature range of 23-25ºC (relative humidity: ~50%). All study procedures and animal handling were performed according to the principles of laboratory animal Care of the National Society of Medical Research and the National Institutes of Animal Care and Use of Laboratory Animals of the National Academy of Sciences (National Institute of Health publication 80-23, revised 1978). Ethical clearance for the study was obtained from the UKZN animal research ethics committee (AREC), ethics registration number AREC/086/016.

5.2.2. Induction of pre-diabetes and experimental design

The control group (n=5) was fed on a low-fat diet containing 10% Kcal fat (Research diet no. D12450J, Research Diets, New Brunswick, NJ, USA). While the high-fat diet experimental groups (n=15/group) were kept on a high-fat diet containing 60% Kcal fat (Research diet no. D12492, Research Diets, New Brunswick, NJ, USA).

5.2.3. Treatment randomization and oral glucose tolerance testing

The high-fat diet (HFD) fed mice (n=15) were then randomized into 3 treatment groups (n=5/group); metformin and low-dose aspirin (150mg/kg metformin + 3mg/kg aspirin); low-dose aspirin (3mg/kg aspirin), and clopidogrel (0.25mg/kg). All treatment drugs were administered daily via oral gavage, once a day daily for 4 weeks. Glucose tolerance was assessed in both control and the HFD-fed mice as previously described (22). Briefly, using oral gavage method, 2g/kg of glucose was administered, and blood glucose levels were measured at varying time intervals (30 minutes, 60 minutes, and 120 minutes) using the Accu-Check active blood glucometer (Roche, Basel, Switzerland).

5.2.4. Blood collection and measurement of baseline haematological parameters

A total of 200µl of venous blood was collected from the lateral tail vein into 3.2% sodium citrate coated microtainer tubes (Sigma Aldrich, St Louis, Missouri, USA). Baseline haematological parameters which included the platelet counts, mean platelet volume, were measured before treatment using the Beckman Coulter AcT diff^{TM} analyser (Beckman Coulter, Brea, CA, USA).

5.2.5. Platelet function and reactivity measurements

5.2.6. Measurements of baseline platelet activation and reactivity

Twenty-five microliters of blood was stained with an antibody cocktail containing CD41-FITC (1:10; clone MWReg30), CD62P-BV421 (1:10; clone RB40.34), and CD36-PE (1:10; clone CRF D-2712) (BD Bioscience, New Jersey, USA. In order to avoid the release of ADP and subsequent artefactual

activation of platelets, the red blood cells were not lysed (23). The samples were then incubated for 10 minutes at room temperature before the samples were suspended in 350µl of PBS. Samples were then immediately analyzed on the BD FACS Canto II flow cytometer (BD Bioscience, NJ, USA).

5.2.7. Assessment of platelet function

Two varying concentrations of ADP were used to assess platelet purinergic receptor-mediated platelet activation. These concentrations are known to induce reversible (4µM) and irreversible (20µM) platelet activation (24). Collagen (0.19mg/mL) and arachidonic acid (500µg/mL) (Bio-data co-operation, Pennsylvania, USA) were used to stimulate irreversible platelet activation via the glycoprotein VI (GPVI) receptor and COX signalling pathway respectively (25). The platelet reactivity index (PRI) for CD62P and CD36 was calculated as follows:

$$\text{PRI}= \left(\frac{\text{(\%levels of CD62P/CD36 post stimulation)-(Basal \% levels of CD62P/CD36)}}{\text{(\%levels of CD62P/CD36 post stimulation)}}\right).$$

5.2.8. Statistical analysis

All statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA). Parametric data were analysed using the repeated measures ANOVA and Bonferroni's Multiple Comparison Test post hoc test. Non-parametric data were analysed using the Kruskal-Wallis test and Dunns post-test. Parametric data were reported as the mean ± standard deviation (SD), and non-parametric data reported as the median and interquartile range (IQR)

5.3. Results

5.3.1. Establishment of a pre-diabetic model

The oral glucose tolerance test (OGTT) showed that the HFD group had a higher postprandial blood glucose levels compared to the control group. This was also illustrated by the higher area under the curve (AUC) observed in the HFD group indicating delayed blood glucose clearance in the HFD pre-diabetic group (p=0.0362). There were no differences in the insulin levels and body weights between the treatment groups (p=0.0675) (Table 5.1).

Table 5.1. Baseline characteristics of pre-diabetic mice and controls

Parameter	Control (n=5)	High-fat diet group	Asp (n=5)	Met + Asp (n=5)	Clo (n=5)	p-value
Body weight (g)	30.0[23.5-31.0]	27.07± 1.49	26.0[26.0-27.0]	30.0[30.0-31.5]	28.0[27.3-29.5]	0.0675
Insulin µIU/ml	16.16[15.43-22.71]	16.43 [14.36-22,46]	23.96[16.11-24.61]	16.65[16.21-17.97]	13.73[13.68-13.78]	0.2667
White cell count (10^3/µl)	6.1[5.1-7.7]	8.471±2.84	8.3[7.7-8.9]	6.7[6.2-9.4]	7.6[4.9-11.1]	0.4269
Red blood cell count (10^6/µl)	7.1[6.1-7.5]	6.24±1.21	7.6[6.9-8.1]	7.6[7.2-7.8]	7.5[6.9-7.8]	0.3597
Mean cell volume (fL)	42.0[41.5-43.0]	5.39± 0.21	41.0[40.5-42.0]	42.0[42.0-43.0]	40.5[39.3-42.5]	0.1049
Haematocrit (%)	30.3[25.7-32.2]	29.00[23.00-31.40]	31.0[28.8-33.3]	31.9[29.9-33.4]	31.0[27.2-32.2]	0.5820
Red blood cell distribution width (%)	10.6[10.4-11.2]	11.05[10.63- 11.88]	11.3[10.8-11.6]	10.4[10.3-12.0]	11.7[10.9-13.4]	0.2821
Neutrophil (%)	13.5[8.6-16.2]	8.00[6.90-9.30]	8.6[6.4-9.3]	11.3[8.6-16.8]	7.4[6.1-8.9]	**0.0363**
Lymphocyte (%)	82.5[79.1-88.8]	89.20[87.80-90.50]	87.2[86.5-89.7]	82.6[77.4-87.0]	87.7[85.8-88.5]	0.1131
Monocyte (%)	3.7[2.4-4.6]	3.95[3.48-5.13]	3.8[3.2-4.4]	4.1[3.5-5.9]	4.9[3.1-6.9]	0.4012

