An Exploration of the Relationship between Cardiovascular Health, Lifestyle Factors and Platelet Function.

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Research was carried out in the School of Health and Human Performance, DCU, under the supervision of Doctor Ronan P. Murphy.

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy, is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Student I.D: 58334765

Date: _____

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Dedication

To my Granddad Jim, my family, and friends.

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Abbreviations

AA	Arachidonic Acid
ADP	Adenosine diphosphate
AF	Atrial Fibrillation
Ago2	Argonaute Two
AS	Aggregate Size
ATP	Adenosine Triphosphate
BF	Body Fat
BMI	Body Mass Index
BP	Blood Pressure
Ca^{2+}	Calcium
CAD	Coronary Artery Disease
cAMP	Cyclic AMP
CBC	Complete Blood Counts
cDNA	Complementary DNA
cGMP	Cyclic GMP
COAT	Collagen and Thrombin Activated
COX	Cyclooxygenase
CRF	Cardiorespiratory Fitness
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
DKK1	Dickkopf Related Protein 1
DTS	Dense Tubular System
EC	Endothelial Cell
ECM	Extracellular Matrix
ELISA	Enzyme Linked Immunosorbent Assay
ENOS	Endothelial Nitric Oxide Synthase
ESA	European Space Agency
Exo	Exosome
GC	Guanylyl Cyclase
GPCRs	G-Protein Coupled Receptors
HAT	Histone Acetyltransferase
Hb	Haemoglobin
HCT	Haematocrit
HDAC	Histone Deacetyltransferase
HDBR	Head-Down Bed-Rest
HGB	Haemoglobin
HR	Heart Rate
HSC	Haematopoietic Stem Cell
Ht	Haematocrit
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intercellular Adhesion Molecule-1
IHF	Irish Heart Foundation
Il	Interleukin
IR	Insulin Resistance
ITAM	Immunoreceptor Tyrosine Based Activation Motif
KEGG	Koyoto Encyclopaedia of Genes and Genomes
LDL	Low Density Lipoprotein
LTA	Light Transmission Aggregometry
MEDES	Institut de Médecine et de Physiologie Spatiales

MI	Myocardial Infarction
miRNA	microRNA
MK	Megakaryocyte
MP	Microparticle
MPV	Mean Platelet Volume
mRNA	Messenger RNA
MSST	Multi-stage Shuttle Test
mtDNA	Mitochondrial DNA
MV	Microvesicle
NcRNA	Non Coding RNA
NO	Nitric Oxide
NPX	Normalised Protein Expression
NSAID	Non-Steroidal Anti-Inflammatory Drug
OCS	Open Canalicular System
Ox-LDL	Oxidised Low Density Lipoprotein
PA	Physical Activity
PAMP	Pathogen Associated Molecular Pattern
PARs	Protease Activated Receptors
PCT	Plateletcrit
PDGF	Platelet Derived Growth Factor
PDW	Platelet Distribution Width
PECAM	Platelet Endothelial Cell Adhesion Molecule
PFA-100	Platelet Function Analyser - 100
PFT	Platelet Function Test
PGE_1	Prostaglandin
PGI_2	Prostacyclin
PI	Physical Inactivity
PI3K	Phosphatidylinositide-3-kinase
PKC	Protein Kinase C
PLC	Phospholipase C
PLCR	Platelet Large Cell Ratio
PLT	Platelet Count
PMP	Platelet-derived Microparticle
PPP	Platelet Poor Plasma
PRI	Platelet Reactivity Index
PRP	Platelet Rich Plasma
PS	Phosphatidylserine
PV	Plasma Volume
RBC	Red Blood Cell
RISC	RNA Induced Silencing Complex
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Reverse Transcription
RT-PCR	Real Time Polymerase Chain reaction
SC	Surface Coverage
SMC	Smooth Muscle Cell
STEMI	ST-Segment Elevation Myocardial Infarction
TBW	Total Body Water
TF	Tissue Factor
TLR	Toll-like Receptor
TPO	Thrombopoeitin

TxA_2	Thromboxane A_2
UTR	Untranslated Region
VAS	Visual Assessment Scale
VASP	Vasodilator stimulated Phosphoprotein
VO_2 max	Maximal Oxygen Consumption
vWF	Von Willebrand Factor
WBA	Whole Blood Aggregometry
WBC	White Blood Cell
WC	Waist Circumference
WHO	World Health Organisation
Wnt	Wingless Protein Family
$\Delta\Psi_m$	Mitochondrial Membrane Potential

Units

bp	Base Pairs
cm	Centimetre
°C	Degree Celsius
KDa	KiloDaltons
Mg	Microgram
µl	Microlitre
µm	Micromolar
g	Grams
h	Hours
kg	Kilogram
fl	Femtoliters
L	Litre
M	Molar
m	Meter
Mg	Milligrams
min	Minutes
ml	Millilitres
mM	Millimolar
ng	Nanograms
nm	Nanometres
pmol	Picomolar
s	Seconds
w/v	Weight per Volume
g/dL	Grams per Decilitre

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PhD Poster Presentations

" The effect of Fibrinogen Adhesion on the microRNA profile of Human Platelets"

Laura C. Twomey, Robert Wallace, Philip Cummins, Ronan Murphy.

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"Investigation of Platelet microRNA as a marker of Epigenetic Drift"

Laura C. Twomey, Robert Wallace, Zainab Ghrefa, Gerardene Meade, Phil Cummins, Niall Moyna, Ronan Murphy.

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'Analysis of Platelets and Microvesicles as Circulating Functional Biomarkers in Blood'.

Laura C. Twomey, Robert Wallace, Zainaib Ghrefa, Aoife Deering, Emma O'Neil, Brian Fitzpatrick, Philip Cummins, Ronan P. Murphy.

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PhD Oral Presentations

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Laura C. Twomey, Robert Wallace and Ronan Murphy.

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PhD Publications

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Abstract

Laura Twomey

An Exploration of the Relationship between Cardiovascular Health, Lifestyle Factors and Platelet Function.

Introduction

Platelets are small, anucleate, cells which travel as resting discoid fragments in the blood circulation. Upon contact with an injured blood vessel, they prevent blood loss through processes of adhesion, activation and aggregation. Platelets play a central role in cardiovascular disease (CVD), both in the development of atherosclerosis and in the development of acute thrombotic events. CVD risk factors can be classed as modifiable or non-modifiable. This thesis focuses on the effect of the modifiable risk factors (physical inactivity, overweight/obesity and low cardiorespiratory fitness (CRF)) on platelet function. We investigate the role of acute exercise on platelet function, and the impact of CRF on platelet responses to acute exercise. We also explore the role of microRNA (miRNA), a subset of small non-coding RNA, in regulating platelet function in response to physical inactivity.

Hypothesis

Physical inactivity, overweight/obesity and low CRF adversely impact platelet function. miRNA expression could influence and modulate the platelet function response to physical inactivity.

Results

We performed a cross-sectional study to investigate the relationship between platelet function and lifestyle factors. High platelet counts and plateletcrit were associated with less favourable cardiovascular risk profiles, in particular with measures of overweight. Platelet adhesion was significantly increased in subjects with an overweight BMI compared to those with a healthy weight BMI. Acute strenuous aerobic exercise resulted in increased platelet adhesion and aggregation in adults and adolescents. This increase was more pronounced in those with a low CRF, particularly in adolescent subjects. Using a Dry Immersion (DI) model, we investigated the effect of acute physical inactivity on platelet function in 12 healthy males. Three days of DI resulted in significant increases in platelet count, plateletcrit, platelet adhesion, aggregation, and an elevation of platelet reactivity index (PRI) and microvesicle concentration. We identified 15 cardiovascular and inflammatory protein biomarkers whose expression levels were altered after physical inactivity. We also identified a set of 22 ‘physical inactivity’ related miRNA, which have potential targets involved in pathways associated with platelet function.

Conclusion

Modifiable CVD risk factors of overweight/obesity and physical inactivity elicit a pro-thrombotic platelet profile. CRF levels affect the platelet response to acute exercise, suggesting that habitual physical activity influences platelet function. miRNA expression can influence and modulate the platelet function response to physical inactivity by negative regulation of gene expression and could act as markers of megakaryocyte epigenetic drift.

Aims and major findings of the thesis

Overall aim

Investigate the effect of the lifestyle factors; physical inactivity, overweight/obesity and low CRF on platelet function.

Main aims

- Investigate the relationship between platelet function and i) overweight/obesity, ii) physical activity, in a diverse population
- Establish the reliability of the Impact-R Cone and Plate device to measure platelet function
- Determine the feasibility of platelet indices as biomarkers of early CVD risk in a disease-free population
- Investigate differences in basal platelet function in males of varying cardiorespiratory fitness (CRF)
- Examine the effect of acute aerobic exercise on platelet adhesion and aggregation in healthy males
- Investigate the relationship between CRF and platelet function
- Explore the effect of physical inactivity on platelet function and cardiovascular and inflammatory protein biomarker expression, in healthy males
- Examine the role of microRNA in regulating the platelet function response to physical inactivity

Major findings

- High platelet counts and plateletcrit were associated with measures of overweight
- Platelet adhesion was significantly increased in subjects with an overweight BMI
- The Impact-R cone and plate analyser could detect platelet function variances in a diverse population
- Varying cardiorespiratory fitness levels had no major influences on basal differences in platelet function
- Acute aerobic exercise resulted in increased platelet adhesion and aggregation
- This increase was more pronounced in those with a low CRF, particularly in adolescents
- Physical inactivity resulted in significant increases in platelet count, plateletcrit and platelet adhesion
- Physical inactivity resulted in a slight elevation in aggregation, platelet reactivity index (PRI) and microvesicle concentration in healthy males
- Physical inactivity resulted in altered expression of 15 protein biomarkers
- We identified a set of 22 ‘physical inactivity’ related miRNA, with potential targets involved in pathways associated with platelet function.

Chapter One: Introduction

1.1 The cardiovascular system

In the broadest sense, the cardiovascular system consists of the heart, blood vessels and blood. Blood is circulated around the body through blood vessels by the pumping action of the heart, which is divided into left and right sides with two chambers in each side, the atrium and ventricle (Baeyens and Schwartz, 2016). During the average human lifespan, the heart beats about 3 billion times, pumping over 200 million litres of blood. Blood vessels are classified into three categories; arteries, veins and capillaries which form intricate vascular networks between tissues and cells, enabling blood perfusion (Figure 1.1). If an adult's blood vessels were laid out continuously, they would spread out over 100,000 km. Arteries and veins share a common three layered structure of a: (i) tunica adventitia; (ii) tunica media and (iii) tunica intima. The endothelium, a continuous dynamic monolayer of endothelial cells (ECs) lines the vasculature, forming a selective interface between circulating blood and the underlying vascular smooth muscle layer.

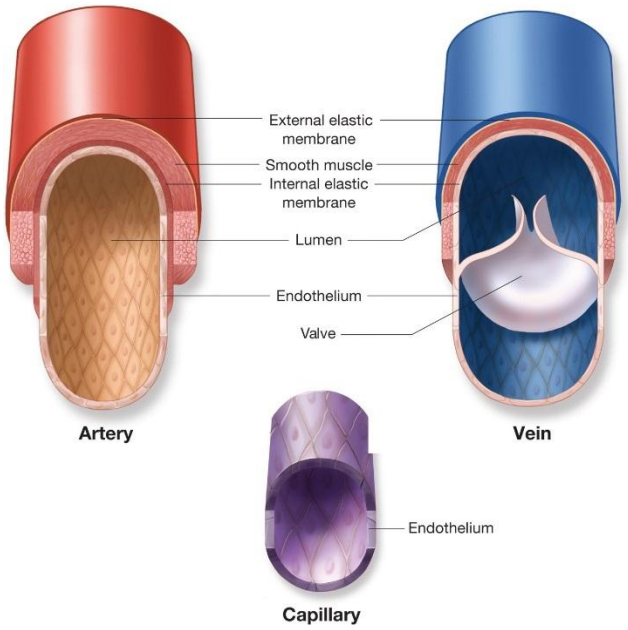


Figure 1.1: The three types of blood vessels. From left to right, a cross-sectional view of an artery, capillary and vein. The main features highlighted include a thicker vessel wall in artery compared to the vein, presence of a valve in the lumen of the vein to prevent backflow of low pressure blood and the single endothelial layer of the capillary, emphasising the delicate structure of this vessel. The endothelium of both the artery and vein is also highlighted. *(Image from http://legacy.owensboro.kctcs.edu/gcaplan/anat2/notes/APIINotes5%20Circulatory%20Anatomy.htm).*

Oxygenated blood returning from the lungs is pumped from the left ventricle into a larger network of arteries and smaller arterioles, each supplying blood to an organ or body region. Returning blood flows through the right atrium, which in turn flows through the tricuspid valve into the right ventricle, and then up to the lungs via the pulmonary artery. Through gaseous exchange, the lungs oxygenate the blood, which then returns to the left atrium via the pulmonary vein (Figure 1.2). The oxygen rich blood now enters the left ventricle and is pumped into the aorta as the entire process begins again (Aaronson *et al.*, 2012). There are two anatomically distinct vascular circuits through which blood is pumped. Circulation of deoxygenated blood flowing from the right ventricle of the heart to lungs and back to the heart is known as the pulmonary circulation. The circulation of blood from the left ventricle of the heart to the rest of the body is known as the systemic circulatory system.

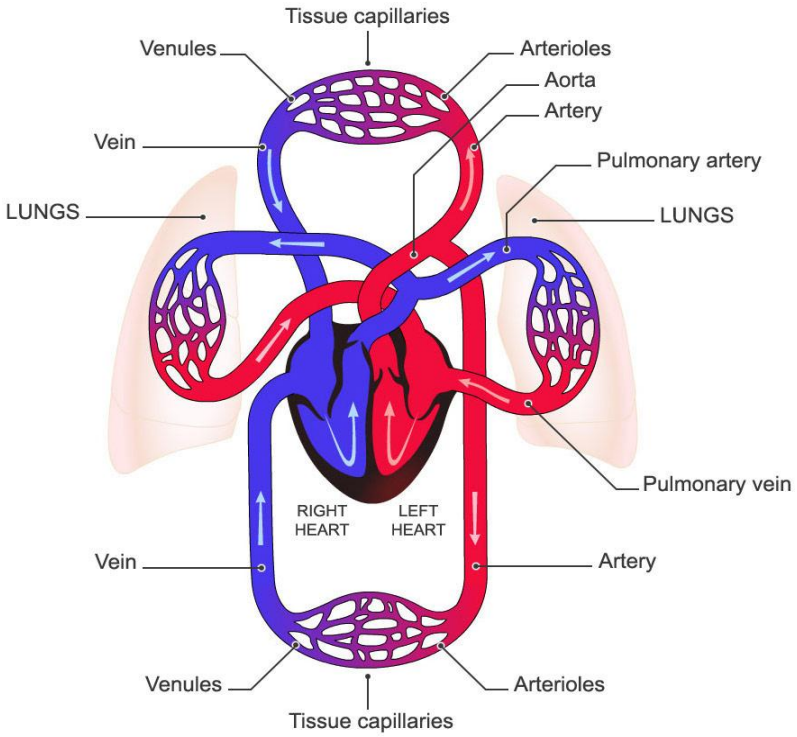


Figure 1.2: The cardiovascular system. The heart functions as a pump to maintain circulation. Oxygenated blood is delivered to tissues via the systemic circulation. Arteries branch into smaller arterioles and capillaries. Deoxygenated blood returns from the capillaries via venules and larger veins in the pulmonary circulation. *(Image from http://humananatomybody.info/neck-arteries-model-labeled/).*

1.2 Blood and its cellular components

The main functions of blood are to supply oxygen and nutrients to tissues and cells, removal of waste, and regulation of pH and body temperature (Ruggeri, 2009). An average adult has approximately five litres of blood, accounting for about 7% of their body weight. Blood is composed of approximately 55% plasma; a pale yellow fluid mainly consisting of water, proteins, sugars and fat particles, and 45% blood cells (Dean *et al.*, 2005). Blood cells include erythrocytes, leukocytes and platelets. Each of these cells are derived from a haematopoietic stem cell (HSC), which reside in the bone marrow and sit at the peak of a developmental hierarchy, with a unique ability to self-renew and give rise to cells of all of the blood lineages (Woolthius and Park, 2016). In adults, nearly a trillion new blood cells are produced daily to sustain steady state in circulation (Huang and Cantor, 2009). In the classic model of haematopoiesis (the production of blood cells), an important bifurcation occurs between the lymphoid and myeloid branches, which then further divide into a number of progenitor cells, shown in Figure 1.3. While the main function of red blood cells (RBCs) and white blood cells (WBCs) is oxygen transport and defences, respectively, this Introduction will focus on platelets, the final product of one of the myeloid cell lines.