Basophil (%)	0.2[0.1-0.3]	0.20[0.10-0.80]	0.2[0.2-0.3]	0.2[0.2-0.3]	0.3[0.2-0.4]	0.3808
Platelet count (10^3/µl)	803.0[669.5-988.5]	771.8± 147.8	956.0[850.5-1027]	805.0[672.5-999.0]	913.0[752.5-1013]	0.6104
Mean platelet volume (fL)	5.4[5.1-5.4]	5.39± 0.21	5.2[5.1-5.2]	5.2[5.2-5.4]	5.4[5.2-5.5]	0.2427
%CD62P	91.87 [58.34- 94.76]	82.69[66.17- 93.15]	84.89[68.53-92.19]	93.40[84.99-95.11]	59.10[49.07-76.98]	0.1419
CD62P MFI	105.2 [105.1- 111.1]	105.3 [105.0- 106.0]	105.5[105.3-112.5]	105.3[105.0-106.1]	105.0[105.0-105.2]	0.1390
%CD36	35.70[25.53-48.37]	25.95[21.71-32.91]	26.98[25.55-50.51]	28.04[24.06-34.84]	19.20[17.29-22.08]	0.0513
CD36 MFI	134.8[121.9-172.3]	199.4[172.7-213.6]	198.7[175.1-201.6]	221.1[165.1-240.1]	191.4[175.4-209.4]	0.0580

$p<0.05$ represented in boldface (control vs treatment groups); values represented as median [IQR] or mean±SD; Met + Asp: metformin and low-dose aspirin dual therapy; MFI: Median fluorescence intensity; Clo: Clopidogrel; MFI: median fluorescence intensity.

5.3.2. P2Y$_{12}$ receptor responses in HFD-fed mice following metformin and low-dose aspirin treatment

In order to assess the P2Y$_{12}$ receptor-mediated response, the levels of P-selectin (%CD62P) were measured post stimulation with 4µM and 20µM of ADP. In the metformin and low-dose aspirin-treated group, the platelet P2Y$_{12}$ receptor-mediated %CD62P expression was only inhibited at 4µM of ADP (p=0.0220). Residual platelet activation was observed at the concentration of 20µM ADP (p=0.0535) (Table 5.2). Whereas the levels of %CD36 expression were only inhibited at a concentration of 20µM ADP (p=0.0002). Furthermore, the glycoprotein GPVI- mediated responses were also inhibited following the 4-week metformin and low-dose aspirin treatment (p=0.0329).

5.3.3. P2Y$_{12}$ receptor responses in HFD-fed mice following on low-dose aspirin treatment

In the low-dose aspirin group, there were no significant variations in the %CD62P and %CD36 expression following stimulation with ADP (p>0.05). Collagen was used to stimulate platelet GPVI receptor-mediated platelet activation. No significant difference in the %CD62P expression from the low-dose aspirin-treated post stimulation with collagen compared to pre-treatment levels (p=0.1396).

5.3.4. P2Y$_{12}$ receptor responses in HFD-fed mice following clopidogrel treatment

Clopidogrel treatment only inhibited the platelet response to 20µM ADP (p=0.0313) (Table 5.2). Variations in P2Y$_{12}$ receptor-mediated platelet responses across the treatment groups were assessed and the %CD62P platelet reactivity index (%CD62P PRI) was compared across the metformin and low-dose aspirin group. A higher %CD62P PRI was observed in the metformin and low-dose aspirin group compared to the low-dose aspirin-treated group, post stimulation with 20µM ADP (p=0.0001) (Figure 5.1).

Table 5.2. %CD62P platelet response in low-dose aspirin, metformin and low-dose aspirin, clopidogrel-treated pre-diabetic mice

	CD62P platelet reactivity index (PRI)					
Agonist	**Pre-treatment**	**Low-dose aspirin**	**Pre-treatment**	**Met + Asp**	**Pre-treatment**	**Clopidogrel**
4µM ADP	2.388[-0.8050-3.490]	-0.6911[-0.7449--0.6272]	0.8182[0.1007-3.531]	-0.7343[-0.7716--0.6956] [a]	7.289[2.561-9.655]	-0.6669[-0.7396--0.6471]
20µM ADP	1.541[-0.8182-4.117]	-0.9516[-0.9633--0.9390]	2.529[0.1007-4.897]	-0.7400[-0.7675--0.7266]	5.974[2.909-6.310]	-0.6821[-0.7452--0.6089] [a]
Collagen	5.987[-0.5918-12.48]	-0.6390[-0.6957--0.5968]	14.64[5.416-28.47]	-0.7029[-0.7593--0.6345] [a]	14.61[11.00-20.21]	-0.8860[-0.9128--0.7689] [a]
Arachidonic acid	2.823[0.3519-0.3519]	-0.9913[-0.9945--0.9639] [a]	11.33[5.178-16.77]	-0.9953[-0.9966--0.9466] [a]	8.310[1.879-23.26]	-0.9712[-0.9915--0.8765]

a: $p<0.05$ pre-treatment vs treated groups (low-dose aspirin, Met + Asp and Clopidogrel). Values represented as median [IQR]; Met + Asp: metformin and low-dose aspirin dual therapy; ADP: adenosine diphosphate.

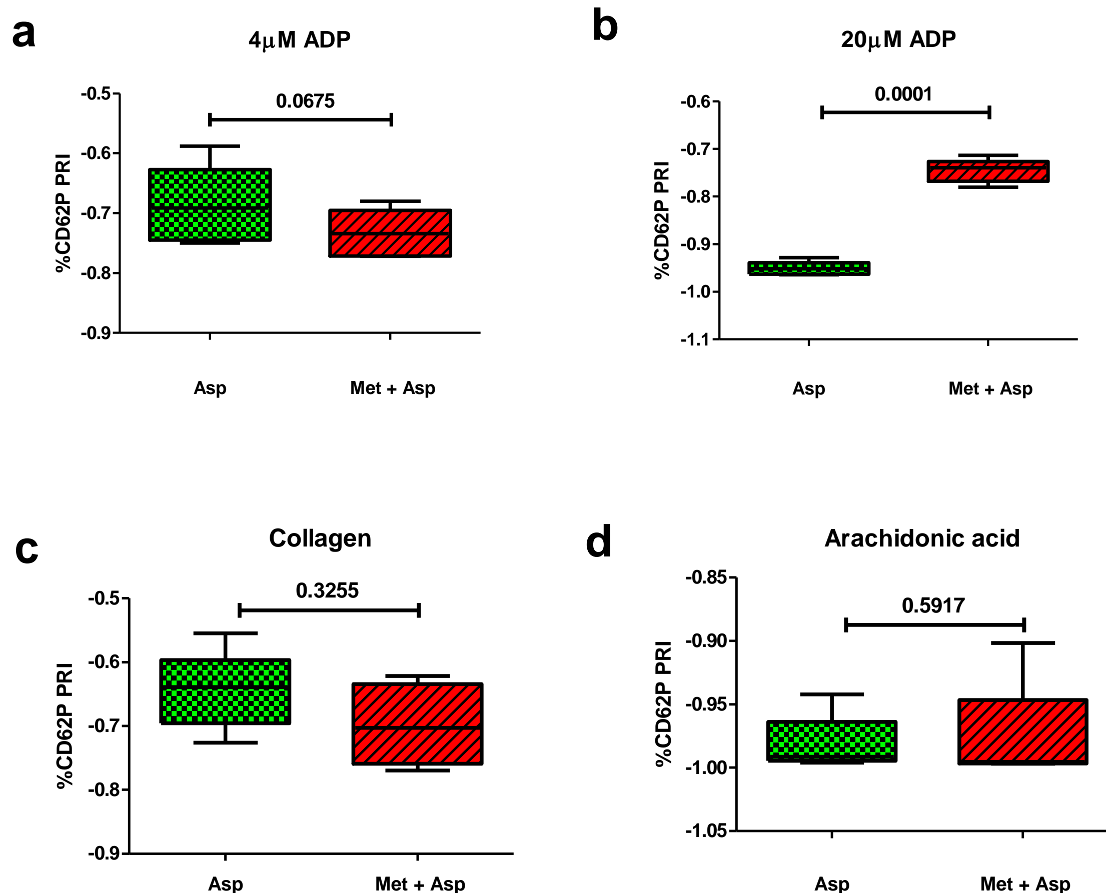

Figure 5.1. Platelet reactivity index in low-dose aspirin (monotherapy) and dual therapy (Metformin + low-dose aspirin). (A) Post-stimulation with 4μM adenosine diphosphate (ADP), there were no significant differences in the platelet reactivity between low-dose aspirin and dual metformin + low-dose aspirin-treated group. (B) Low-dose aspirin significantly lowered the platelet reactivity index compared to dual metformin + low-dose aspirin therapy at post stimulation with 20μM ADP (p=0.0001). There were no significant differences between the two therapies post stimulation with collagen and arachidonic acid (p>0.05), respectively (C) and (D).

5.3.5. Glycoprotein VI mediated responses in HFD-fed mice following low-dose aspirin and clopidogrel treatment

Clopidogrel treatment, similar to the other therapies, inhibited the response to collagen stimulation (p=0.0270) (Table 5.2). Clopidogrel inhibited the platelet response to both 4μM (p=0.0165) and 20μM ADP (p=0.0108) (Table 5.3). Clopidogrel did not significantly inhibit the %CD36 expression (p=0.1949). Low-dose aspirin significantly inhibited %CD36 PRI post stimulation with collagen compared to combined therapy (p=0.0416) (Figure 5.2).

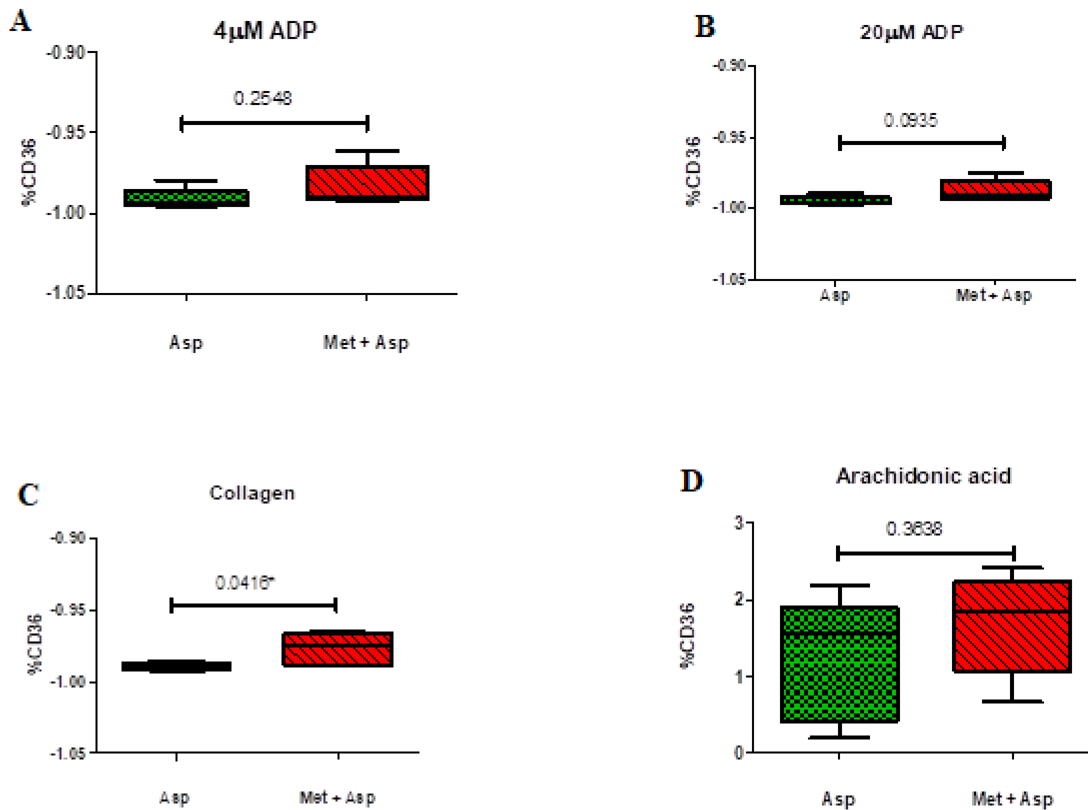

Figure 5.2. Effects of monotherapy and dual therapy platelet on reactivity. (A) and (B) Post stimulation with 4µM and 20 µM ADP, there was no significant difference between monotherapy and dual therapy treated group. (C) There was a significant decrease in reactivity in the low-dose aspirin monotherapy group compared to the metformin and low-dose aspirin dual therapy treated group. (D) There were no significant differences between the two therapies post stimulation with arachidonic acid. *p<0.05; ADP: adenosine diphosphate, Asp: low-dose aspirin monotherapy; Met + Asp: metformin and low-dose aspirin dual therapy.

5.3.6. Cyclooxygenase pathway response

In the low-dose aspirin group, no significant inhibition of %CD36 expression was observed post stimulation with arachidonic acid (p=0.2509). Interestingly, in the metformin and low-dose aspirin group, the %CD36 PRI post stimulation with arachidonic acid was significantly lower compared to baseline (p<0.0001). Similar findings were observed in the clopidogrel group where %CD36 PRI post stimulation with arachidonic acid was lower than baseline pretreatment PRI (p=0.0108) (Table 5.3). In the low-dose aspirin group, the COX-dependent platelet activation was also inhibited as shown by the reduced %CD62P expression (p=0.0106) (table 5.2), whereas the levels of %CD36 were not affected (p=0.2509) (table 5.3).