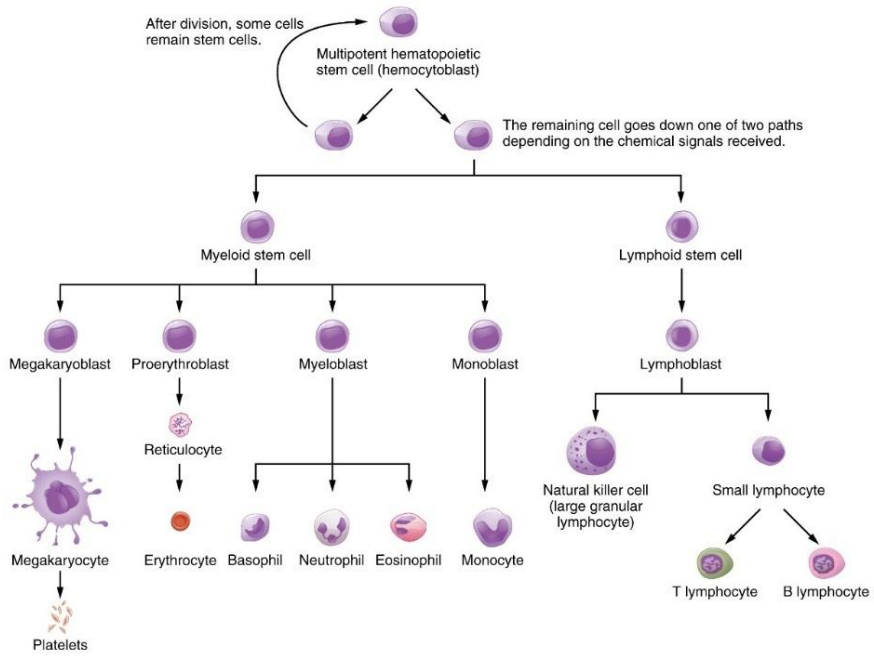


Figure 1.3: Blood cell lineage and development from haematopoietic stem cells. HSCs are self-renewing cells found in the bone marrow. While diverse in function, all blood cell components are derived from (HSCs). The myeloid stem cell leads to the production of megakaryoblasts, and subsequently megakaryocytes, the precursor to the mature platelet. *(Image from Betts et al., 2013).*

1.3 Introduction to platelets

Gulio Bizzozero first described platelets as ‘spherules piastrine’ (little plates), small cell fragments that clumped together at an injured blood vessel site. He also showed that these blood elements did not have a nucleus (Bizzozero, 1882). Circulating anucleate platelets are now described as dynamic specialised cells, formed in an elaborate style from their precursor cell, the megakaryocyte. Normal platelet counts ranges between 150 - 450 x 10^3 per microliter of blood, constituting the second most abundant cell type in blood after red blood cells. Platelets travel as resting (quiescent) discoid fragments in the circulation, but an elaborate internal cytoskeleton allows shape changes to be made when they come in contact with an injured blood vessel (Figure 1.4).

The size of a mature platelet is approximately 2-4 µm, making them the smallest cells in circulation, while their average thickness is 0.5 µm (Xu *et al.,* 2016) and their volume about 7 fl. The small size of platelets facilitates in their role as ‘guardians of the vasculature’, as under laminar flow environments, platelets are pushed to the periphery by larger white and red blood cells. Consequently, they are then in close proximity to the blood vessel wall where they can quickly detect any vascular damage (Zucker & Nachmias 1985; McFadyen and Kaplan, 2015). This enables platelets to perform their main physiological function to prevent blood loss in primary haemostasis by the formation of a ‘platelet plug’ (Cimmino and Golino 2013).

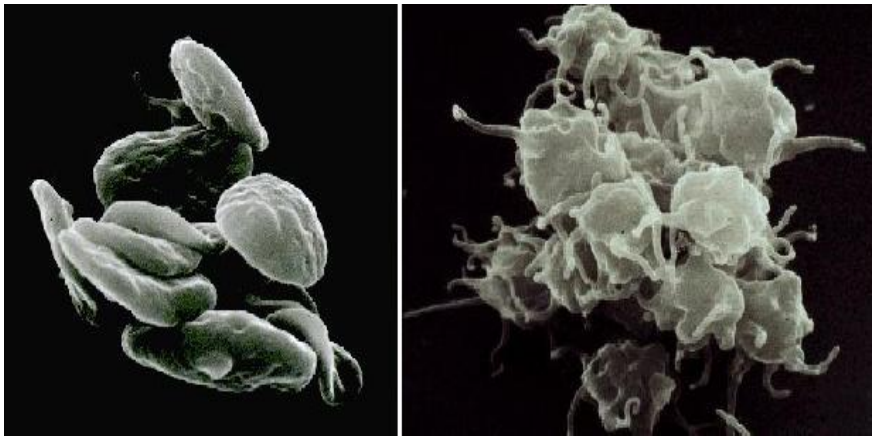


Figure 1.4: Quiescent (resting) and activated platelets. The left image shows, quiescent, inactivated platelets in their travelling discoid shape. The right image depicts activated platelets aggregating together after vascular damage. *(http://www.ouhsc.edu/platelets).*

1.3.1 Platelet production

Platelets are formed and released into the bloodstream in an elaborate manner by rare precursor cells called megakaryocytes (MKs), which reside in the bone marrow, mainly of the ribs and thighs (Patel *et al.,* 2005; Thon *et al., 2*013). Their production is arguably the most elegant and distinct developmental process in eukaryotes (Simon *et al.,* 2014). While accounting for only 0.01% of nucleated bone marrow cells, MKs are also the largest cells, measuring between 50 – 100 µm (Machlus and Italiano, 2013).

Both MK and platelet production are regulated by multiple cytokines, with thrombopoeitin (TPO), a hormone produced by the liver and kidneys, being the key regulator. In response to TPO, HSCs differentiate into MKs by differential expression of various transcription factors. This maturation is characterised by a growth in MK size and DNA ploidy levels due to a process called endomitosis (DNA replication without cell division) enabling the accumulation of RNA, protein and organelles in the MK for packaging into platelets. MKs then migrate to the sinusoidal blood vessels in the vascular niche and the bulk of their cytoplasm and its contents are bundled into numerous long processes called proplatelets. MKs can extend as many as 20 proplatelets which branch repeatedly over time. Platelets form at the tips of proplatelets, receiving organelles, genetic material and granule contents that are transported from the MK cell body. The final point of platelet production occurs in circulation whereby anucleate fragments of proplatelets bud into preplatelets (Thon *et al.,* 2010) and barbell-shaped platelets (Schwartz *et al.,* 2010) that are subsequently converted into single platelets in a microtubule-driven process (Poulter and Thomas, 2015) aided by the shear forces of the bloodstream (Thon *et al.,* 2012). The process of platelet production from MKs is illustrated in Figure 1.5.

After leaving the bone marrow and entering the blood stream, platelets then have an average lifespan of 8-10 days, after which they are cleared via phagocytic cells such as macrophages in the spleen. Apoptosis (programmed cell death) is also well recognised in the anucleate platelet (Gyulkhandanyan *et al.,* 2013). The constant number of platelets in circulation is a consequence of a balance between their production and destruction/clearance.

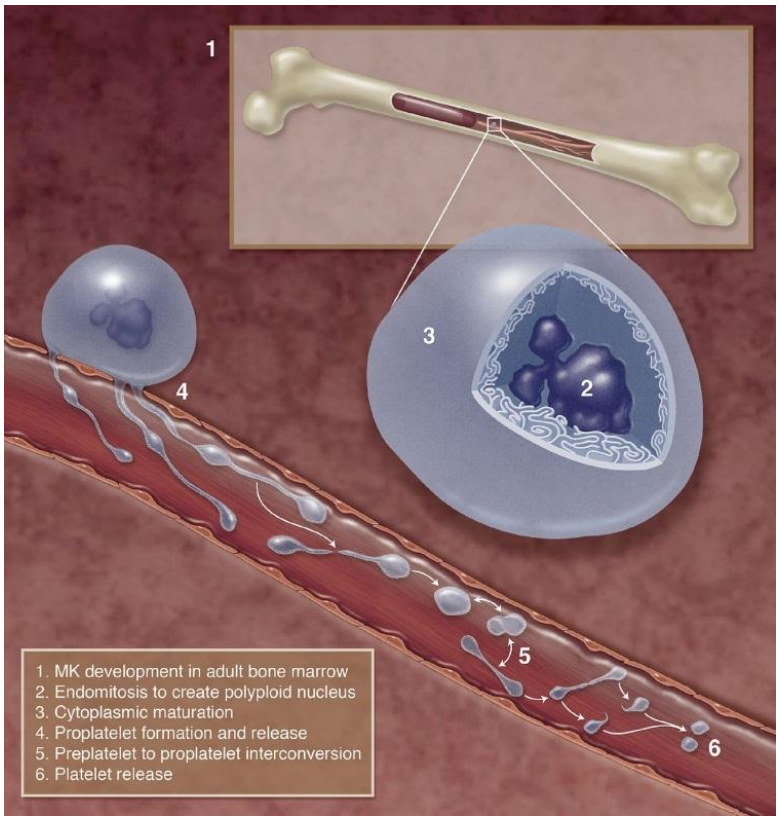


Figure 1.5: Platelet production from megakaryocytes. 1: In response to thrombopoeitin, HSCs residing in the bone marrow differentiate into MKs. 2: MK size increases and they undergo DNA replication (endomitosis), generating nuclei of which can reach 128n. 3: Maturation of MKs involves the development of an elaborate membrane which acts as a reservoir for proplatelet formation. 4: MKs migrate to the vascular beds where they extend proplatelets and release them into sinusoidal vessels. The whole MK is transformed into pre/proplatelets and its nucleus is phagocytosed. 5: Once entered into the blood vessel, proplatelets convert to preplatelets. 6: A microtubule fission event produces two platelets from a barbell proplatelet. *(Image from Machlus and Italiano, 2013).*

1.3.2 Platelet structure

1.3.2.1 Internal

Platelets are unique in their structural composition and, while anucleate, contain a large variety of cellular organelles, granules and distinct mitochondria (Figure 1.6). Granules are generally secretory vesicles that release their contents either to the platelet surface or to extracellular fluid by endocytosis. An excess of 300 proteins from platelet granules has been identified in the platelet releasate after strong activation (Coppinger *et al.,* 2004; Wijten *et al.,* 2013). There are 3 types of platelet granules: α-granules, dense granules, and lysomal granules all of which derive their cargo from MKs. α-granules are the largest and most numerous (50-80 per platelet) encompassing roughly 10% of the platelet volume (Blair, 2009).

α-granule function originates from their contents. They harbour a vast assortment of sizable proteins important for primary haemostasis including integrins (αIIbβ3) immunoglobulin family receptors (e.g. GPVI, PECAM), leucine-rich repeat family receptors (e.g., GPIb-IX-V complex), tetraspanins (e.g., CD9) and other adhesive proteins such as von Willebrand Factor (vWF), fibrinogen, and coagulation factors (Factors V, XI) that participate in secondary haemostasis. While it was previously assumed that platelet α-granules were homogenous populations, Italiano *et al.,* (2008) suggested that platelets have distinct subpopulations of alpha granules which differentially release their cargo in a context-dependent manner; for example, encouraging angiogenesis by releasing VEGF from one granule subset under one condition and inhibiting angiogenesis in other conditions by releasing angiostatin from a different granule subset. However, the regulation of the secretory processes in response to certain agonists is not fully understood.

Dense granules are smaller in size and number (3-8 per platelet) storing high concentrations of non-protein molecules that potentiate platelet activation such as adenosine diphosphate (ADP, adenosine triphosphate (ATP), calcium, histamine and serotonin (Morell *et al.,* 2014). Dense granules also contain polyphosphate which can be secreted upon platelet activation (Ruiz *et al.,* 2004; Senis *et al.,* 2013). Lysosomal granules are sparse and harbour enzymes such as acid hydrolases and proteases (Italiano *et al.,* 2009). They function in the digestion of cytosolic components, similarly to nucleated cells. Secretion of the lysosomal content has key extracellular functions including receptor cleavage, fibrinolysis and degradation of extracellular matrix (ECM) (Heijnen and Van der Sluijs, 2015); however, they are the least researched of the platelet granules. A recent report has described a possible new type of secretory granule termed a T-granule, after their tubular morphology (Thon *et al.,* 2012). These novel electron-dense granules have been proposed to function in toll-like receptor (TLR) organisation and signalling. Platelet granule deficiencies or defects such as the Gray Platelet Syndrome (α-granule deficiency) or Hermansky-Pudlack Syndrome (dense granule deficiency) can cause mild to severe bleeding disorders (Nurden *et al.,* 2007).

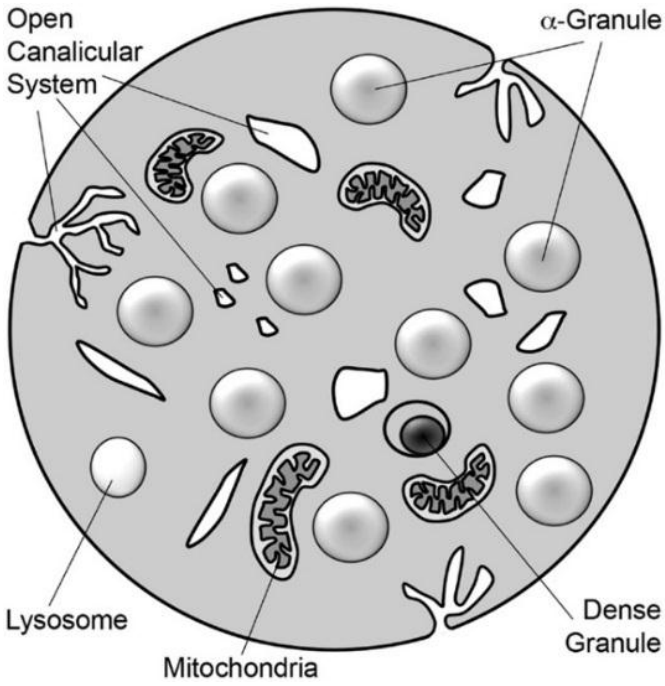


Figure 1.6: Platelet structure highlighting the granules present in platelets. Platelets contain three types of granules: α-granules, dense granules, and lysosomes. They also contain a small number of functionally active mitochondria. The open canalicular system (OCS) is a tunnel of invaginations of the plasma membrane, allowing for trafficking of platelet granules. ***(****Image from Tewfik and Flaumenhaft, 2013).*

Platelets also contain an important cytoskeleton complex of microtubules and actin filaments that is central to all of their functions (Sorrentin *et al.*, 2016). The cytoskeleton of resting platelets has approximately 2,000 actin filaments (0.5 mM) accounting for approximately one fifth of total protein in platelets. In resting platelets, only ~ 35% of actin molecules are assembled into filaments (F-actin) (Boyles *et al.*, 1985) growing to ~70% upon activation (Fox and Philips, 1981). These cytoskeletal proteins create a structural framework that responds to stimuli by forming dynamic morphological changes. In resting platelets, the cytoskeleton bears resemblance to a spoked wheel, whereas upon activation, platelets undergo a morphological transformation from a discoid to round to fully spread shape. The actin framework is completely rearranged to enable the extension of filipodia and lamelipodial from the platelet, increasing their area of surface contact. Shape change is mediated by a rapid remodelling of the resting platelet cytoskeleton and the assembly of filamentous actin (F-actin) induced by receptor interaction and phosphorylation of the platelet protein myosin (Hartwig, 1992; Zucker and Nachmias, 2013; Sorrentino *et al.*, 2015).

Platelets possess an intricate membranous system referred to as the open canalicular system (OCS) which acts as a connection between the cytosol and surrounding medium (Escolar and White, 1991). The OCS is an extravagant system of tunnelling invaginations of the cell membrane unique to platelets that increases their surface area by 2-4 fold when spreading is required. The OCS enables secretion of platelet granules by exocytosis and represents a pivotal means for uptake and trafficking of proteins from plasma to granules by endocytosis (Heijnen *et al.,* 2015). Upon platelet activation, granules journey to the OCS or plasma membrane for fusion and content release. It also enables the formation of filipodia during platelet activation.

Platelets also retain a dense tubular system (DTS) that originates from the rough endoplasmic reticulum in the MK and is dispersed through the platelet cytoplasm as thin elongated membranes. It acts as a storage base for Ca^{2+} and enzymes such as ATPases and cyclooxygenase (Gerard *et al.,* 1978; Ebbeling *et al.,* 1992) and has integral roles in platelet activation. Upon platelet activation, calcium is rapidly released from the DTS to increase Ca^{2+} levels required for regulation of platelet function.

Platelets contain functional mitochondria which, despite being few in number, have higher rates of ATP turnover than resting mammalian muscle, suggesting they are very metabolically active (Zharikov and Shiva 2013). The traditional role of mitochondria in the platelet was considered to supply energy in the form of ATP for primary platelet functions. However, novel functions for mitochondria continue to emerge. Dual activation of platelets with collagen and thrombin results in a subtype of platelets known as collagen and thrombin activated (COAT) platelets. COAT platelets display striking alterations in function and structure to typical "activated platelets", by exhibiting a myriad of features such as phosphatidylserine exposure due to cytoskeletal reorganisation, high microparticle release, and increased levels of fibrinogen on the platelet surface (Remenyi *et al.,* 2005; Dale *et al.,* 2005; Jobe *et al.,* 2008). Mitochondrial membrane potential ($\Delta\Psi_m$) is reduced in (COAT) platelets and decreases in parallel with elevated mitochondrial ROS levels that are necessary for facilitating platelet PS exposure upon activation (Choo *et al.,* 2012). Mitochondria are involved in the process of platelet apoptosis (Hayashi *et al.,* 2011; Yang *et al.,* 2014) and can be released from platelets as potential inflammatory mediators (Bordreau *et al.,* 2014).