Table 5.3: %CD36 platelet reactivity in response to agonist stimulation

Agonist	Baseline Pre-treatment	Low-dose aspirin	p-value
4µM ADP	-0.2739[-0.5367-2.337]	-0.9937[-0.9956--0.9858]	0.1956
20µM ADP	-0.2659[-0.4537-2.216]	-0.9957[-0.9969--0.9925]	0.1767
Collagen	0.2442 [-0.3304-2.931]	-0.9889[-0.9908--0.9871]	0.1387
Arachidonic acid	5.976[4.635-33.01]	1.570[0.4057-1.893]	0.2509
	Pre-treatment	**Met + Asp**	
4µM ADP	-0.1985[-0.2835--0.9970]	-0.9904[-0.9915--0.9715]	0.0579
20µM ADP	0.02397[-0.1375-0.1628]	-0.9903[-0.9937--0.9806]	**0.0002**
Collagen	0.4084[0.3092-0.5472]	-0.9748[-0.9881--0.9662]	**P<0.0001**
Arachidonic acid	8.920[7.973-9.555]	1.848[1.061-2.227]	**P<0.0001**
	Pre-treatment	**Clopidogrel**	
4µM ADP	-0.3844[-0.3936--0.04524]	-0.9893[-0.9906--0.9881]	**0.0251**
20µM ADP	-0.2498[-0.3202--0.1291]	-0.9859[-0.9912--0.9832]	**0.0053**
Collagen	0.4724[0.06032-0.6927]	-0.9859[-0.9898--0.9771]	**0.0165**
Arachidonic acid	7.670[6.951-7.776]	1.323[0.9795-2.118]	**0.0108**

p<0.05 represented in boldface; values represented as median [IQR]; Met + Asp: metformin and low-dose aspirin dual therapy; ADP: adenosine diphosphate.

5.4. Discussion

The aim of the study was to investigate the effects of dual of metformin and low-dose aspirin therapy in modulating platelet function and reactivity in pre-diabetic mice. High-fat diet-induced pre-diabetes is used to study diabetes-associated complications (26). Previous studies have shown that the C57BL/6 animal strain demonstrated thrombopoiesis mechanisms and antigen localization is analogous to humans (27). Although there was no significant difference in insulin levels or body weights, in our study the HFD-fed mice presented with delayed glucose clearance with no weight gain after 4-weeks on an HFD which is similar to findings by Toye et. al (28). Most importantly, the pre-diabetic mice showed abnormal platelet reactivity and function, an important pathophysiological sign in the development of thrombotic complications in pre-diabetes (29). Interestingly, treatment with dual therapy was effective in improving platelet function in our model. We highlight the synergistic function of metformin and aspirin in inhibiting platelet activation. Metformin improves the inhibitory function

of aspirin by lowering the platelet reactivity during dual therapy, which may prompt further investigation into the metformin mechanism of action on platelet function.

Currently, various thrombo-inflammatory molecules that can be modulated by some drug targets such as P-selectin are continually investigated for their potential role platelet physiology (30). P-selectin, which is localized in the α-granules, is translocated to the surface of activated platelets where plays a role in stabilizing platelet aggregates as well as their interaction with leukocytes (31,32). However, low-dose aspirin had no effect on the expression of CD36 (glycoprotein IV) indicating possible platelet activation mediated by COX-independent pathways in pre-diabetes. Activation of the COX-pathways is dependent on an increase in calcium levels. This is a consequence of the binding of ADP, collagen and TXA_2 to their respective receptors (33). Cyclooxygenase oxidises arachidonic acid converting it into prostaglandins G_2 and H_2 leading to TxA_2 production. This leads to the activation of platelet phospholipase C by the binding of TxA_2 to the TxA_2 receptor (TP) (34–36). We assume irreversible acetylation of cyclooxygenase by low-dose aspirin inhibits this process of conversion, resulting in decreased platelet activation and consequently decreased hypercoagulability (37,38). However, aspirin resistance has been reported in 41.9% of patients with T2DM (10). The failure of aspirin to inhibit platelet TxA_2 production and other thromboembolic ischemic events is common in T2DM and may be attributed to high platelet turnover, as well as glycation and acetylation of platelet membrane proteins (39–41). In this current study, the purinergic receptor and GPVI mediated pathways were not inhibited in the low-dose aspirin group, which may result in high on-treatment platelet reactivity (HTPR). Similar findings were reported by Duzenli et. al (18), who demonstrated in a cohort of patients with T2DM that low-dose aspirin did not adequately inhibit ADP and collagen-induced platelet activation (18). However, the use of combination therapy consisting of low-dose aspirin and a $P2Y_{12}$ receptor antagonist such as clopidogrel could offer partial and variable inhibition (42). This suggests that the combination of a well-known antidiabetic drug such as metformin could also reduce cardiovascular complications in people living with T2DM, although additional investigations are needed to establish the mechanisms involved (43).

In our study, the use of metformin and low-dose aspirin as a dual therapy significantly reduced platelet activation and the exocytosis of P-selectin from the alpha granule, by altering the $P2Y_{12}$ receptor, GPVI and the cyclooxygenase-dependent pathways. Furthermore, the use of metformin in combination with low-dose aspirin further inhibited the expression of CD36, a receptor that is known to be increased in diabetic and insulin-resistant patients. A study by Handberg et.al (44), showed an increased level of soluble CD36 in overt T2DM. Metformin, on the other hand, has been shown to enhance the effect of aspirin mediated cyclooxygenase inhibition by decreasing the levels of 11-dehydrothromboxane B_2 urinary excretion (13). This is agreement with a previous study by Formoso et. al (45), demonstrating reduced levels of urinary 11-dehydrothromboxane B_2 in metformin-treated T2DM patients. In addition, metformin inhibits platelet activation, aggregation and decreases the mean platelet volume which is

associated with vascular complications in T2DM (46). Although our results support the use of metformin in combination a low-dose aspirin, the direct impact of this antidiabetic drug on platelets still remains unclear. However, it is suggested that metformin may inhibit platelet mitochondrial DNA release in platelets activated with either ADP, arachidonic acid or thrombin, leading to platelet aggregation (43). In our study, we observed reduced levels of platelet degranulation and the exocytosis of P-selectin in the response to post-treatment with metformin and low-dose aspirin.

We further assessed platelet function following clopidogrel treatment, which is a known antagonist of the $P2Y_{12}$ receptor that is activated in the liver by cytochrome P450 34A (17,19,47). The $P2Y_{12}$ receptor antagonist reduced platelet degranulation and the exocytosis of P-selectin, leading to inhibition of both GPVI and $P2Y_{12}$ pathways. However, residual platelet activation in response to activation of the cyclooxygenase pathway by arachidonic acid was observed. In addition, a variable dose-dependent response to ADP in the clopidogrel group was also observed, which may be due to the variability in the platelet response to endogenous agonists following clopidogrel treatment (17,48). An accelerated platelet turnover or single nucleotide polymorphism (SNPs) in the $P2Y_1$ and $P2Y_{12}$ receptors, as well as variations in surface density of the receptors, may be responsible for the observed variability (17,49). This was a limitation to our study, however, clopidogrel dose used in this study significantly reduced the expression of CD36 which may indicate inhibition of platelet shape change caused by increased intracellular calcium levels (50).