1.3.2.2 Surface receptors

Platelets express a wide variety of receptors on their membrane, which are constantly at the vanguard of platelet research and are fundamental to platelet function and downstream signalling (Cimmino and Golini, 2013). Major receptors include integrins, leucine-rich repeat receptors (Glycoprotein GPIb/IX/V, Toll-like receptors), C-type lectin receptors (P-Selectin, CLEC-2), tyrosine kinase receptors (Ephrins and Eph kinases), proteins belonging to the immunoglobulin superfamily (GPVI, FcγRIIA) and other receptors shared with vascular cells (TNF receptor type, CD63, CD36, PSGL-1). C-type lectin receptor -2 (CLEC-2), is a hemITAM receptor (Hemi- immunoreceptor tyrosine-based activation motif) expressed at high levels on the platelet surface. Binding of CLEC-2 to its endogenous ligand, Podoplanin, induces strong platelet activation and plays major roles in thrombus stability under flow conditions (Navarro-Nunez *et al.*, 2013).

Integrins are a major family of cell adhesion receptors (Hynes *et al.*, 2000). They are a type I transmembrane molecule consisting of a short intracellular and larger extracellular domain. All integrins contain an α subunit and a β subunit. During signal transduction, they transmit information concerning the chemical and mechanical status of the ECM to the cell. Hence, they are involved in cell signaling and regulation of cell shape and motility (Cosemans *et al.*, 2008; Bennet *et al.*, 2009). Platelets express five integrins; αIIbβ3 (fibrinogen receptor), α2β1(collagen receptor), α5β1(fibronectin receptor), αVβ3 (vitronectin receptor) and α6β1 (laminin receptor), all of which share related signal transduction processes (Shattil and Newman, 2004). On the cytoplasmic face of the plasma membrane, integrins organise and coordinate the assembly of cytoskeletal polymers and signaling complexes. On the extracellular side, integrins bind with high affinity to either ECM ligands or counter receptors on adjacent cell surfaces (Humphries *et al.*, 2006). αIIbβ3 is the most abundant platelet integrin, with copy numbers of ~50,000 per platelet, and is present in both alpha granules and the platelet surface.

Platelets express a number of G-protein coupled receptors (GPCRs) which constitute a large family of receptors that can sense molecules outside the cell and initiate signal transduction pathways and ultimately cell function. The main GPCRs present on platelets include thrombin receptors called protease-activated receptors (PARs) (PAR1 and PAR4), ADP receptors ($P2Y_1$, $P2Y_{12}$,), of which approximately 150 $P2Y_1$ receptors are present on the platelet (Ohlmann *et al.,* 2010), thromboxane receptors (TPα and TPβ) and glycoprotein receptors (Rivera *et al.,* 2009). The main platelet integrins and GPCRs are highlighted in Figure 1.7.

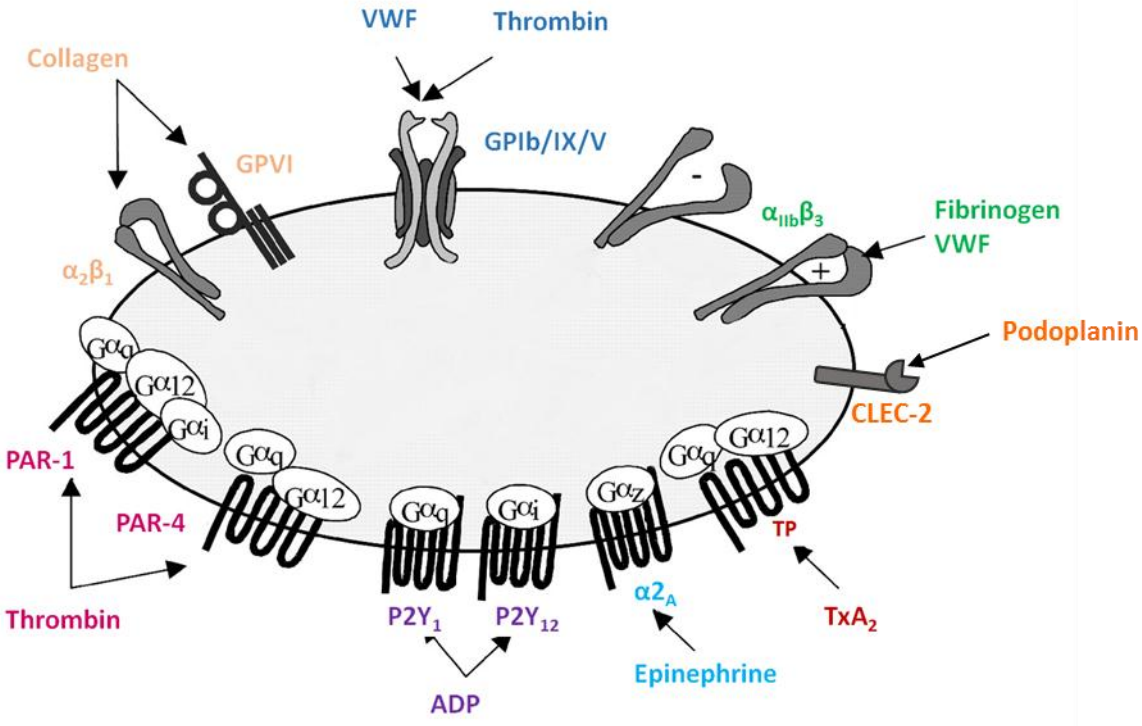


Figure 1.7: Major platelet receptor-ligand interactions. Shown in this diagram are platelet integrins α2β1 (yellow) and αIIbβ3 (Green) and their ligands collagen and fibrinogen/vWF respectively. Platelets express G-protein coupled receptors such as thrombin receptors called protease activated receptors (PARs) (pink) and ADP receptors (Purple). Receptors for Epinephrine and TxA_2 are also shown. In orange is the platelet receptor CLEC-2 and its ligand podoplanin. *(Image adapted from Rivera et al., 2009).*

1.3.3 Platelet function in primary haemostasis

Haemostasis is the physiological process which stops bleeding at an injured blood vessel, while maintaining normal blood flow elsewhere in circulation. Haemostasis can be subdivided into primary haemostasis, secondary haemostasis and fibrinolysis (Gale, 2013). The main physiological function of blood platelets is to prevent blood loss in primary haemostasis by the formation of a 'platelet plug'. Therefore, platelets have been labelled as "Band-Aids" of the bloodstream (Thon *et al.,* 2012). Secondary haemostasis refers to the deposition of insoluble fibrin that is generated by the coagulation cascade. Finally, fibrinolysis results in the breakdown of blood clots during wound healing involving the interplay between a number of enzymes (Gale *et al.,* 2011).

Under physiological conditions, a healthy endothelium provides a non-adhesive surface for platelets and therefore they do not normally interact with the surface of healthy vessels. However, in areas of vascular injury, the subendothelium is exposed and platelets may adhere quickly to different extracellular matrix components, and then form a platelet plug. This process is achieved through three distinct processes – platelet adhesion, platelet activation and secretion, and platelet aggregation (Clemetson, 2012).

1.3.3.1 Platelet adhesion

Depending on the matrix proteins exposed to blood and the hemodynamic conditions, platelet adhesion entails a collaborative effort of various platelet receptors, fundamentally leading to platelet activation and aggregation. The ECM elements that platelets adhere to include proteins such as collagen, vWF, fibronectin, laminin and fibrinogen amongst others (Ruggeri, 2007; Broos *et al.,* 2011). Among these subendothelial substrates, the thrombogenic fibrillar collagens type I and III are the most powerful intermediaries of platelet adhesion due to their robust activating potential and affinity for vWF (Farndale *et al.,* 2003).

Early adhesive interactions between platelets and the ECM are dictated by local rheological environments. At areas of low shear ($<1000s^{-1}$) in veins and large arteries, platelet adhesion mainly involves collagen, fibronectin and laminin. At areas of high shear rates ($>1000s^{-1}$) such as small arteries or atherosclerotic vessels, the interaction between vWF and platelet receptor GPIbα is required to slow down fast flowing platelets, enabling the formation of additional bonds and definitive arrest of platelets.

Following vascular damage, initial platelet 'tethering' is mediated by the interaction between the A1 domain of vWF deposited in the subendothelial matrix of the damaged vessel wall, and the GPIbα in the platelet receptor GPIb-IX-V (Figure 1.8, (A)). This interaction is particularly important at high shear rates supporting platelet translocation (i.e. decelerating platelets and keeping them in close contact with the endothelium) over the subendothelium, but not stable adhesion (Ruggeri and Jackson, 2013)**.** This interaction allows engagement of other platelet receptors. vWF/GPIb-IX interface also induces platelet activation signalling events, resulting in integrin activation (Li *et al.*, 2010).

Following platelet tethering, platelet collagen receptors, GPVI and α2β1, interact with exposed collagen and promote platelet adhesion and activation. GPVI is non-covalently coupled to the Fc Receptor chain (FcRγ) (Tsuji *et al.*, 1997; Jarvis *et al.*, 2002) and has been acknowledged as the major signalling receptor for collagen. FcRγ has an immunoreceptor tyrosine-based activation motif (ITAM) on its cytoplasmic sphere. After collagen binding to GPVI, the ITAM motif on the GPVI/FcRγ complex is phosphorylated, resulting in activation of the Syk kinase pathways that phosphorylate downstream targets, ultimately resulting in increased cytosolic Ca^{2+} and subsequent platelet shape change, granule secretion and integrin activation.

GPVI has a low affinity for collagen, rendering it unable to mediate stable adhesion alone. The α2β1 integrin then maintains stable adhesion to collagen. α2β1 stimulates downstream steps indirectly by reinforcing GPVI-collagen interactions (Broos *et al.*, 2011) and by direct signalling leading to activation of αIIbβ3. The importance of α2β1 in platelet adhesion has been a topic of deliberation for decades (Nieswandt *et al.*, 2001; Mazzucato *et al.*, 2009) as the predominant thought was that GPVI was solely responsible for signalling. However, platelet Ca^{2+} signalling is markedly dissimilar between GPVI and α2β1 (Mazzucato *et al.*, 2009) and it seems that the alliance regarding GPVI and α2β1 supports optimal platelet adhesion.

The final step of stable platelet adhesion occurs via binding of platelets to other ECM components such as fibronectin, laminin and immobilised vWF. Platelets bind to fibronectin via the α5β1 receptor and αIIbβ3, whilst adhesion to laminin is mediated by their α6β1 receptor. Stable binding of platelets elicits activation pathways involving tyrosine kinases and signal transduction GPCR receptor signalling, cumulatively resulting in elevated cytosolic Ca^{2+} levels, cytoskeletal reorganisation and integrin activation as discussed below.

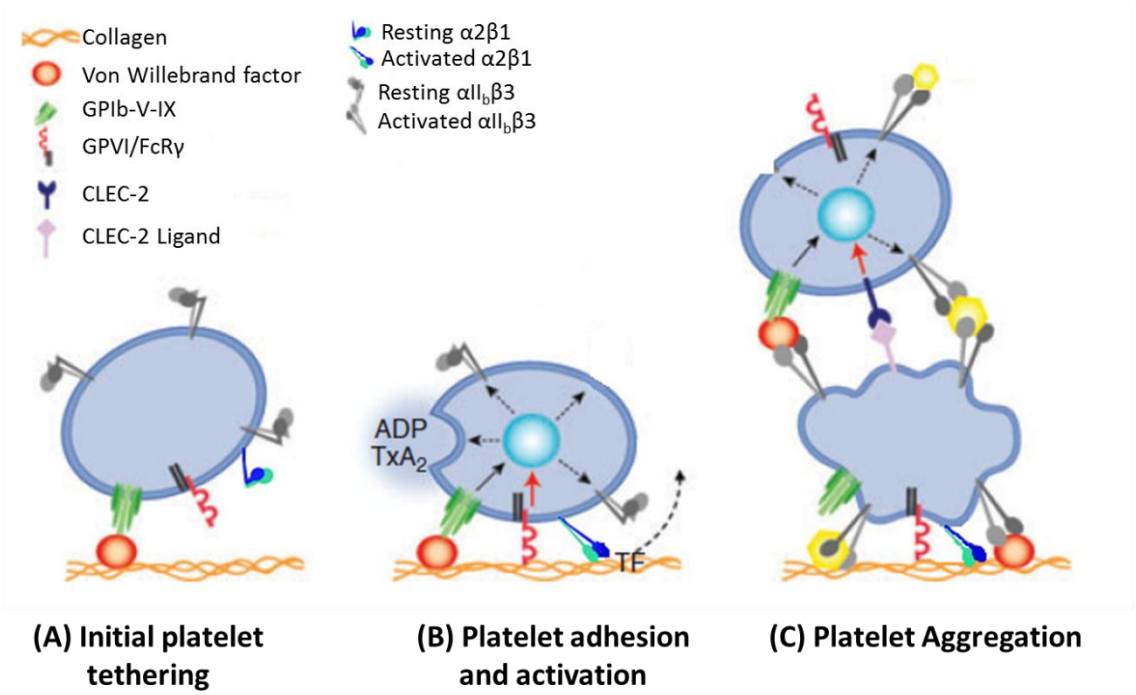


Figure 1.8: Platelet function in primary haemostasis. Vascular wall damage leads to exposure of collagen and subendothelial proteins. Platelet binding to immobilised von Willebrand factor (vWF) initiates adhesion to the ECM, thereby allowing the collagen receptors GPVI and α2β1 to interact with their ligands and mediate platelet activation. Platelet activation is reinforced by locally formed thrombin and platelet-derived ancillary mediators such as adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2). These series of events encourage a shift in the β1 and β3 integrins from a low affinity resting state to a high affinity activated state through 'inside out signalling' enabling firm platelet adhesion and aggregation. Platelet aggregates are stabilised by fibrin formation and signalling events between neighbouring platelets comprising numerous platelet receptors *(Image adapted from Nieswandt et al., 2011).*

1.3.3.2 Platelet activation and secretion

Once platelet adhesion has occurred at the site of vessel wall damage, and subsequent signaling to the platelet cytoplasm, platelet activation needs to be maintained for haemostasis to continue (Figure 1.8, (B)). Essential for the amplification of platelet activation is the production and release of soluble agonists at the site of damage (Zharikov and Shiva 2013), which act in an autocrine and paracrine manner to amplify platelet activation and recruit further circulating platelets.

These agonists consist of TxA_2, ADP, epinephrine and thrombin. ADP is secreted from platelet dense granules and binds to its relevant receptors, $P2Y_{12}$ and $P2Y_1$ on the platelet surface (Kaplan and Jackson, 2011). ADP is also released from red blood cells at the site of vascular damage (Rivera *et al.,* 2009). Binding of ADP initiates a full complement of activation events such as elevation of intracellular platelet Ca^{2+}, TxA_2 synthesis, protein phosphorylation, shape change, granule release, and most importantly, activation of αIIbβ3 (Andrews and Berntd, 2004). $P2Y_{12}$ is also the target of a class of antiplatelet drugs called thienopyridines (ticlopidine, clopidogrel, prasugrel), widely used in the prevention of vascular events in patients with CVD.

TxA_2 is a potent platelet agonist synthesised from arachidonic acid through the COX pathway and TxA_2 synthase enzymes. It subsequently binds to TPα and TPβ receptors that differ in their cytoplasmic tails, causing vasoconstriction, shape change, protein phosphorylation, secretion and platelet aggregation (Nakahata *et al.,* 2009; Angiolillo *et al.,* 2010). Indeed, high levels of TxA_2 has been implicated in CVD, whilst inhibition of TxA_2 synthesis through aspirin-mediated COX inhibition is a major antiplatelet target.

The agonist thrombin rapidly accumulates at sites of vascular damage and has major functions in promoting and stabilizing thrombus formation. Platelets release factors that support the activation of prothrombin, which after a complex series of sequential events in the coagulation cascade, results in the generation of thrombin (Monroe *et al.,* 2000). The increase in cytosolic Ca^{2+} after platelet activation results in platelet phosphatidylserine (PS) exposure on the activated platelet membrane providing a procoagulant surface for thrombin to interact with its PAR1 and PAR4 (G protein-coupled) receptors.

Unlike every other ligand involved in platelet activation that function by binding in a reversible mode to their individual receptors, thrombin activates its PAR-receptors by cleaving an N-terminal part at a consensus site. Cleavage exposes a new binding site that acts as a ligand to activate the receptor. Thrombin is the most powerful platelet activator, initiating an entire complement of platelet responses (shape change, granule secretion, TxA_2 synthesis, aggregation etc.) (Candia, 2013). Thrombin can activate platelets at extremely low concentrations and within seconds, increases the cytosolic level of Ca^{2+}, eliciting downstream signalling events. Unlike ADP, TxA_2 or thrombin, the catecholamine epinephrine is a weak agonist unable to cause shape change alone. However, it works collectively with the other agonists increasing their activatory potential. Epinephrine's mode of action is to inhibit cAMP formation by the platelet α_2A-adrenergic receptor (Horn *et al.*, 2005).