Platelet activation is orchestrated by multiple pathways that converge and result in morphological changes, degranulation and aggregation (51,52). In T2DM, there is increased platelet reactivity in response to endogenous agonists such as ADP, arachidonic acid and collagen (9). The complex nature of platelet signalling creates for a challenge in tailoring patient treatment options. Residual platelet activation during aspirin and clopidogrel treatment at the pre-diabetic stage may be useful in the thrombotic risk stratification of pre-diabetic individuals. In addition, resistance to both anti-platelet therapies has been reported in T2DM (17,49). The therapeutic targeting of these pathways plays a pivotal cardioprotective role and may reduce the risk of thrombotic complications (53). In our study, the combination therapy consisting of metformin and low-dose aspirin inhibited residual platelet activation in pre-diabetic mice. The high on-treatment platelet reactivity highlights the involvement of complex platelet signalling pathways and may suggest the importance of dual-therapy approach in preventing thrombotic complications in pre-diabetes.

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5.8. Disclosure statement

The authors declare no competing financial interests to disclose.

5.9. References

1. Leon BM. Diabetes and cardiovascular disease: Epidemiology, biological mechanisms, treatment recommendations and future research. World J Diabetes [Internet]. 2015;6(13):1246. Available from: http://www.wjgnet.com/1948-9358/full/v6/i13/1246.htm

2. Szuszkiewicz-Garcia MM, Davidson JA. Cardiovascular disease in diabetes mellitus: Risk factors and medical therapy. Endocrinol Metab Clin North Am [Internet]. 2014;43(1):25–40. Available from: http://dx.doi.org/10.1016/j.ecl.2013.09.001

3. Papazafiropoulou AK, Melidonis A. Diabetes drugs and cardiovascular safety. Arch Hell Med. 2017;34(3):321–6.

4. Udell JA, Scirica BM, Braunwald E, Raz I, Gabriel Steg P, Davidson J, et al. Statin and aspirin therapy for the prevention of cardiovascular events in patients with type 2 diabetes mellitus. Clin Cardiol. 2012;35(12):722–9.

5. Li Z, Yang F, Dunn S, Gross AK, Smyth SS. Platelets as immune mediators: Their role in host defense responses and sepsis [Internet]. Vol. 127, Thrombosis Research. Elsevier B.V.; 2011. p. 184–8. Available from: http://dx.doi.org/10.1016/j.thromres.2010.10.010

6. Wang Y, Li Z, Wang W. Platelet-Leukocyte Interaction in Atherosclerosis and Atherothrombosis : What We Have Learnt From Human Studies and Animal Models. J Cardiol Ther [Internet]. 2014;1(5):92–7. Available from: http://ghrnet.org/index.php/jct/article/view/710

7. Schrottmaier WC, Kral JB, Badrnya S, Assinger A. Aspirin and P2Y12 Inhibitors in platelet-mediated activation of neutrophils and monocytes. Thromb Haemost. 2015;114(3):478–89.

8. Evangelista V, De Berardis G, Totani L, Avanzini F, Giorda CB, Brero L, et al. Persistent

platelet activation in patients with type 2 diabetes treated with low doses of aspirin. J Thromb Haemost. 2007;5(11):2197–203.

9. Natarajan A, Zaman AG, Marshall SM. Platelet hyperactivity in type 2 diabetes: role of antiplatelet agents. Diabetes Vasc Dis Res [Internet]. 2008;5(2):138–44. Available from: http://journals.sagepub.com/doi/10.3132/dvdr.2008.023

10. Tasdemir E, Toptas T, Demir C, Esen R, Atmaca M. Aspirin resistance in patients with type II diabetes mellitus. Ups J Med Sci. 2014;119(1):25–31.

11. Lemkes B, Bähler L, Kamphuisen PW, Stroobants a K, Van Den Dool EJ, Hoekstra JB, et al. The influence of aspirin dose and glycemic control on platelet inhibition in patients with type 2 diabetes mellitus. J Thromb Haemost [Internet]. 2012;10(4):639–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22252020

12. Ford RJ, Fullerton MD, Pinkosky SL, Day EA, Scott JW, Oakhill JS, et al. Metformin and salicylate synergistically activate liver AMPK, inhibit lipogenesis and improve insulin sensitivity. Pediatr Emerg Care. 2010;26(2):952–62.

13. Gonçalves LH, Silva MVF, Duarte RCF, Dusse LMS, Fernandes AP, Bosco AA, et al. Acetylsalicylic acid therapy: Influence of metformin use and other variables on urinary 11-dehydrothromboxane B2 levels. Clin Chim Acta [Internet]. 2014;429:76–8. Available from: http://dx.doi.org/10.1016/j.cca.2013.11.028

14. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. Diabetologia. 2017;60(9):1577–85.

15. MACDONALD MR, EURICH DT, R.MAJUMDAR S, LEWSEY JD, BHAGRA S, JHUND PS, et al. Treatment of Type 2 Diabetes and Outcomes in Patients With Heart Failure: A Nested Case–Control Study From the U.K. General Practice Research Database. Cardiovasc Res. 2010;33(6).

16. Schöndorf T, Musholt PB, Hohberg C, Forst T, Lehmann U, Fuchs W, et al. The fixed combination of pioglitazone and metformin improves biomarkers of platelet function and chronic inflammation in type 2 diabetes patients: results from the PIOfix study. J Diabetes Sci Technol [Internet]. 2011;5(2):426–32. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3125938&tool=pmcentrez&rendertype=abstract

17. Michelson AD, Linden MD, Furman MI, Li Y, Barnard MR, Fox ML, et al. Evidence that pre-existent variability in platelet response to ADP accounts for "clopidogrel resistance." J Thromb Haemost. 2007;5(1):75–81.

18. Duzenli MA, Ozdemir K, Aygul N, Soylu A, Tokac M. Comparison of Increased Aspirin Dose Versus Combined Aspirin Plus Clopidogrel Therapy in Patients With Diabetes Mellitus and Coronary Heart Disease and Impaired Antiplatelet Response to Low-Dose Aspirin. Am J Cardiol. 2008;102(4):396–400.

19. Diehl P, Olivier C, Haischeid C, Helbing T, Bode C, Moser M. Clopidogrel affects leukocyte dependent platelet aggregation by P2Y 12 expressing leukocytes. Basic Res Cardiol [Internet]. 2010 May [cited 2015 Feb 25];105(3):379–87. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19943165

20. Mathewkutty S, Mcguire DK. Platelet perturbations in diabetes : implications for cardiovascular disease risk and treatment. 2009;541–50.

21. Angiolillo DJ, Bernardo E, Sabat?? M, Jimenez-Quevedo P, Costa MA, Palazuelos J, et al. Impact of Platelet Reactivity on Cardiovascular Outcomes in Patients With Type 2 Diabetes Mellitus and Coronary Artery Disease. J Am Coll Cardiol. 2007;50(16):1541–7.