1.3.3.3 Platelet aggregation

Following platelet adhesion and activation, the ultimate step in primary haemostasis is platelet aggregation, caused by crosslinking of αIIbβ3 on adjacent platelets by fibrinogen (Figure 1.8, (C)). While platelet aggregation is a complex process involving different receptors (αIIbβ3 and GPIbα) and ligands (fibrinogen, fibronectin, and vWF), the main process revolves around the integrin αIIbβ3. On resting platelets, integrin αIIbβ3 has a low affinity for its ligands fibrinogen and vWF, which dramatically increases upon platelet activation.

Binding of the main agonists to their respective receptors described above induce intracellular signals that disrupt the complex between the cytoplasmic tails of αIIbβ3. This ultimately leads to a conformational change in its extracellular globular head domains from a low affinity resting state to a high affinity activated state in order to bind extracellular ligands such as fibrinogen and vWF. This irreversible activation of αIIbβ3 is a prerequisite for the development of irreversible platelet aggregates. Due to the symmetrical nature of fibrinogen, platelets can be 'bridged' and platelet aggregates are formed enabling the formation of a platelet plug (Ruggeri, 2007).

While αIIbβ3 is the major player in platelet aggregation, couplings between other platelet receptors and their ligands could be incorporated in aggregation (Figure 1.9). Some of these comprise CD40 ligand (Andre *et al.,* 2002) interaction with αIIbβ3, the vWF–GPIb complex (Kulkarni *et al.,* 2000), and an involvement of fibronectin in stabilising platelet aggregation (Ni *et al.,* 2000; Cho and Mosher, 2006), yet the roles of these mechanisms (and others) in platelet aggregation remain to be clearly elucidated. Cadherin-6 was recently acknowledged as a new counter-receptor for αIIbβ3 that could be involved in platelet aggregation (Dunne *et al.,* 2012). Furthermore, GPVI, has been identified as a receptor for fibrin (Alsheri *et al.,* 2015) and has therefore been suggested as a central player not only in platelet adhesion to collagen, but also in both thrombus formation and stability. Importantly, multiple adhesive ligands regulate platelet aggregation, however further investigation is required to elucidate their contribution to haemostasis and thrombosis under different conditions.

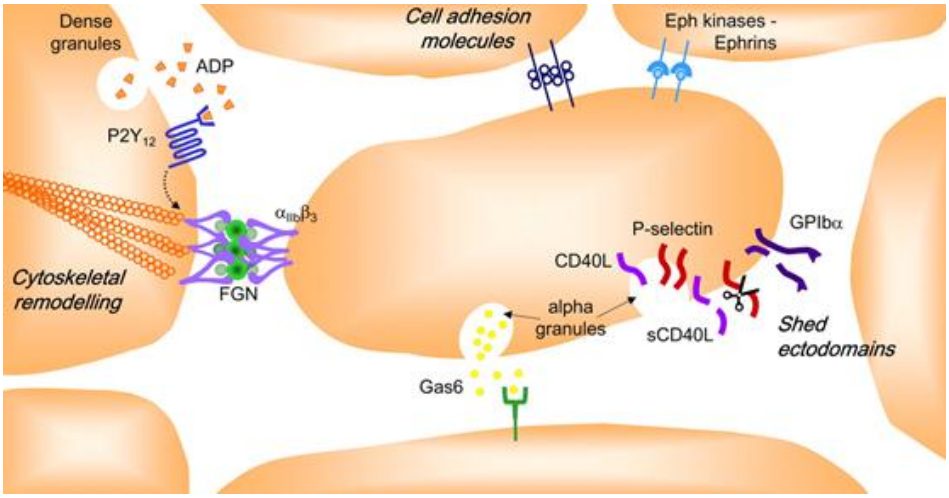


Figure 1.9: Multiple platelet receptors and ligands involved in aggregation. While αIIbβ3 and fibrinogen (FGN) interaction is the key to platelet aggregation, there are interactions between multiple adhesive receptors and their ligands such as cell surface ephrins and CD40L. The intercellular space between aggregating platelets facilitates a shielding environment for the accumulation of soluble agonists (ADP, thrombin, and TxA_2) *(Image from Jackson et al., 2007).*

Bi-directional ‘inside out’ and ‘outside in’ signals are transferred by the two subunits of integrins that mediate integrin conformation and platelet function (Li *et al.*, 2010). These important context-specific concepts are explained below. After platelet stimulation with ADP, the signal from the ADP receptors is conducted to the intracellular domain and the cytoplasmic tail of αIIbβ3 and subsequently transmitted through a series of events to the extracellular domain (inside-out) causing a conformational change in the extracellular domain that binds to its ligand (Figure 1.10). Inside-out signaling necessitates binding of talin and kindlins to the cytoplasmic domain of β3. After ligand binding (to fibrinogen or vWF, for example) a signal is sent to the cell (outside-in) to control platelet function such as filopodia and lamellipodia extension to dull platelet spreading (Shattil *et al.*, 2004; Li *et al.*, 2010). Furthermore, αIIbβ3 outside-in signals can also act as a break to curb excessive platelet activation by activated SHIP-1 (Dai *et al.*, 2016). While the early signalling mechanisms of copious platelet receptors differ during adhesion and aggregation, they ultimately congregate into common intracellular signalling events (Li *et al.*, 2011).

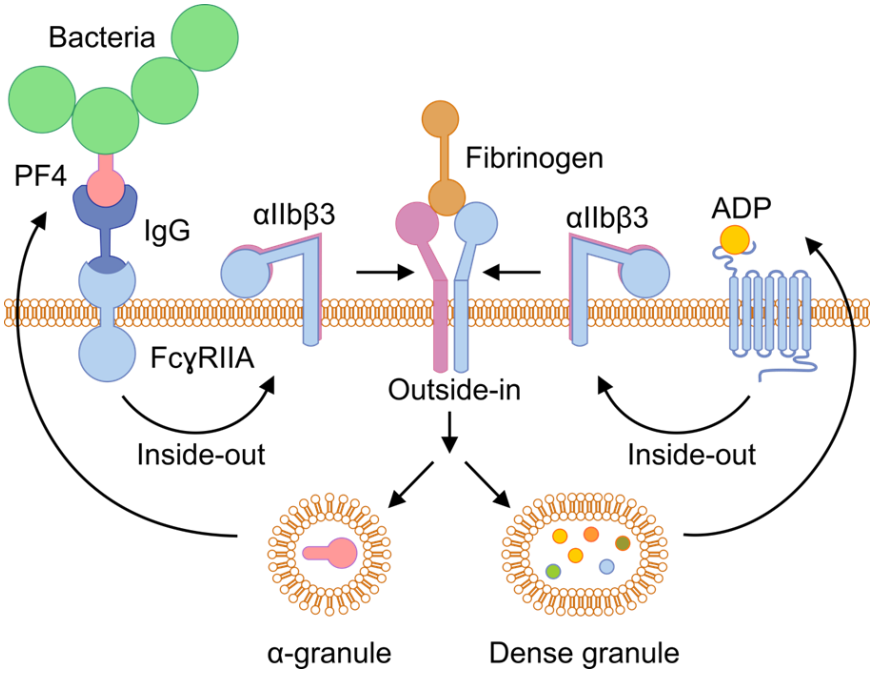


Figure 1.10: Outside-in and inside-out signalling in platelets. Bacteria-bound platelet factor four (PF4) is recognised by plasma IgG. This complex binds to the platelet receptor FcγRIIA, which causes inside-out signalling resulting in a conformational change in integrin αIIbβ3 to an activated state. Binding of fibrinogen to the activated αIIbβ3 induces outside-in signalling and release of platelet granule contents. Dense granule secreted ADP provides positive feedback by binding to the ADP receptors and stimulating more inside-out signalling. *(Image from Naik et al., 2014).*

1.3.3.4 Influencing factor in platelet aggregation

A significant factor influencing platelet aggregation is the distinct shear environment experienced within the vascular system. Platelets are subjected to fluctuating haemodynamic (blood flow parameters) conditions *in vivo* such as shear stress and shear rate. The latter refers to the rate of increase of blood flow velocity, whereas the former denotes the force per unit area on the vessel wall (Sakariassen, 2015). Shear rates experienced by platelets range from slow flow environment in veins (shear rate 10 s^{-1} to 500 s^{-1}) to small arteries (approx. 2000 s^{-1}) to diseased or pathological arteries, where extremely high shear rates (up to 40,000 s^{-1}) have been described (Jackson, 2007; Sakariassen, 2015). Increasing shear rate both activates platelets itself and promotes their relocation toward the vessel wall/thrombus

At low shear rates (< 1000 s^{-1}), platelet aggregation is primarily facilitated by αIIbβ3–fibrinogen interactions. At shear rates typically over 5000 s^{-1} (but between 1000-10000), a two-step sequential process occurs. The first depends on the adhesive properties of GP1bα and αIIbβ3 and is facilitated by the formation of reversible platelet aggregates. The second relies on the generation of platelet agonists and involves the irreversible activation of αIIbβ3 to form stable aggregates (Munnix *et al.*, 2009). In stenotic arteries, at pathological shear rates (>10,000 s^{-1}), platelet aggregation can occur autonomously of platelet activation, given that soluble vWF is present (Ruggeri *et al.*, 2006). This is characterised by the construction of unstable platelet aggregates which roll over the vWF surface, facilitated by vWF-GP1bα interactions. Accordingly, substrates fibrinogen and vWF, and receptors GPIbα and αIIbβ3, have distinctive but complementary roles in platelet aggregation subject to the haemodynamic environment.

1.3.4 Signals during platelet function

The role of platelets in haemostasis is reliant on the equilibrium between activatory and inhibitory signals (Jones *et al.*, 2012). Inhibitory signals from the vasculature prevent platelet activation in healthy vessels, ensuring a resting state is maintained. Without inhibitory signals, platelets would become activated even in the absence of activating signals. Activatory signals present at an injured blood vessel initiate platelet activation and thrombosis, and endogenous negative signalling regulators diminish activatory signals to manage thrombus development. The interplay between these signals is illustrated in Figure 1.11.

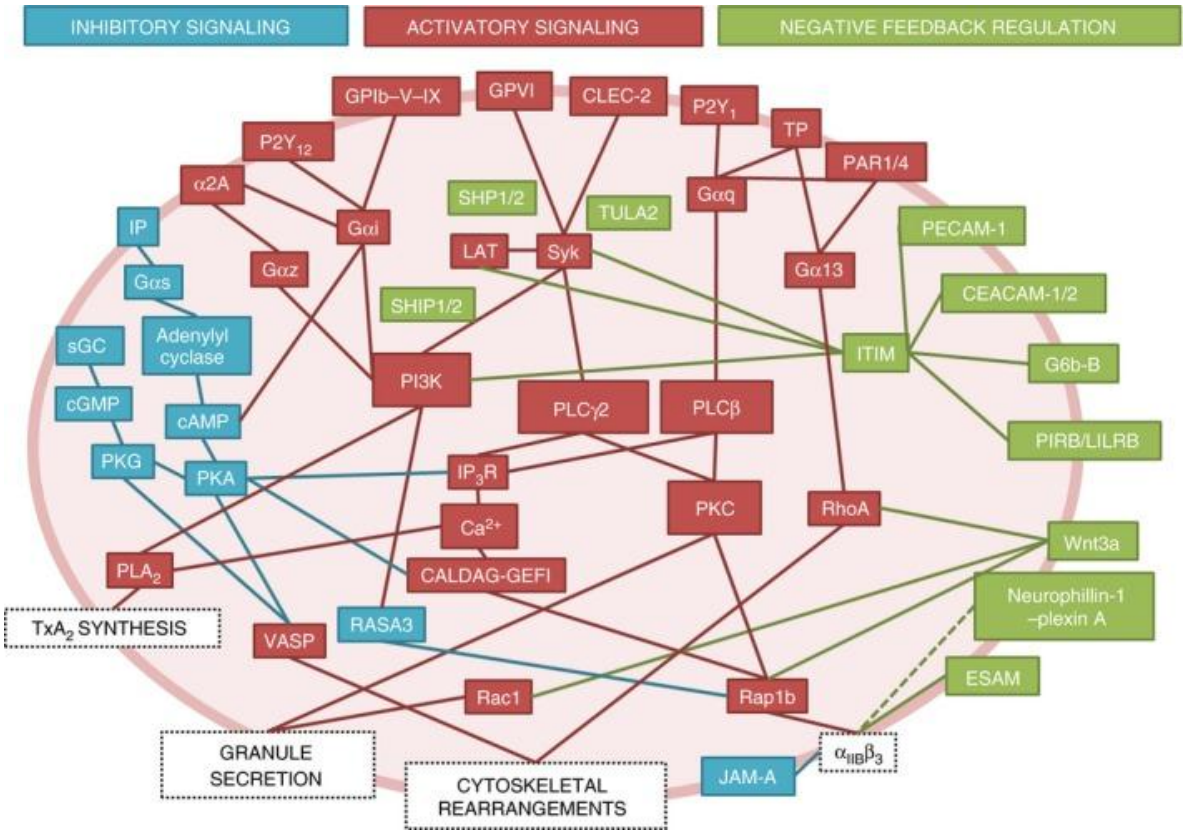


Figure 1.11: The complex network of platelet signals. Platelet signalling is an extremely complex process, with multiple agonists and negative regulators at play. Central signalling molecules common to most pathways include phospholipase C (PLC), protein kinase C (PKC), and phosphatidylinositide-3-kinase (PI3K) acting as hubs of platelet regulation. Inhibitory signals that suppress platelet function are represented by the blue boxes and lines, e.g. binding of PGI_2 to its IP receptors on the platelet surface activates guanylyl cyclase (GC) to elevate intracellular cAMP levels which ultimately suppress platelet adhesion and activation. Activation signals are represented by the red boxes and lines, e.g. platelet adhesion to collagen through GPVI initiates a signalling cascade involving a number of downstream targets, resulting in αIIbβ3 activation. The green boxes and lines represent negative feedback and inhibitory signals which limit platelet activation. For example, Wnt3a binding to platelets regulates RhoA and inhibits platelet adhesion. *(Image from Bye et al., 2016).*

The primary platelet inhibiting signals produced by healthy ECs are nitric oxide (NO) and prostacyclin. Both NO and prostacyclin relax blood vessels and prevent platelet activation (Broos *et al.,* 2011; Smolenski *et al.,* 2012). NO is synthesised from several cells including platelets and ECs and plays vital roles in maintaining platelets in a resting state (Gkaliagkousi and Ferro 2011; Gambaryan *et al.,* 2016). While ECs provide the main source of NO, RBCs are also implicated in NO-dependent regulation of cell function (Benz and Fleming, 2016). Endothelial-derived NO disperses throughout the platelet membrane where it's effects are mainly mediated by a cGMP pathway. NO binds to its intracellular receptor soluble guanylyl cyclase (sGC), which causes an increase in intracellular cGMP levels. cGMP effects are primarily mediated by the cGMP-dependent protein kinase (PKG). Activation of PKG results in protein phosphorylation. These proteins inhibit increases in intracellular Ca^{2+} cytoskeletal reorganisation, integrin activation (Subramanian *et al.,* 2013), activation of G-proteins (RAP1), eventually leading to inhibition of platelet adhesion and granule secretion and aggregation (Smolenski *et al.,* 2012).

Prostacyclin (PGI_2) is a physiological anti-aggregating agent produced constitutively by ECs as a result of arachidonic acid metabolism by cyclooxygenase (COX) enzymes. Binding of PGI_2 to its IP receptors on the platelet surface activates guanylyl cyclase (GC) to elevate intracellular cAMP levels and ultimately activation of the cAMP-dependent protein kinase A (PKA). Activated PKA can phosphorylate several regulatory proteins including vasodilator-stimulated phosphoprotein (VASP). Phosphorylated VASP inhibits the reorganisation of the actin cytoskeleton, which is associated with platelet spreading and aggregation. Furthermore, VASP is associated with focal adhesions where its phosphorylation prevents the inside-out activation of αIIbβ3 and therefore platelet aggregation. Elevation of platelet cyclic nucleotides obstructs nearly all platelet activation signalling pathways, efficiently preventing complex intracellular signalling cascades, cytoskeletal reorganisation, integrin activation, granule release and expression of pro-inflammatory molecules (Schwarz *et al.,* 2011).