22. Andrikopoulos S, Blair AR, Deluca N, Fam BC, Proietto J. Evaluating the glucose tolerance test in mice. 2008;1323–32.

23. Helms CC, Marvel M, Zhao W, Stahle M, Vest R, Kato GJ, et al. Mechanisms of hemolysis-associated platelet activation. J Thromb Haemost. 2013;11(12):2148–54.

24. Cosemans JMEM, Angelillo-Scherrer A, Mattheij NJA, Heemskerk JWM. The effects of arterial flow on platelet activation, thrombus growth, and stabilization [Internet]. Vol. 99, Cardiovascular Research. 2013 [cited 2015 Feb 25]. p. 342–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23667186

25. Nkambule BB, Davison G, Ipp H. The value of flow cytometry in the measurement of platelet activation and aggregation in human immunodeficiency virus infection. Platelets. 2015;26(3):250–7.

26. King AJF. The use of animal models in diabetes research. Br J Pharmacol. 2012;166(3):877–94.

27. Schmitt A, Guichard J, Massé J, Debili N, Cramer EM. Of mice and men : Comparison of the ultrastructure of megakaryocytes and platelets. 2001;29:1295–302.

28. Toye AA, Lippiat JD, Proks P, Shimomura K, Bentley L, Hugill A, et al. A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice. Diabetologia. 2005;48(4):675–86.

29. Kakouros N, Rade JJ, Kourliouros A, Resar JR. Platelet function in patients with diabetes mellitus: From a theoretical to a practical perspective. Int J Endocrinol. 2011;2011.

30. Théorêt JF, Yacoub D, Hachem A, Gillis MA, Merhi Y. P-selectin ligation induces platelet activation and enhances microaggregate and thrombus formation. Thromb Res [Internet]. 2011;128(3):243–50. Available from: http://dx.doi.org/10.1016/j.thromres.2011.04.018

31. Merten M, Thiagarajan P. P-selectin expression on platelets determines size and stability of platelet aggregates. Circulation. 2000;102(16):1931–6.

32. Polgar J, Matuskova J, Wagner DD. The P-selectin, tissue factor, coagulation triad. J Thromb Haemost. 2005;3(8):1590–6.

33. Xiang YZ, Xia Y, Gao XM, Shang HC, Kang LY, Zhang BL. Platelet activation, and antiplatelet targets and agents: Current and novel strategies. Drugs. 2008;68(12):1647–64.

34. Offermanns S. Activation of platelet function through G protein-coupled receptors. Circ Res. 2006;99(12):1293–304.

35. Korbecki J, Baranowska-Bosiacka I, Gutowska I, Chlubek D. Cyclooxygenase pathways. Acta Biochim Pol. 2014;61(4):639–49.

36. Khan N, Farooq AD, Sadek B. Investigation of cyclooxygenase and signaling pathways involved in human platelet aggregation mediated by synergistic interaction of various agonists. Drug Des Devel Ther. 2015;9(Figure 1):3497–509.

37. Smith JP, Haddad E V., Taylor MB, Oram D, Blakemore D, Chen Q, et al. Suboptimal inhibition of platelet cyclooxygenase-1 by aspirin in metabolic syndrome. Hypertension. 2012;59(3):719–25.

38. Li R, Diamond SL. Detection of platelet sensitivity to inhibitors of COX-1, P2Y1, and P2Y12 using a whole blood microfluidic flow assay. Thromb Res. 2014;133(2):203–10.

39. Hankey GJ, Eikelboom JW. Aspirin resistance. Lancet. 2006;367(9510):606–17.

40. Floyd CN, Ferro A. Mechanisms of aspirin resistance. Pharmacol Ther [Internet]. 2014;141(1):69–78. Available from: http://dx.doi.org/10.1016/j.pharmthera.2013.08.005

41. Kimura Y, Takano K, Satoh K, Aida K, Kobayashi T, Ozaki Y. Aspirin half maximal inhibitory concentration value on platelet cyclooxygenase1 in severe type-2 diabetes mellitus is not significantly different from that of healthy individuals. Clin Appl Thromb. 2014;20(6):629–36.

42. Warner TD, Nylander S, Whatling C. Anti-platelet therapy: Cyclo-oxygenase inhibition and the use of aspirin with particular regard to dual anti-platelet therapy. Br J Clin Pharmacol. 2011;72(4):619–33.

43. Huang W, Xin G, Wei Z, Ji C, Zheng H, Gu J, et al. Metformin Uniquely Prevents Thrombosis

by Inhibiting Platelet Activation and mtDNA Release. Sci Rep. 2016;6(July):1–12.

44. Handberg A, Norberg M, Stenlund H, Hallmans G, Attermann J, Eriksson JW. Soluble CD36 (sCD36) clusters with markers of insulin resistance, and high sCD36 is associated with increased type 2 diabetes risk. J Clin Endocrinol Metab. 2010;95(4):1939–45.

45. Formoso G, De Filippis E, Di Fulvio P, Pandolfi A, Bucciarelli T, Ciabattoni G, et al. Decreased in vivo oxidative stress and decreased platelet activation following metformin treatment in newly diagnosed type 2 diabetic subjects. Diabetes Metab Res Rev [Internet]. 2014;32(30):13–23. Available from: http://libweb.anglia.ac.uk/

46. Papazafiropoulou A, Papanas N, Pappas S, Maltezos E, Mikhailidis DP. Effects of oral hypoglycemic agents on platelet function. J Diabetes Complications. 2015;29(6):846–51.

47. Hall HM, Banerjee S, McGuire DK. Variability of clopidogrel response in patients with type 2 diabetes mellitus. Diabetes Vasc Dis Res. 2011;8(4):245–53.

48. Damman P, Woudstra P, Kuijt WJ, De Winter RJ, James SK. P2Y12 platelet inhibition in clinical practice. Vol. 33, Journal of Thrombosis and Thrombolysis. 2012. p. 143–53.

49. Feher G, Feher A, Pusch G, Koltai K, Tibold A, Gasztonyi B, et al. Clinical importance of aspirin and clopidogrel resistance. World J Cardiol [Internet]. 2010;2(7):171–86. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2998916&tool=pmcentrez&rendertype=abstract

50. Rauchfuss S, Geiger J, Walter U, Renne T, Gambaryan S. Insulin inhibition of platelet-endothelial interaction is mediated by insulin effects on endothelial cells without direct effects on platelets. 2008;(January):856–64.

51. Jurk K, Kehrel BE. Platelets: Physiology and biochemistry. Semin Thromb Hemost. 2005;31(4):381–92.

52. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. Arterioscler Thromb Vasc Biol. 2010;30(12):2341–9.