Following endothelial damage, endogenous inhibitory signals are lost and platelets react rapidly to limit blood loss as described in section 1.3.3. The activating stimuli, such as collagen, vWF, ADP, TxA_2 and thrombin, which induce platelet adhesion, activation and aggregation ultimately regulate a central set of signalling mediators that support activation. Three core mediator families of platelet activation are phospholipase C (PLC), protein kinase C (PKC) and phosphatidylinositide-3-kinase (PI3K), which are well characterised and underlie two crucial events in platelet activation – secretion of amplifying mediators and activation of integrin αIIbβ3. Evidently there are other regulators of platelet function, however these three represent main hubs in the multifaceted network of platelet signalling that in turn regulates other aspects of signalling such as calcium signalling

After initiation of platelet activation and thrombus development, negative signalling mechanisms manage activation to ensure that platelet aggregation does not escalade out of control, thus avoiding excessive thrombus formation. Examples of such negative regulators and pathways include the immunoreceptor tyrosine-based inhibition motif (ITIM) containing receptors that are postulated to reduce activation of PLC, PI3K and integrin αIIbβ3 (Jones *et al.*, 2012). Platelets contain a number of ITIM receptors such as G6b-B and platelet-endothelial cell adhesion molecule 1 (PECAM-1), highlighted in Figure 1.11 (Senis *et al.*, 2014). The Wnt–β-catenin pathway has recently gained attention as negative regulator of platelet function (Steele *et al.*, 2009; MacCauley *et al.*, 2013). The Wnt family is comprised of 19 Wnt glycoproteins. Wnt3a is one of these glycoproteins which is secreted from activated platelets, enabling the self-regulation of platelet activation. Wnt3a is a GP secreted from activated platelets permitting self-regulation of activation (Steele *et al.*, 2009). Wnt3a has been suggested to activate the canonical Wnt–β-catenin pathway as constituents of this pathway have been recognised in platelets. Wnt binding results in the inhibition of all aspects of platelet function – adhesion, shape change, granule release, integrin αIIbβ3 activation and aggregation. The regulation of small GTPases such as Rap1, Rac1, RhoA and Cdc42 have been suggested as players in this inhibition of platelet function (Steele *et al.*, 2009). These pathways are less well characterised than the classical activation pathways (Bye *et al.*, 2016).

1.3.5 Platelet function beyond haemostasis

Besides their long-established roles in haemostasis, platelets are increasingly recognized as pivotal players in numerous other processes ranging from inflammation and atherosclerosis, fighting microbial infection and tumour growth and metastasis (shown in Figure 1.12)**.**

Platelets are equipped to influence inflammation and the innate immune response and infection. They express a collection of pattern recognition receptors called toll-like receptors (TLRs) that identify molecular motifs called pathogen associated molecular patterns (PAMPs) and initiate immune responses (Cognasse *et al.,* 2015). Platelets express functional TLR 1-9, whilst TLR2 stimulation in platelets by bacteria through the activation of the PI3K signalling pathway induce a pro-inflammatory response (Blair *et al.*, 2009). Platelet TLR9 activation has been associated with thrombosis and oxidative stress, and is found within the previously mentioned novel T-granules in platelets (Thon *et al.,* 2012).

Platelets interact with numerous white blood cells, such as leukocytes, monocytes and granulocytes through different receptor-ligand interactions (P-Selectin and PSGL-1 interaction) enabling communication between these cell types (Zago *et al.,* 2008). The capacity of platelets to store and release copious inflammatory cytokines and chemokines is intimately associated to their role in inflammation. Platelets release microparticles linked to inflammatory pathways and which are associated with inflammatory diseases such as rheumatoid arthritis (Boilard *et al.,* 2010). Platelets play key roles in infection, acting as sentinels in pathogen detection. They are the first cell type to arrive at areas of vascular infection and have been demonstrated to aid the immune system against bacterial and viral infections (Metcalf *et al.,* 2014). Thrombocytopenia (low platelet count) is a well-established manifestation of sepsis and experimental work suggests that platelets play a functional role in the pathogenesis of sepsis and multi-organ failure (Guclu *et al.,* 2013).

Platelets have significant roles in the pathogenesis of metastasis (Labelle *et al.*, 2011). Tumour cells can aggregate platelets *in vitro* and it has been proposed that platelet adhesion to metastatic cells can act as a 'cloak' around circulating tumour cells therefore acting as a shield for immune clearance. This phenomenon of platelet cloaking has resulted in pro-survival, pro-angiogenic and epithelial mesenchymal transition (EMT) in cancer cells (Cooke *et al.*, 2015). Platelets also release growth factors such as VEGF and PDGF that can expedite tumour growth (Erpenbeck and Schon, 2010). Platelets are deeply implicated in wound healing and bone health, and indeed the use of platelet rich plasma (PRP) therapy (i.e. rich in growth factors and bioactive substances) is effective in osteoarthritis (Patel *et al.*, 2013) and in muscle damage such as rotator cuff tendinopathy (Kesikburun *et al.*, 2013).

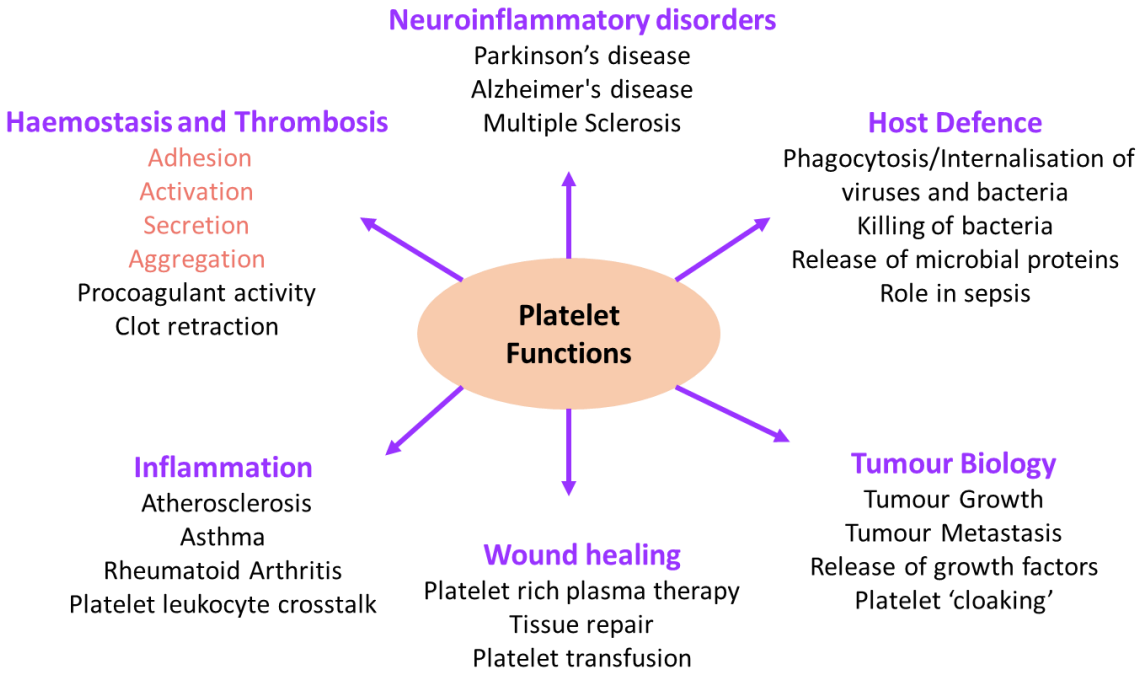


Figure 1.12: The multifunctional platelet. Besides their main functions in haemostasis and thrombosis, which are highlighted in pink above, platelets have a multitude of functions in other processes. These include roles in inflammation, immune response and fighting infection, tumour biology, wound healing and involvement in Neuroinflammatory disorders. *(Figure adapted from Harrison, 2005).*

1.3.6 Platelet function tests (PFT)

The different functions of platelets may be reliably detected with a wide spectrum of tests (Table 1.1). These can be utilised to identify inherited or acquired platelet dysfunction, monitor antiplatelet therapy, manage various aspects of platelet banking and transfusion, and to aid in the understanding of platelet physiology in basic research. PFTs are centred around principles of platelet function such as platelet adhesion and aggregation, platelet function under shear conditions, and measurement of the platelet releasate (Paniccia *et al.*, 2014).

Platelet function testing began with the evaluation of the bleeding time (the time taken for platelets to occlude an *in vitro*wound), using the Duke procedure (Duke *et al.*, 1910), before the development of light transmission aggregometry (LTA) revolutionised the study of platelet function. Considered the historical gold standard, LTA is a relatively easy technique that involves stirring a suspension of platelet rich plasma in a cuvette in the presence of a platelet agonist (such as ADP or collagen). The cuvette is placed between a light source and photocell. Agonist addition causes *in vitro* platelet aggregation and changes in light absorbance, which is detected by the photocell (Born, 1960).

1.3.6.1 Tests based on platelet adhesion under shear stress

Investigation of platelet function in the environmental milieu of whole blood under conditions that take into account most of the physiological parameters that influence platelet adhesion and aggregation (red blood cells, white blood cells, plasma) is important. The PFA-100, The Impact-R Cone and Plate analyser and the global thrombosis test are examples of such assays. The PFA-100 assesses platelet aggregation under high shear where platelets are activated in whole blood by an amalgamation of shear stress and agonists, resulting in closure of an aperture. In this assay, blood samples are aspirated at high shear ($5000\text{-}6000s^{-1}$) through a capillary in the instrument cartridge which is coated with agonists such as collagen and ADP. This causes platelet aggregation and occlusion of the cartridge (Karger *et al.*, 2012).

The Impact-R Cone and Plate analyser is a point of care (POC) device which measures global platelet function by testing platelet adhesion and aggregation in whole blood under close to arterial shear conditions (Varon *et al.,* 1997; Peerschke *et al.,* 2007). In this assay, platelet adhesion is dependent on plasma proteins vWF, fibrinogen and RBCs and WBCs.

The Impact-R has shown to correlate effectively with other platelet function tests. A study by Gremmel *et al.,* (2009) compared five platelet function tests in in 225 patients on dual antiplatelet therapy. Included were the gold standard LTA, the VerifyNow aspirin assay, the PFA-100, Multiplate electrode and the Impact-R. They reported that only the correlation between results from LTA and Impact-R were statistically significant. Furthermore, results from a comparative study of platelet reactivity in healthy subjects and subjects with metabolic syndrome (metS) showed that Impact-R analysis correlated with the measurement of agonist induced P-selectin expression using flow cytometry. In this study, increased SC % (platelet adhesion) correlated with higher levels of P-selectin expression in metS subjects, reflecting activation dependent expression of adhesion receptors

Furthermore, the addition of platelet agonists such as arachidonic acid (AA) and ADP in the system has enabled the evaluation of dual antiplatelet therapy (Savion and Varon, 2006; Anand *et al.,* 2007; Koshy *et al.,* 2014). The system is effective in the assessment of platelet function disorders in adults, (Goldschmidt *et al,* .2008) children (Revel-Vilk *et al.,* 2007) and new-borns (Levy-Shraga *et al.,* 2006), the latter of which demonstrated that the device is a convenient screening assay before more labour intensive diagnostic steps are engaged.

Table 1.1: Platelet function tests. Typical platelet function tests range from assessment of their primary haemostatic function including measurement of granule secretion using lumi-aggregometry to whole blood shear based assays, which measure platelet function under flow conditions. Platelet function can be tested in washed platelets, whole blood or platelet rich plasma.

Method	Sample	Principle	Reference
Platelet aggregation assays			
Light transmission aggregometry (LTA)	Citrated PRP	Measurement of light transmission in response to agonist-induced platelet activation	Born, (1962)
Whole blood aggregometry (WBA)	Citrated WB	Monitors changes in electrical impedance in relation to agonist-induced platelet aggregation	Toth *et al.*, (2006)
VerifyNow	Citrated WB	Turbidimetric optical detection of platelet aggregation in whole blood in response to agonists/inhibitors	Smith *et al.*, (1999)
Plateletworks	Citrated WB	Platelet counting pre- and post-activation	Campbell *et al.*, (2008)
Assays measuring release reactions			
Lumi-aggregometry	Citrated WB	Combination of LTA/WBA with nucleotide release	Cattaneo *et al.*, (2009)
Soluble platelet release markers (E.g. PF4, scD40L)	Urine, serum, citrated plasma	Typically measured by ligand binding ELISA immunoassays	Chung *et al.*, (2009)
Shear-based assays			
PFA-100/200	Citrated WB	High-shear platelet adhesion and aggregation during formation of a platelet plug	Koessler *et al.*, (2012)
Impact R Cone and Plate Analyser	Citrated WB	Shear-induced platelet adhesion–aggregation upon specific surface	Varon *et al.*, (2008)
Global thrombosis test	Native WB	High-shear platelet plug formation – measurement of time cessation of WB flow	Saraf *et al.*, (2009)
Platelet activation based assays			
VASP phosphorylation	Citrated WB	Flow cytometry or ELISA measurement of VASP phosphorylation	Aleil *et al.*, (2005)
Flow cytometry	Citrated WB, PRP, Washed platelets	Measurement of platelet glycoproteins, activation markers, platelet leukocyte aggregates, platelet microvesicles	Michelson *et al.*, (2007)

1.3.6.2 Platelet indices

In addition to platelet function tests, platelet indices are useful as inexpensive non-invasive biomarkers for assessing platelet activation (Budak *et al.,* 2016). Platelet indices are straightforwardly measured by semi-automated counters in complete blood counts (CBC) and usually include four factors; platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), and depending on the analyser, platelet large cell ratio (PLCR). PLT is a universal indicator of haemostasis in a clinical setting and is utilised as a sensitive biomarker for a range of diseases. High PLT, even within the physiological range of 150 - 450 µl, is associated with a greater risk of thrombosis and CVD suggesting that enhanced PLT encourage platelet hyperactivity and a proinflammatory state (Thaulow *et al.,* 1991; Khandekar, 2006). However, the consequence of high platelet numbers that are still within physiologic ranges remains unclear (Madjiid and Fatemi, 2013).

The indices MPV, PDW and PLCR are quantitative measures of the variability in platelet size. MPV reflects the average platelet size while PDW reflects the volume variability in platelet size (Khandekar *et al.,* 2006; Vagdatli *et al.,* 2010). The volume of circulating platelets is heterogeneous with subsequent functional differences. Some authors suggest that larger platelets are metabolically more active than smaller platelets, that they have faster rates of aggregation and release higher quantities of prothrombotic elements such as TxA_2 and ADP (Thompson *et al.*, 1983, Mangalpally *et al,* .2010). MPV and PDW levels can be altered in several diseases including T2DM (Jindal *et al.,* 2011; Yilmaz 2016), CVD (Beckler *et al.,* 2015) and atherosclerosis (Berger *et al.,* 2010), and in this regard they have been suggested as markers of subclinical platelet activation.

PLCR and PCT may serve as sensitive biomarkers of platelet health (Kaito *et al.,* 2004; Yilmaz, 2016). PLCR indicates the percentage of large platelets present in blood (Budak *et al.,* 2016). PLCR is significantly higher in subjects with dyslipidaemia compared to healthy subjects (Grotto and Noronha, 2004) and higher in children with T2DM compared to healthy children (Malachowska *et al.,* 2015). Moreover, Rechcnski *et al.,* (2013) believe that PLCR has the potential to be a prognostic biomarker, but more research into its relationship with platelet function is required. Importantly, thrombogenicity of large platelets may put individuals at higher risk of acute cardiovascular events.

PCT is the volume of blood occupied by platelets as a percentage, similar to the erythrocyte measurement of haematocrit (HCT). PCT reflects total platelet mass and is calculated as PLT x MPV/10^7, providing comprehensive information about platelet activity. PCT has been proposed as a novel predictor of cardiovascular risk and higher PCT is associated with the risk of reinfarction and long-term mortality in CVD patients (Gul *et al.,* 2016). However, the clinical significance, reference values and efficacy of some of these parameters are still under exploration.

1.3.6.3 Measurement of other blood cell components

Also measured by CBC analysis are RBC and WBC components of blood such as Haematocrit (HCT) and Haemoglobin (Hgb). In peripheral blood, there is ample interplay between RBCs, WBCs and platelets (Ho, 2004) and altered levels of blood cells and their morphology have been associated with CVD (Maajid 2013; Cetin *et al.,* 2014). Platelet adhesion and aggregate size, as assessed by the Impact-R system, is influenced by platelet indices, RBC and WBC (Shenkman *et al.,* 2000; Peerschke *et al.,* 2007). RBCs are known to encourage platelets toward the vessel wall (Chestnut and Han, 2013), which can affect platelet adhesion and aggregation. In this context, it is important to investigate the associations between the various indices of each blood cell to interpret the multicellular contribution to both thrombogenesis and CVD risk.

1.3.6.4 Novel platelet function testing

Identification of primary platelet function changes in activation, aggregation, and secretion are typically measured by the aforementioned techniques. However, novel techniques continue to emerge and develop the knowledge surrounding the regulation of platelet function. The modern "omics" revolution enables simultaneous quantification of hundreds of molecules (e.g. protein or mRNA) from a single sample and their signatures may be reflective of platelet function changes. The amalgamation of transcriptomic and proteomic data and subsequent bioinformatic analysis will lead to a more complete characterisation of platelet function in response to environmental stimuli (Mc Redmond *et al.,* 2004; Ferroni *et al.,* 2012). Epigenetics and its ancillary elements, including platelet secreted microvesicles (MVs), microRNA (miRNA), and regulation of the platelet mitochondrial genome are new avenues of investigation and testing in platelet research (Bacarelli and Byun, 2015; Dahiya *et al.,* 2016; Kaudevitz *et al.,* 2016).

1.4 Epigenetics

Epigenetics describes modifiable changes that occur to genes, *via* chemical modifications and/or varying states of chromatin organisation and structure, which alter gene expression without altering the actual DNA sequence (Pozharny *et al.*, 2010). Smoking habits, obesity, aging, and physical fitness among others are examples of environmental factors that have been suggested to have a long-term influence on epigenetic changes (Fraga *et al.,* 2005). Epigenetics can be classed as three distinct but highly interconnected processes; DNA methylation, histone modification and RNA-associated silencing, shown in Figure 1.13. DNA methylation and histone modification alter DNA accessibility for transcriptional machinery and chromatin structure (Figure 1.13). These changes are heritable and can be passed down between generations through either mitosis or meiosis.

1.4.1 Epigenetic mechanisms

DNA methylation involves the addition of a methyl group to the 5-position of cytosine by DNA methyltransferases. This generally occurs at areas known as CpG islands – where a guanine is directly followed by a cytosine on the same DNA strand. Methyl groups control gene expression by binding to promotor sites of the gene and act like a switch if the gene is required to be turned off. This changes the affinity of methylation-sensitive binding proteins, and is associated with transcriptional gene silencing. (Freson *et al.,* 2012). Whilst required for normal development, changes in DNA methylation have been linked to CVD conditions such as atherosclerosis, for example, the atheroprotective estrogen receptor genes ESR1 and ESR2 that are usually expressed in smooth muscle cells (SMCs) are hypermethylated in atherosclerosis (Weinhold, 2006; Lund and Zaina, 2011).

Unlike the platelet transcriptome and proteome, the investigation of epigenetic processes is almost a completely unexplored area in platelet biology, as analysis of these mechanisms requires DNA (Freson, Izzi and Van Geet, 2012). Platelets have functionally active mitochondria (Antony *et al.,* 2012). Like nuclear DNA, mitochondrial DNA (mtDNA) can also be methylated, moderating control of mitochondrial gene expression.

Understanding epigenetic regulation of mitochondrial genes in platelets is proving crucial to understanding their implication in CVD development. Novel research by Baccarelli and Byun (2015) showed that CVD patients had significantly higher platelet mtDNA methylation than healthy individuals in MT-CO1, MT-CO2, MT-CO3, and MT-TL1, genes involved in ATP synthesis. These results suggest that DNA methylation in platelet mitochondria could be a potential contributor to CVD development through the regulation of platelet function.

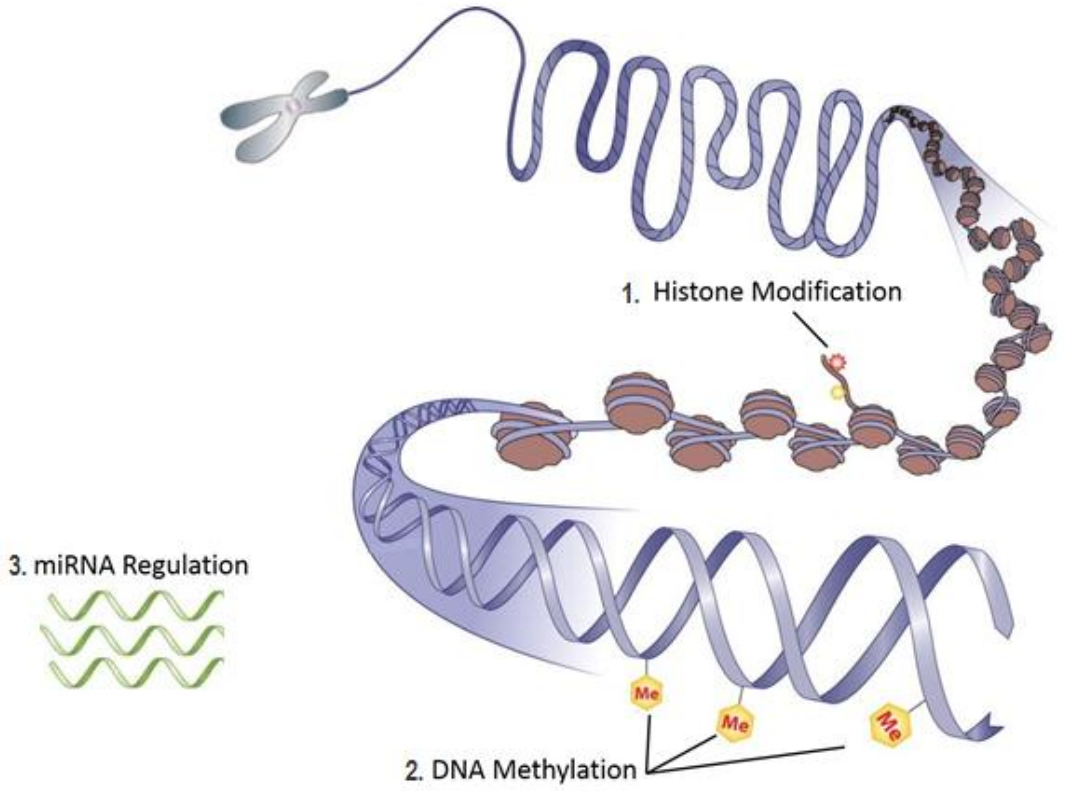


Figure 1.13: The three epigenetic mechanisms. DNA methylation involves the addition of a methyl group to the 5-position of cytosine. Histone modifications refer to the posttranslational alterations to the N-terminals of histone tails, which subsequently modify histone-DNA interactions, controlling gene transcription. miRNA regulate gene expression at a post-transcriptional level. Crosstalk between miRNA and epigenetic pathways form an epigenetic-miRNA regulatory circuit, arranging the whole gene expression profile. *(Figure adapted from Wade et al., 2015).*

Histone modifications are another epigenetic mechanism (Figure 1.13). Histones are proteins that condense and structure DNA into units known as nucleosomes. Nucleosomes consist of 8 core histone proteins that contain modifiable amino-acid tails. Histone modifications refer to the posttranslational alterations of the N-termini of these tails that subsequently modify histone-DNA interactions (Webster *et al.,* 2013). Acetylation is a major type of histone modification involving the addition or removal of an acetyl group. This process is catalysed by proteins known as histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs). This mechanism changes chromatin structure to influence gene expression. Tightly wound bounded chromatin tends to reduce gene expression, whereas loosely wound DNA is more highly expressed (heterochromatin versus euchromatin) (Weinhold, 2006).

RNA-based processes are the third type of epigenetic mechanism. Non-coding RNA (ncRNA) has emerged as a factor in the chromatin-based regulation of gene expression (Zaratiegui *et al.,* 2007). ncRNAs can be classified as either long or short. Whilst long ncRNAs are a major form of RNA-based epigenetic regulation, some small ncRNAs also have a function in chromatin-based silencing. For example, microRNA (miRNA) are a subset of ncRNA that negatively regulate gene transcription by degrading or repressing target mRNA (Calin *et al.,* 2007). miRNA can control the expression of important epigenetic regulators such as histone deactylases and DNA methyltransferases and similarly, DNA methylation and histone modification can control the expression of some miRNA, thereby forming a feedback loop (Sato *et al.,* 2011; Osello *et al.,* 2014). This complex crosstalk between miRNA and epigenetic pathways forms an epigenetic-miRNA regulatory circuit, arranging the whole gene expression profile. Disruption of this circuit interferes with normal physiological functions and can contribute to disease process. miRNA will be discussed in detail in Section 1.5.

1.4.2 Epigenetic drift

Individuals age differently and lifestyle factors such as exercise or smoking have been shown to delay or accelerate the aging process, respectively (Blair *et al.*1989). These observations have resulted in the search for molecular markers to predict and monitor age-associated disease. DNA methylation is associated with chronological age over time (Bollati *et al*, .2009; Ailish *et al.,* 2012). Epigenetic drift is the term given to epigenetic modifications as they occur as a direct consequence of age (Tan *et al.,* 2016). This was previously observed when DNA methylation marks in identical twins differed increasingly as a function of age (Fraga *et al.,* 2009). Monozygous twins share a common genotype and while this study found that the twins were epigenetically synonymous during childhood, older twins showed significant differences in their total content and dispersal of histone acetylation and DNA methylation, subsequently impacting their gene expression. Disparity in these epigenetic marks between twins can be as a result of lifestyle influences such as diet, physical activity levels and smoking.

Epigenetic drift affects the majority of the genome. The aging effects of epigenetic drift over time are highlighted in Figure 1.14. Aging is a natural process associated with de-regulation of histone tags, senescence-associated ncRNA, a gradual deregulation of DNA methylation, in a potential linear fashion depicted by age-predictive linear models (Hannum *et al.,* 2013). However, an individual exposed to either environmental or genetic risk factors may show signs of premature aging as a result of either lifestyle or environmental risk factors. This can be characterised by altered or increased deregulation of DNA methylation, increasing the susceptibility to chronic diseases like CVD. Furthermore, it has been hypothesised that a healthy lifestyle may reserve a more intact epigenome and longevity (Tschendorff *et al.,* 2013).

DNA methylation undergoes extensive changes during differentiation of self-renewing stem cells (Broske *et al.,* 2009; Oshima and Iwama, 2014; Choudry *et al.,* 2016). Indeed, DNA methylation is involved in the production of MKs and their subsequent transcription (Georgantas *et al.,* 2007). Lifestyle components such as physical inactivity and obesity may incur epigenetic changes in the production of platelets from megakaryocytes.

Thus, platelets could signify a marker of megakaryocyte epigenetic drift, holding substantial predictive potential of disease. Epigenetic changes in the megakaryocyte genome such as hypomethylation of genes determining PLT or changes in histone acetylation with aging have been suggested to play an important role in platelet function (Daly, 2011).

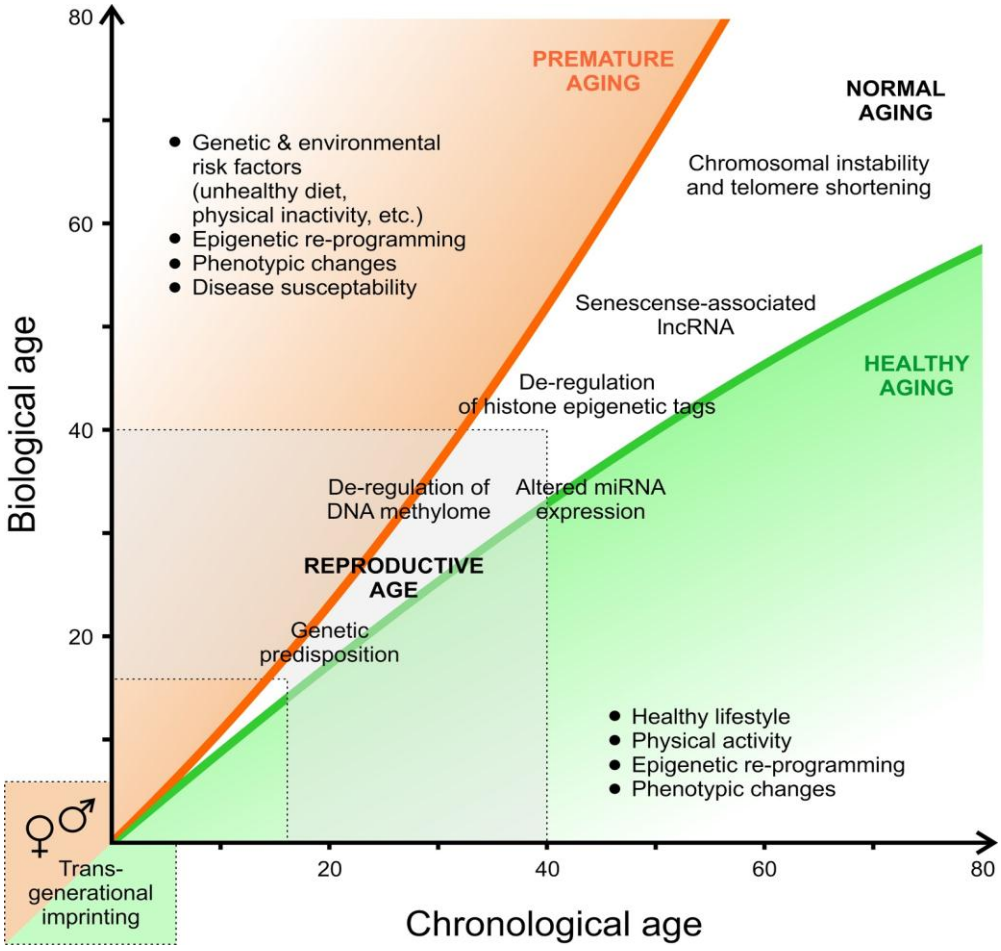


Figure 1.14: Epigenetic drift. Epigenetic drift represents changes in DNA methylation as a function of time. Normal aging (white) results in de-regulation of DNA methylation amongst other features. Premature aging as a result of exposure to risk factors can display altered DNA methylation. By contrast, healthy aging as a result of physical activity can result in a different epigenetic profile. Epigenetic Drift over time can result in measurable differences between biological and chronological age. *(Adapted from Teschendorf et al., 2013).*

1.5 MicroRNA

1.5.1 Introduction to miRNA

miRNAs are short (18-24) nucleotide long non-coding RNAs that function in post transcriptional regulation of gene expression. They inhibit translation by binding with the 3′-untranslated (UTR) regions of their target mRNA. Here, the miRNA promotes either translational repression or degradation of target mRNA, depending on complementarity with the mRNA strand, resulting in silencing of various genes (Van Rooij and Olsen, 2007). Genetic screening of the nematode, *Caenorhabditis elegans*, gave rise to the identification of the first miRNAs, lin-4 and let-7 (Lee *et al.,* 1993), which functioned as regulators of developmental timing. However, miRNAs were not recognised as a distinct category of biological regulators until the early 2000's. Since their discovery, huge progress has been made in the field and they are now labelled as "fine-tuners" of cellular phenotypes by repressing expression of proteins that are inappropriate for particular cell types.

To date, more than 4000 miRNA sequences have been identified across a variety of species, of which over 2000 are encoded by the human genome (Van Dongen *et al.,* 2008; Kozomara *et al.,* 2011). They are thought to be involved in the regulation of ~ 60 percent of human genes and are convoluted in most physiological and pathological processes (Ple *et al.,* 2012; Laffont *et al.,* 2013). miRNA can be classed as intronic, exonic and intergenic miRNA, according to the location of their encoding genes (Wang *et al.,* 2009). Intronic miRNA account for approximately 70 percent of all transcribed miRNA *(*Bartel *et al.,* 2004; Rodriguez *et al.,* 2004). Intergenic miRNA are found between two protein-coding genes and employ their own promoters and regulatory molecules (Sarnow *et al.,* 2006). As miRNA target mRNA by imperfect binding, each miRNA has numerous targets, enabling miRNA to regulate over half of the human genome (Bartel *et al.,* 2009; Rigoutous *et al.,* 2009). Various online algorithms are available to predict miRNA binding sites such as Miranda, TargetScan and miRBase (Peterson *et al.,* 2014). Importantly, a single miRNA can regulate hundreds of different mRNA, whilst each mRNA can be regulated by several miRNA (Edelstein and Bray, 2011).

1.5.2 miRNA biogenesis and function

The miRNA population within a cell can be highly concentrated, with tens of thousands of miRNA copies per cell. They possess a long half-life (a half-life of between 28-220 hours has been reported) and are very stable (Van Rooij *et.al., 2007*; Zhang *et al.*, 2012). miRNA biogenesis beings with transcription, followed by several processing steps to generate the mature miRNA. Each step of the miRNA biogenesis pathway is subject to regulation (Finnegan and Pasquinelli, 2013). Encoded by the genome of nucleated cells, miRNA are transcribed by RNA polymerase II from miRNA coding genes into long, hairpin stem-looped primary miRNAs (pri-miRNAs) of approximately 70 nucleotides. They are 5'7-methylguanosine capped and poly-adenylated at the 3'end, the latter allowing recognition by the microprocessor complex (Bartel, 2004).

miRNA biogenesis begins in the nucleus with transcription by RNA polymerase II to produce the long primary miRNA (Pri-miRNA). This houses a hairpin that holds the mature sequence (Winter *et al.,* 2009). The hairpin is then cleaved at the 3' poly-A tail and the 5' cap by a microprocessor processing complex that includes the endonuclease III type protein, Drosha, acting in conjunction with the protein DGCR8 and an RNase III enzyme. This results in a hairpin looped precursor miRNA (pre-miRNA) of about 60-70 base pairs in length. RAN-GTPase and the transport protein Exportin-5 then actively export the pre-miRNA from the nucleus.

The following step to maturation is processing by the RNAse III protein, Dicer, which removes the hairpin loop and cleaves the pre-miRNA into a 21-24 nucleotide duplex miRNA. The duplex is then unwound by the Argonaute 2 (Ago2) complex producing a mature and complimentary minor strand, sometimes termed the passenger or star (*) strand. The Ago2 complex incorporates the mature strand into the multiprotein complex RNA-Induced Silencing Complex (RISC) (Osman and Falker, 2011).

Using imperfect pairing, the RISC then binds the miRNA to the 3'UTR of the target mRNA. The binding occurs on what is identified as the "seed region" of the miRNA that is normally 6-8 nucleotides long, beginning at the second nucleotide. Usually, the star strand is degraded quickly after the mature strand has been incorporated into RISC, although it is sometimes possible for both strands to be cleaved into mature, functional miRNA, depending on the tissue or cell type (Bhayani *et al.,* 2012). Depending on which strand side (either 3' or 5') is incorporated into RISC, the miRNA is subsequently labelled as miRNA 3' or miRNA 5'.