53. Patrono C. Low-dose aspirin in primary prevention: Cardioprotection, chemoprevention, both, or neither? Eur Heart J. 2013;34(44):3403–11.

Chapter 6. General discussion

The project investigated platelet activation and function in pre-diabetes following treatment with anti-inflammatory and anti-thrombotic drugs. We further assessed the association of PMAs as a possible link between the pro-thrombotic and pro-inflammatory condition reported in pre-diabetes. The effect of low-dose aspirin and clopidogrel as monotherapy were compared to the dual therapy consisting of metformin and low-dose aspirin. This was conducted to assess the levels of residual high on-treatment platelet reactivity (HTPR), that contributes to residual platelet activation associated with adverse cardiovascular disease (CVD) outcomes in type 2 diabetes mellitus (T2DM) (1, 2). Residual platelet reactivity has been reported in obese individuals (3) whereas we report on the presence of HTPR using a non-obese pre-diabetic mouse model.

6.1. Increased platelet activation in pre-diabetes

P-selectin is expressed on the membrane surface due to the degranulation of α-granules when platelets are activated (4). It binds to P-selectin glycoprotein ligand-1 (PSGL-1) on the surface of monocyte and neutrophils prompting a pro-inflammatory response thereby highlighting the role of platelets in the inflammatory process (4, 5). Platelet-monocyte aggregates (PMAs) are more robust markers for platelet activation and function which contribute to the increased thrombotic and pro-inflammatory environment in T2DM (6–8). Our study demonstrated comparable levels of PMA formation in the pre-diabetic condition in post-stimulation with adenosine diphosphate (ADP). This highlights an important finding that in the pre-diabetic condition, platelets are already at their maximal activation state hence can bind to monocytes and other leukocytes to form aggregates. Platelet binding also increases the pro-coagulant activity of monocytes with increased expression of tissue factor (9). Platelets from the pre-diabetes group are hyperreactive and exhibit high affinity to monocyte binding giving rise to PMAs which are sensitive markers of in vivo platelet activation (10–12). An increase in the formation of PMAs is also regarded as an early marker for T2DM that occurs independently of inflammation (13). Our study was able to demonstrate the formation of PMA providing evidence of the interaction between inflammatory and thrombotic mechanisms at the pre-diabetic stage.

6.2. Platelet function in pre-diabetes

Platelet activation is mediated by various signalling pathways activated through the interaction of ligands and surface receptors which include, the purinergic receptor ($P2Y_{12}$) (14), thromboxane A_2 receptor (TP) (15, 16) and collagen receptor (GPVI) (17). The common activation pathways involve receptor agonist binding on the platelet surface leading to the activation of phospholipase-Cγ (PLCγ), phospholipase-Cβ (PLCβ), phosphoinositide-3 kinase (PI3K) and adenyl cyclase. Activation of these receptors culminates in elevated calcium levels which play a role in shape change and secretion of platelet granules. The pathophysiology of platelet hyperreactivity in pre-diabetes and T2DM is well documented. Increase in platelet reactivity is attributed to glycation of the membrane proteins, increased

surface expression of P-selectin, increased intracellular calcium concentration, activation of protein kinase C (PKC) and increased cellular adhesion molecules (18). Metabolic changes which include impaired glucose metabolism contribute to increased platelet reactivity in T2DM (2, 19). In healthy individuals, acute hyperglycaemia results in the increased expression of P-selectin and CD40-ligand as well as activation of the PKC (2). Insulin deficiency, which was not exhibited by the pre-diabetic mice in this study, enhances the platelet response to collagen and ADP agonist (19).

It is currently known that ADP is an endogenous agonist capable of reversible or irreversible platelet activation at varying concentrations (20). In our study, in the pre-diabetic group, platelets were hyperreactive to stimulation with ADP which resulted in dense and α granule release and consequently increased expression of P-selectin from the α-granules on the platelet surface (21). In this manner, stimulation of the $P2Y_{12}$ receptor maintains and amplifies platelet activation, as well as stabilizes platelet aggregates (22, 23). In addition, the $P2Y_{12}$ receptor plays a role in ADP mediated thromboxane A_2 generation which in turn irreversibly activates the receptor itself (21). Cyclooxygenase plays a role in the metabolization of arachidonic acid into short-lived prostaglandins which are later converted to bioactive thromboxane A_2, an activator of platelet aggregation (4, 24). In brief, cyclooxygenase transforms arachidonic acid into prostaglandin H_2 which is transformed into prostaglandins and thromboxanes by synthases including cytosolic prostaglandin E synthase (cPGES), microsomal prostaglandin E synthase-1 (mPGES-1), prostaglandin I synthase (PGIS) and thromboxane synthase (TxS) (5). In our study, we demonstrated that stimulation of the cyclooxygenase pathway increased platelet reactivity in the pre-diabetic condition. Thromboxane A_2 is a potent vasoconstrictor and induces platelet adhesion, shape change and aggregation which may contribute to vascular occlusion and the development of cardiovascular disorders (4). In the pre-diabetes group, platelets were hyperreactive to activation of the GPVI receptor post stimulation with collagen. Once collagen is bound to GPVI, enzyme phospholipase Cγ (PLCγ) is activated, which hydrolyzes the lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) into DAG and IP_3 (6). As a result, intracellular calcium levels are increased leading to shape change and granule secretion (7, 16). Increased PKC levels lead to the expression of the GP IIb/IIIa complex and consequently platelet aggregation via the binding of fibrinogen to its co-receptor (8). Increased fibrinogen binding, as well as expression of granule secreted P-selectin, have been demonstrated in hyperglycaemia and in diabetic patients with CVD (8, 9).

6.3. High on-treatment platelet reactivity (HTPR) in pre-diabetes

We assessed whether the use of clopidogrel, a P2Y receptor antagonist that blocks the binding of ADP (10), could inhibit residual platelet reactivity in pre-diabetes. Our study demonstrated the inhibition of the P2Y12 receptor-mediated platelet activation in clopidogrel-treated pre-diabetic mice. We further demonstrated the synergistic effect of metformin and low-dose aspirin dual therapy in inhibiting platelet activation and function. The combination of metformin and low-dose aspirin reduced platelet reactivity

in response to ADP stimulation compared to low-dose aspirin monotherapy. Low-dose aspirin monotherapy reduced platelet reactivity in pre-diabetic platelets stimulated with arachidonic acid providing evidence of its COX inhibitory function. Similarly, the addition of metformin to low-dose aspirin therapy also inhibited COX activation. Whereas in the clopidogrel-treated group, platelet hyperreactivity to arachidonic acid was observed in the pre-diabetic group. The continued platelet hyperreactivity despite clopidogrel treatment, illustrates that the calcium levels can still be elevated even after inhibition of the GPVI receptor. Therefore, the COX pathway is still activated due to increased calcium levels. Treatment with metformin and low-dose aspirin dual therapy reduces platelet hyperreactivity post stimulation with collagen, which was not observed following low-dose aspirin treatment. This may suggest the synergistic properties of dual therapy in pre-diabetes and a possible GPVI downstream inhibitory effect of metformin.