Once incorporated into RISC, miRNAs regulate gene expression by either of two posttranscriptional mechanisms: mRNA degradation or translational repression. The degree of complementarity between the target mRNA and miRNA decides which mechanism occurs. If full complementarity between the strands is achieved, mRNA degradation will be the process of choice. Otherwise, translation will be repressed with sufficient complementarity (Bartel 2004). In the majority of mammals, miRNA induces mRNA degradation in over 80 % of cases (Guo *et al.,* 2010). Although deeply conserved, many miRNA express a modest effect on their target mRNA often less than 2 fold (Bartel *et al.,* 2004). However, several miRNAs often work together to co-target an mRNA. This is possible as many mRNA 3’UTRs have more than four highly conserved seed sequences. The full biogenesis of miRNA is depicted in Figure 1.15.

Turnover of mature miRNA is required for rapid changes in miRNA expression profiles. Regulation of miRNA maturation occurs during various steps throughout their biogenesis at both a transcriptional and post-transcriptional level (Finnegan and Pasquinelli *et al.,* 2013). Transcriptional regulation involves alterations to the expression of a host gene such as epigenetic regulation (where miRNA genes located near CpG islands in the genome are found to be hypermethylated). Post-transcriptional mechanisms define modifications in miRNA processing and stability (Gulyaeva and Kushlinskiy, 2016).

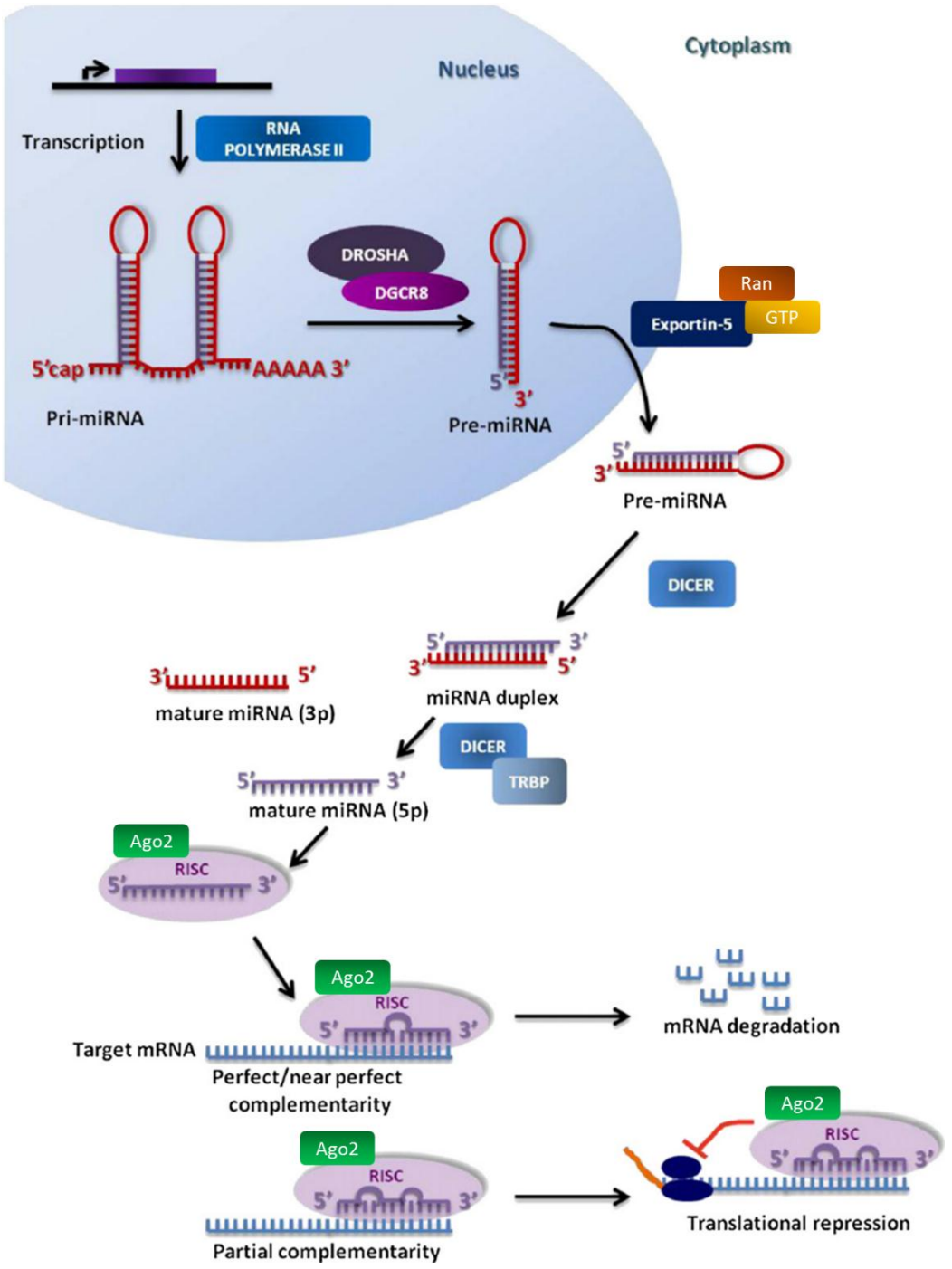


Figure 1.15: The biogenesis of microRNA. miRNA genes are usually transcribed by RNA polymerase II. This transcript folds into a hairpin loop structure called the pri-miRNA. The pri-miRNA is processed in the nucleus by the microprocessor complex of Drosha/DCGR8 to form an approximate 70 bp pre-miRNA. The pre-miRNA is exported to the cytoplasm by Exportin-5 and processed by Dicer to generate the miRNA-miRNA* duplex. The mature miRNA strand is incorporated into the RISC by Ago2. Here, the seed region of the miRNA binds to the 3'-untranslated region of the target mRNA resulting in either mRNA degradation or translational inhibition. *(Figure adapted from Winter et al., 2009).*

1.5.3 Platelet miRNA

Platelet function is a highly regulated process. Despite their anucleate nature, platelets accommodate a small but competent transcriptome that is employed for translation of various proteins with significant physiological functions. Platelets have been shown to retain genetic material derived from their megakaryocyte precursor (Figure 1.16). Approximately 32% of all human genes are present in platelets at the mRNA level (Ple *et al.,* 2012). It is well accepted that platelets contain the necessary splicing machinery, rough ER and polyribosomes that allow the synthesis of proteins required for their functions in haemostasis and beyond (Bijak *et al.,* 2013). Perhaps due to the requirement of sustaining a proteome over an ~ 8-day life span, the fact that the average half-life of a cellular protein is 46 hours, or the necessity to adapt to environmental stimuli, it is equitable to assume that the platelet must also retain its transcriptome, as well as processes of nucleated cells such as splicing, translating and posttranscriptional RNA mechanisms (Corey and Edelstein 2016).

The fact that platelets contain mRNA and are capable of protein synthesis has raised the issue of how these mRNAs are regulated. Notably, stored platelets in blood banks can synthesis integrin β3 (Thon and Devine, 2007). The existence and functionality of a miRNA pathway in the anucleate human platelet was first described in a landmark study by Landry and co-workers (2009), who showed by locked nucleic acid (LNA) microarray profiling, that platelets harboured an impressive number (219) of miRNA. They reported that the three most abundant miRNA in platelets were miR-223, miR-19a and Let-7c. Further analysis discovered the presence of functional processing miRNA machinery in platelets – Dicer and Ago2, suggesting that partial biogenesis of mature miRNA from pre-miRNA could occur within platelets themselves (Landry *et al.,* 2009; Thum and Dangwal, 2013) as pre-miRNAs have been identified at low levels (21 transcripts) in platelets (Ple *et al.,* 2012). Star or passenger strand miRNA have also been identified (Bray *et al.,* 2013). Accordingly, the detection of nuclear miRNA microprocessor Drosha and DGCR8 in platelets has not been observed, consistent with their anucleate nature. Moreover, miRNA-associated Ago2 complexes were identified, in addition to the presence of $P2Y_{12}$ in Ago2 precipitates, suggesting a regulation of $P2Y_{12}$ by miRNAs (Landry *et al.,* 2009).

The next breakthrough study in platelet miRNA biology revealed that a protein involved in platelet granule release, platelet vesicle-associated membrane protein 8 (VAMP8), was associated with distinctly different platelet aggregation responses to epinephrine in healthy donors, and that VAMP8 was regulated by miR-86 (Kondkar *et al.,* 2011). Since then the platelet-miRNA field has grown exponentially, whereby a number of studies have suggested a physiological role for miRNA in the regulation of platelet function. Most notably, research by Nagalla and colleagues (2011), who focused on the roles of miRNA as biomarkers of platelet reactivity and controllers of platelet mRNA disparity, demonstrated that miRNA profiles of healthy subjects (n=19) were associated with the response of platelet aggregation to epinephrine. They also employed a computational approach to produce possible miRNA-mRNA pairs (miR-200b: PRKAR2B, miR-495: KLHL5 and miR-107: CLOCK), pairings which were experimentally validated in cell lines. Networks of miRNA-mRNA pairs also associated with age and gender and race (Simon *et al.,* 2013; Simon *et al.,* 2014). Other reports on agonist-induced platelet activation by thrombin (Osman and Falker, 2011) and ADP (Cimmino *et al.,* 2012) show differential expression of platelet miRNA compared to resting platelets.

Progression in miRNA detection techniques has led to the revelation of 40 new miRNA sequences, expanding the total amount of platelet expressed miRNAs to more than twice that (544) of the initial finding (Ple *et al.,* 2012; Thum and Dangwal, 2013; Teruel Montoya *et al.,* 2014). Transcriptomic approaches show that miRNA make up the majority (80 %) of all small RNAs in platelets (Ple *et al.,* 2012). Furthermore, comparison of RNA and miRNA by cell type showed that despite low RNA yields, platelets express high quantities of miRNA compared to their nucleated counterparts (Teruel-Montoya *et al.,* 2014).

1.5.3.1 Abundantly expressed platelet miRNA

Evidently, a number of highly expressed miRNA have been characterised in human platelets, some of which are involved in myeloid cell differentiation, megakaryocytopoiesis and thrombopoeisis (Ple *et al.,* 2012). miR-223 has been identified as the most highly expressed platelet miRNA (Nagalla *et al.,* 2011; Edelstein and Bray, 201l; Halkein and Windt, 2013) and has roles in thrombopoeisis and megakaryocyte differentiation (Laffont *et al.,* 2013). miR-223 regulates ADP $P2Y_{12}$, a target for existing antiplatelet drug therapy. The 3'-UTR of $P2Y_{12}$ mRNA has been identified as complementary to the miR-223 seed region. Platelet miR-223 has also been observed to be decreased in subjects who show high levels of platelet activation whilst on clopidogrel therapy. Furthermore, miR-223-deficient mice show

reduced bleeding times, larger thrombi, and elevated sensitivity to low doses of thrombin, suggesting an important role of miR-223 in modulating platelet function (Landry *et al.,* 2009; Shi *et al.,* 2015).

miR-126 plays central roles in vascular inflammation and is thought to be the second most highly expressed miRNA in platelets (Fish *et al.,* 2008; Gatsiou *et al.,* 2012). miR-126 was found to correlate with circulating P-Selectin levels in T2DM subjects and this level was sensitive to aspirin treatment, signifying a platelet origin. miR-126 is postulated to regulate ADAM9 and $P2Y_{12}$ receptor expression in platelets and inhibition of miR-126 in mice distinctly reduces platelet aggregation (Kaudevitz *et al.,* 2016).

Existence of miRNA in platelets is multifaceted. Besides their obvious function as regulators of platelet protein expression, platelet miRNAs have been labelled as biomarkers of disease and platelet activation, markers of mature megakaryocyte miRNA and as a means of understanding megakaryocyte/platelet gene expression (Emmrich *et al.,* 2012; Edelstein *et al.,* 2013). The majority of platelet miRNA are supposedly formed in the megakaryocyte and packaged into platelets upon formation. Identification of some specific miRNA involved in megakaryopoieses and thrombopoeisis has been enabled by using either megakaryocyte-like cell lines and megakaryocytes generated from human CD34+ hematopoietic stem cells *in vitro*.

For example, miR-146b positively regulates megakaryopoieses by targeting and down regulating the megakaryopoieses inhibitor PDGFRA (Zhai *et al.,* 2014; Kamat *et al.,* 2014). miR-142 has also been reported to inhibit megakaryocyte production. In miR-142 knockout mice, platelet counts are decreased and MK differentiation is modified, including reduced proplatelet network establishment (Chapnik *et al.,* 2014). The total extent to which MK and platelet mature miRNA patterns correlate remains an area of active investigation. A significant correlation between the miRNA levels was found using three separate studies (Huessein *et al.,* 2009; Landry *et al.,* 2009; Edelestein *et al.,* 2013). However, as platelets are more accessible than megakaryocytes, they provide an efficient tool in which to study the relationship between the two cells (Freedman *et al.,* 2015).

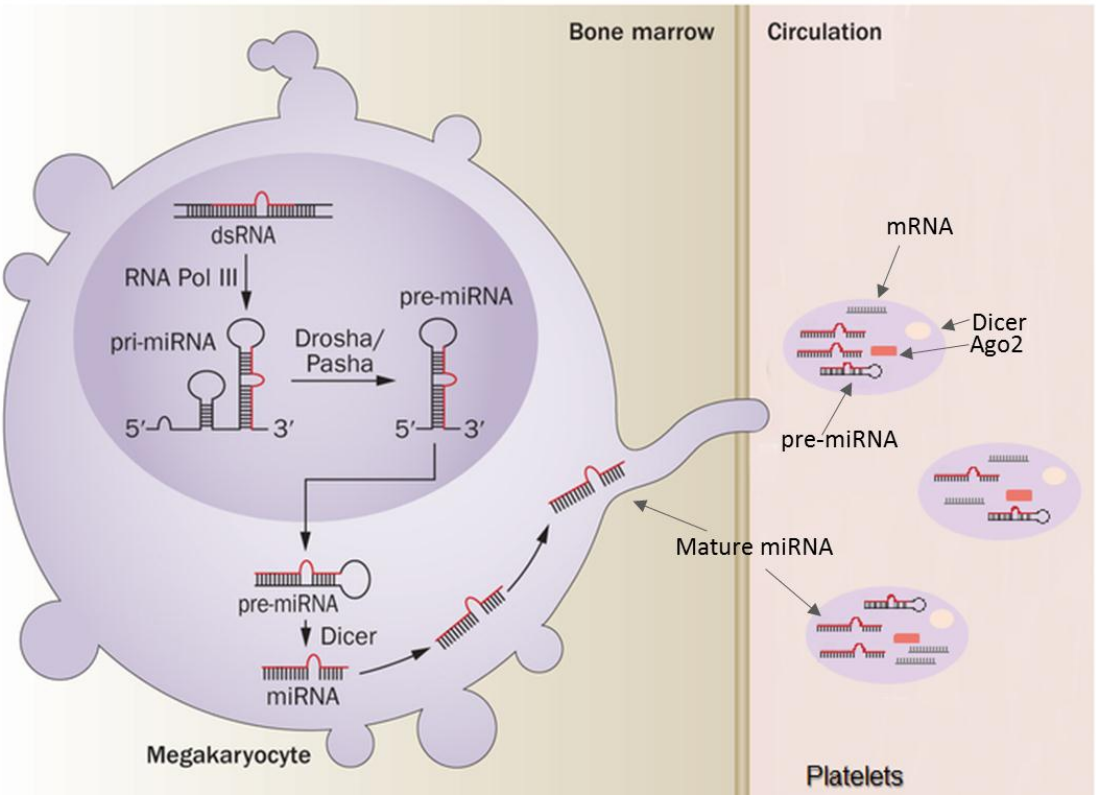


Figure 1.16: MicroRNA regulation in platelets. Megakaryocytes produce miRNAs according to the basic principles of miRNA biogenesis and maturation processes. Platelets are produced from megakaryocytes and retain both mature and pre-miRNA in addition to miRNA machinery Argonaute2 and Dicer. *(Adapted image from Freedman et al., 2016).*

1.5.4 Microvesicles and miRNA

Cells release various types of microvesicles (MVs) – defined as exosomes and microparticles (MPs) – from different origins, into the extracellular environment (Loyer *et al.*, 2014), where they play important physiological roles. Typically, Exosomes are described as 30-100nm in diameter, with MPs being larger and in the range of 100nm-1µm in diameter. Importantly, MVs carry both plasma membrane and cytosolic constituents (Burger *et al.*, 2013; Torreggiani *et al.*, 2014) and maintain a similar antigenic autograph to their parent cell, e.g. platelet and megakaryocyte specific MVs both express CD41, whereas red blood cell MVs express CD235a. MV release can be either pathologic or physiologic (Goubran *et al.*, 2015). MVs can be released under a plethora of different conditions, for example, during the natural processes of apoptosis or cell activation, under shear stress, cell damage or agonist interaction with cell surface receptors (Herring *et al.*, 2013). The two main types of MVs are shown in Figure 1.17.

Activated platelets release MPs by surface shedding of the plasma membrane, while exosomes are released by exocytosis of multivesicular bodies and alpha granules (Heijnen *et al.*, 1999; Gnatenko *et al.*, 2006). Platelet MPs are the most abundant cell-derived MPs, accounting for between 70-90% of all circulating MVs. Platelet MVs have been linked with both procoagulant activity and their levels are altered in pathological states including obesity and atherosclerosis (Pap *et al.*, 2009), whilst physical activity (Sossdorf *et al.*, 2011; Chaar *et al.*, 2011) and high stress conditions and are biomarkers of platelet activation (Nunes-Franca *et al.*, 2015).