Overall, the current study demonstrated increased platelet activation and function in pre-diabetes. We further highlight the interaction between monocytes and activated platelets. In addition, we demonstrated variable significant HTPR in the dual metformin and low-dose aspirin therapy, low-dose aspirin and clopidogrel monotherapy indicating residual platelet activity. Our findings provide evidence of interaction of pro-inflammatory and pro-coagulant mechanisms and the role of platelets in increasing the risk of CVD in as early as the pre-diabetes stage. We also highlighted the pathways involved in platelet activation in response to agonists, as well as the increased platelet hyperreactivity to endogenous agonists in pre-diabetes. Importantly, we demonstrate a variant platelet inhibitory response to anti-glycaemic and anti-platelet therapy. This further highlighted the variable inhibition of the PKC and COX-1 mediated pathways by the anti-platelet therapy giving rise to persistent HTPR. This may explain the occurrence of CVD in pre-diabetic and diabetic patients on either anti-glycaemic and anti-platelet treatment (11–13). Further work is required to investigate the efficacy of the combination of anti-platelet therapy and anti-inflammatory treatment regimens in a CVD model. Prospective work will investigate other coagulation factors such as tissue factor involved in the progression of thrombotic conditions in pre-diabetes. The targeting of the scavenger receptor sCD36 may provide important therapeutic impact in reducing the hypercoagulable state in T2DM. Further molecular work targeted at inhibiting or overexpression of PKC, including modulation of calcium homeostasis in pre-diabetes is planned for future experiments.

6.4. References

1. Feher G, Feher A, Pusch G, Koltai K, Tibold A, Gasztonyi B, Papp E, Szapary L, Kesmarky G, Toth K (2010) Clinical importance of aspirin and clopidogrel resistance. World J Cardiol 2:171–86 . doi: 10.4330/wjc.v2.i7.171

2. Kakouros N, Rade JJ, Kourliouros A, Resar JR (2011) Platelet function in patients with diabetes mellitus: From a theoretical to a practical perspective. Int J Endocrinol 2011:1–14 .

doi: 10.1155/2011/742719

3. Badimon L, Hernández Vera R, Padró T, Vilahur G (2013) Antithrombotic therapy in obesity. Thromb Haemost 110:681–688

4. Khan N, Farooq AD, Sadek B (2015) Investigation of cyclooxygenase and signaling pathways involved in human platelet aggregation mediated by synergistic interaction of various agonists. Drug Des Devel Ther 9:3497–3509 . doi: 10.2147/DDDT.S84335

5. Korbecki J, Baranowska-Bosiacka I, Gutowska I, Chlubek D (2014) Cyclooxygenase pathways. Acta Biochim Pol 61:639–649

6. Xiang YZ, Xia Y, Gao XM, Shang HC, Kang LY, Zhang BL (2008) Platelet activation, and antiplatelet targets and agents: Current and novel strategies. Drugs 68:1647–1664

7. Clemetson KJ (2012) Platelets and primary haemostasis. Thromb Res 129:220–224 . doi: 10.1016/j.thromres.2011.11.036

8. Sudic D, Razmara M, Forslund M, Ji Q, Hjemdahl P, Li N (2006) High glucose levels enhance platelet activation: Involvement of multiple mechanisms. Br J Haematol 133:315–322 . doi: 10.1111/j.1365-2141.2006.06012.x

9. Soma P, Swanepoel AC, du Plooy JN, Mqoco T, Pretorius E (2016) Flow cytometric analysis of platelets type 2 diabetes mellitus reveals ‘angry’ platelets. Cardiovasc Diabetol 15:1–7 . doi: 10.1186/s12933-016-0373-x

10. Diehl P, Olivier C, Haischeid C, Helbing T, Bode C, Moser M (2010) Clopidogrel affects leukocyte dependent platelet aggregation by P2Y 12 expressing leukocytes. Basic Res Cardiol 105:379–387 . doi: 10.1007/s00395-009-0073-8

11. Boussageon R, Supper I, Bejan-Angoulvant T, Kellou N, Cucherat M, Boissel JP, Kassai B, Moreau A, Gueyffier F, Cornu C (2012) Reappraisal of metformin efficacy in the treatment of type 2 diabetes: A meta-analysis of randomised controlled trials. PLoS Med 9: . doi: 10.1371/journal.pmed.1001204

12. Hall HM, Banerjee S, McGuire DK (2011) Variability of clopidogrel response in patients with type 2 diabetes mellitus. Diabetes Vasc Dis Res 8:245–253 . doi: 10.1177/1479164111420890

13. Lorber D (2014) Importance of cardiovascular disease risk management in patients with type 2 diabetes mellitus. Diabetes, Metab Syndr Obes Targets Ther 7:169–183 . doi: 10.2147/DMSO.S61438

14. Burnstock G, Novak I (2013) Purinergic signalling and diabetes. Purinergic Signal 9:307–324

15. Jurk K, Kehrel BE (2005) Platelets: Physiology and biochemistry. Semin Thromb Hemost 31:381–392 . doi: 10.1055/s-2005-916671

16. Ghoshal K, Bhattacharyya M (2014) Overview of platelet physiology: Its hemostatic and nonhemostatic role in disease pathogenesis. Sci World J 2014: . doi: 10.1155/2014/781857

17. Furihata K, Nugent DJ, Kunicki TJ (2002) Influence of platelet collagen receptor polymorphisms on risk for arterial thrombosis. Arch Pathol Lab Med 126:305–309 . doi: 10.1043/0003-9985(2002)126<0305:iopcrp>2.0.co;2

18. Hess K, Grant PJ (2011) Inflammation and thrombosis in diabetes. Thromb Haemost 105:43–54 . doi: 10.1160/THS10-11-0739

19. Schneider DJ (2009) Factors Contributing to Increased Platelet Reactivity in People With Diabetes. Diabetes Care 32:525–527 . doi: 10.2337/dc08-1865

20. Cattaneo M (2011) The platelet P2Y12 receptor for adenosine diphosphate: congenital and drug-induced defects. Syst Rev 4:2102–2112

21. Dorsam RT, Kunapuli SP (2004) Central role of the P2Y 12 receptor in platelet activation. J Clin Invest 113:10–15 . doi: 10.1172/JCI200420986.The

22. Damman P, Woudstra P, Kuijt WJ, De Winter RJ, James SK (2012) P2Y12 platelet inhibition in clinical practice. J Thromb Thrombolysis 33:143–153

23. Nicholas RA (2015) Insights into platelet P2Y 12 receptor activation. 125:893–896

24. Grove EL (2012) Antiplatelet effect of aspirin in patients with coronary artery disease. Dan Med J 59:B4506