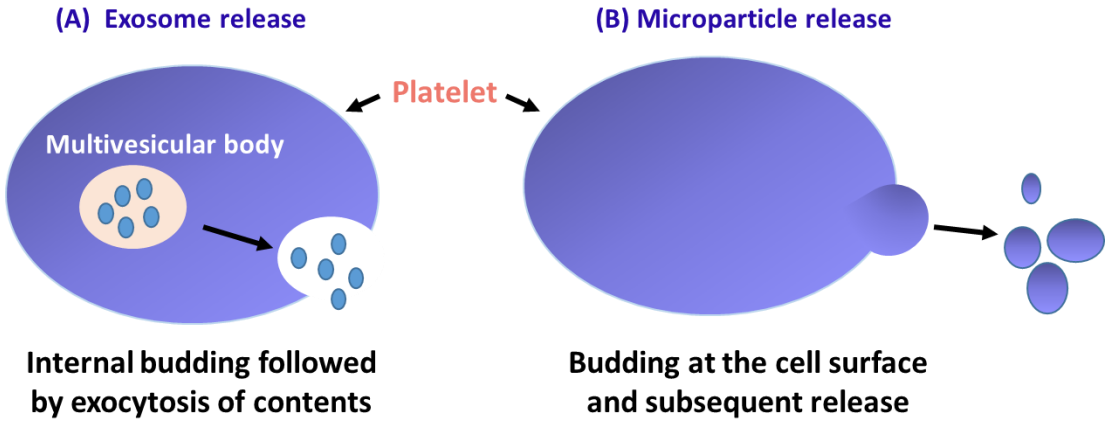


Figure 1.17: Two types of microvesicles released from platelets. Schematic depicts activated platelets releasing exosomes by exocytosis of multivesicular endosomal bodies and alpha granules (A), while microparticles are released by surface shedding of the plasma membrane (B). *(Image adapted from Kanada and Backman, 2015).*

However, their function in intercellular communication is perhaps the most interesting. Platelet MVs harbour a multitude of cell components including proteins and genetic material. Different activation pathways regulate both the quantity and type of MV subpopulations produced from platelets (Aatonen *et al.*, 2014), thus governing their molecular profiles and facilitating tailor-made participation in intercellular communication (Diehl *et al.*, 2012; Mooberry and Key 2016).

In this respect, perhaps the most intriguing feature regarding platelet miRNA is their extracellular function. miRNA can be packaged and delivered to distant cells in the form of platelet microvesicles, fulfilling novel processes of gene regulation in target cells (Diehl *et al.*, 2012). Figure 1.18 illustrates this process. Initial studies by Laffont *et al.,* (2013) and Gidlöf *et al.,* (2013) demonstrate the functionality of platelet miRNA. Functional complexes of miR-223 and Argonaute 2 protein (Ago2) packaged in MVs from activated platelets was found to modulate the expression of targeted endothelial cell endogenous mRNA transcripts FBXW7 and EFNA1 (Laffont *et al.,* 2013). This miR-223/Ago2 complex has also been shown to reduce expression levels of insulin-like growth factor 1 receptor in endothelial cells, and to promote human umbilical vein endothelial cell (HUVEC) apoptosis (Pan *et al.,* 2014). This type of platelet MV-cell interaction is illustrated in Figure 1.18.

Gidlof *et al.,* (2013) suggested that platelet miRNA could modulate vascular endothelial inflammatory responses. They described a down regulation of intercellular adhesion molecule 1 (ICAM-1) gene expression in cultured human microvascular endothelial cells after exposure to miR-320b, which is secreted upon platelet activation and reduced in platelet thrombi aspirated from patients with ST-segment elevation myocardial infarction (STEMI). The relevance of this intercellular transfer was further reinforced when Liang *et al.,* (2015) showed that platelet-released miR-223 through platelet MPs can encourage lung cancer cell invasion by targeting the tumour suppressor EPB41L3.

Novel research has shown that platelet MPs containing miRNA can also be internalised by primary human macrophages and deliver functional miR-126-3p. miR-126-3p caused a downregulation in the expression of four predicted mRNA targets of miR-126-3p and a reduction in macrophage cytokine release. This suggests that platelet miRNA-containing MPs can modify the macrophage transcriptome and potentially reprogram their function (Laffont *et al.*, 2016). Finally, platelet-derived exosomes have recently been shown to carry miR-223, miR-339 and miR-21, which can be transferred to SMCs affecting PDGRFβ (Tan *et al.,* 2016).

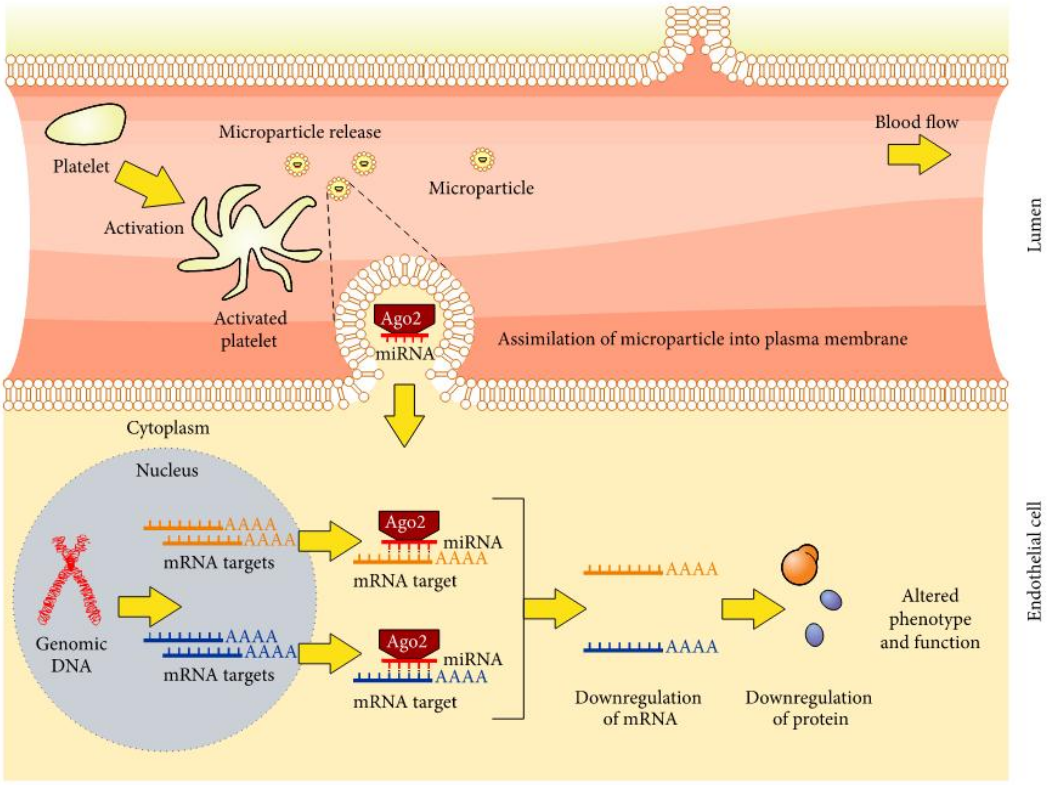


Figure 1.18: Platelet MVs act as intercellular transporters of functional Ago2-miRNA complexes. Activated platelets release MV packages with functional Ago2-miRNA complexes that can be up taken by human aortic endothelial cells. Platelet-derived miRNA accumulates within the cell, regulating expression of endothelial genes at the mRNA and protein level, resulting in altered phenotype and function.

1.6 Cardiovascular disease (CVD)

CVD is an umbrella term used to describe a broad group of disorders of the circulatory system. It includes atherosclerosis, myocardial infarction (MI), arrhythmia, angina, hypertension, and stroke amongst others. Types of CVD are varied and complex; hence there are multiple pathological mechanisms through which disease may arise. CVD often exists as an underlying complication where the initiation of CVD will appear well before any clinical threshold, such as acute conditions of myocardial infarction or stroke. The World Health Organisation (WHO) predict that the number of people who die from CVDs, will reach 23.3 million by 2030. In Ireland, CVD accounts for over 33% of all deaths in those over 65 years of age (Irish Heart Foundation, IHF), as seen in Figure 1.19.

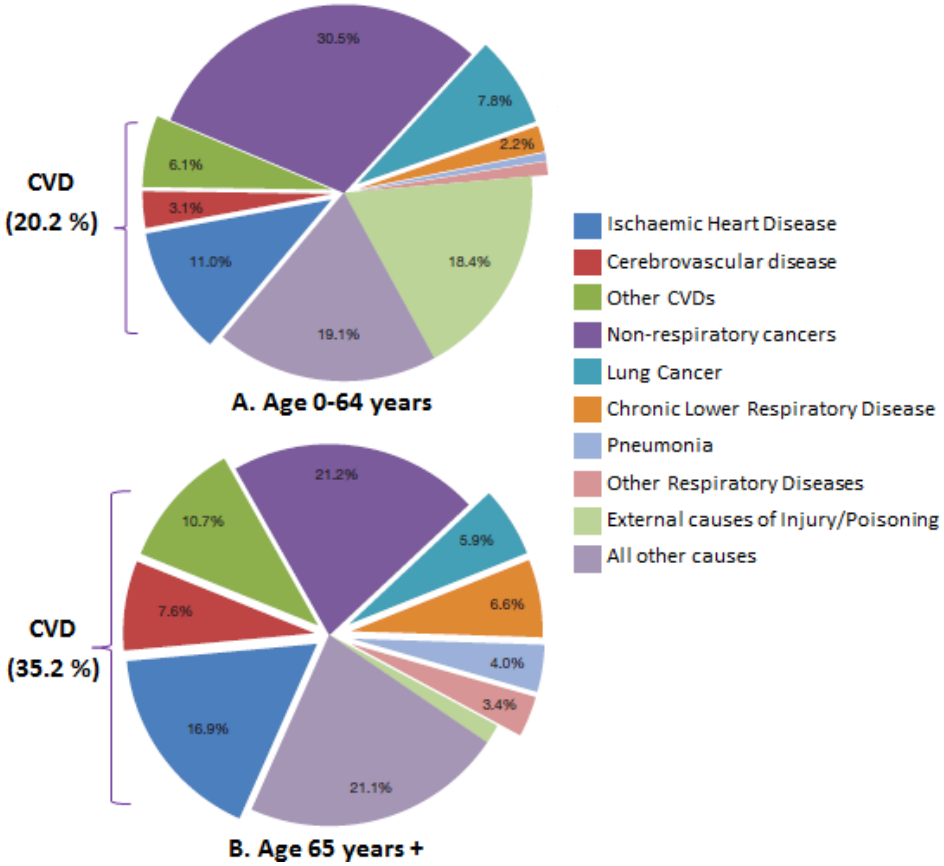


Figure 1.19. Causes of mortality in Ireland in 2014. A shows a pie chart representing age 0-64 years, while B represents the older population. CVD accounted for over 33 percent of deaths in those over 65 years. *(Department of Health, 2014).*

Atherosclerosis is the most common CVD and the main cause of coronary heart disease (CHD) and stroke (Figure 1.20). It is regarded as a chronic inflammatory condition, specifically affecting the intimal layer of arterial blood vessels and is characterised by arterial remodelling after the sub-endothelial accumulation of fatty substances called plaques. Atherosclerosis is a complex disease involving the dynamic deregulation of different cell types including SMCs, ECs, platelets and white blood cells (Wierda *et al.*, 2010; Lusis, 2012). The pathogenic process of atherosclerosis begins early in life, specifically during childhood and adolescence.

Whilst multi-factorial in its development, a brief synopsis of atherosclerosis would describe a modulation of haemodynamics resulting in endothelial dysfunction, platelet activation and inflammation, with elevated expression of inflammatory cytokines, and recruitment of leukocytes (monocytes) to the endothelium, concomitant with a reduction in NO generation. Migration of monocytes into the sub-endothelium follows, leading to their activation and transformation into macrophages that digest oxidised LDL cholesterol molecules to form foam cells. Foam cells, the main component of 'fatty streaks' subsequently undergo apoptosis, releasing stored lipids and generating a necrotic lipid core within the vessel intima.

Expansion of this core increases its thrombogenicity and is separated from the blood by a fibrous ECM cap. Atherosclerotic plaques can be classified as either stable or unstable, depending on their composition. The stable plaque can expand until it occludes the vessel, or it can rupture, releasing its thrombotic content with subsequent thrombosis (Hopkins, 2013).

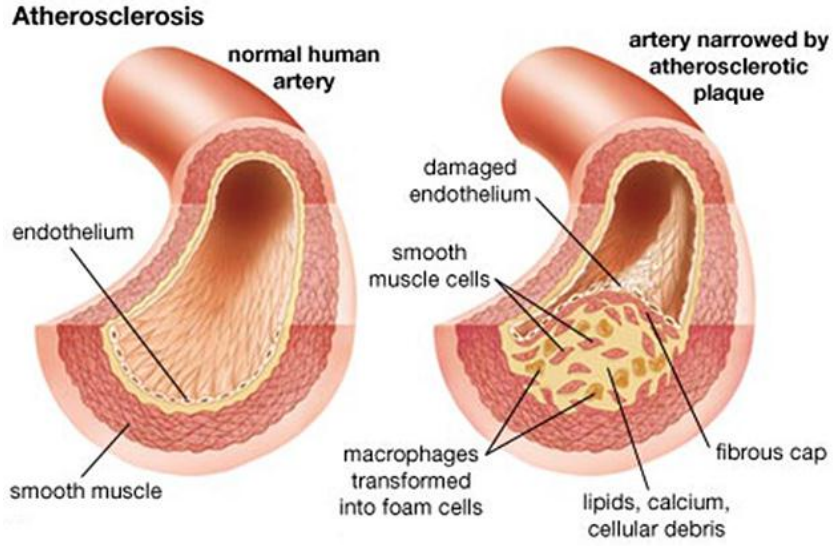


Figure 1.20: Atherosclerosis. Figure shows a schematic of a blood vessel on the right with a ruptured atherosclerotic plaque, which is occluding the lumen resulting in restricted blood flow. Activated platelets adhere to the ruptured plaque, resulting in thrombus formation. The healthy artery on the left is visualised with an unobstructed lumen. (*(http://www.pharmaceutical-networking.com/merck-mk-0524b-treatment-of-atherosclerosis/).*

1.6.1 Role of platelets in CVD and atherosclerosis

Platelets have central roles in CVD such as stroke (Kirkpatrick *et al.,* 2014), coronary artery disease (CAD) (Angiolillo *et al.,* 2014), and acute coronary syndromes (ACS) (Massberg *et al.,* 2003) contributing to the development of acute thrombotic events. In atherosclerosis, platelets contribute to both early stages of endothelial dysfunction and advanced stages of the plaque rupture (Badimon *et al.,* 2012) as summarised in Figure 1.21. Platelets participate in early stage disease initiation through multiple mechanisms that enable adhesion to the dysfunctional endothelium. Activated platelets express high levels of adhesion receptors (e.g. ICAM1, P-Selectin, CD40L) associated with oxidised-LDL (ox-LDL) that contributes to vascular inflammation (Daub *et al.,* 2010). TLR signalling may also play a role in the progression of atherosclerosis by binding of lipopolysaccharides (LPS) to TLR4 on platelets and also mediating platelet-neutrophil interactions.

Direct cell-cell communication through platelet P-Selectin and CD40 ligand (CD40L) encourages inflammatory processes (Berger, 2003; Lievens *et al.,* 2010). CD40L is thought to be at the heart of the atherosclerotic process. Studies have suggested that 90% of circulating CD40L resides in platelets. CD40L is abundant in platelets and is sent to the platelet surface upon activation, where it can initiate numerous inflammatory processes. The release of CD40L is intrinsically linked to αIIbβ3 as αIIbβ3 antagonists can block the release of sCD40L from activated platelets *in vitro* (Nannizzi-Alaimo *et al.,* 2001). Recently, platelet CD40 was shown to mediate the formation of platelet-leukocyte aggregates (stimulates leukocyte activation) and release inflammatory chemokines that activate endothelial cells, supporting atherosclerosis (Gerdes *et al.,* 2016).

The significance of P-Selectin in atherosclerosis has been demonstrated in P-Selectin deficient animals that were protected from the disease. The role of platelet P-Selectin was clarified further by Huo *et al.,* (2003) who illustrated that the introduction of P-Selectin-expressing platelets into ApoE (-/-) mice accelerated atherosclerosis, whereas mice injected with platelets lacking P-Selectin formed smaller plaques (Berger and Wagner, 2003).

Platelet-derived microparticles, (one type of platelet MV) released upon activation can further amplify the progression of atherosclerosis through processes of adhesion, coagulation, inflammation and lipid metabolism (Wang *et al.,* 2016). Platelets also provide a huge repertoire of additional inflammatory mediators including a vast array of chemokines and cytokines that contribute to the crosstalk of platelets with other inflammatory cells – e.g.

endothelial cells, monocytes, neutrophils, dendritic cells, T-cells (Lievens *et al.*, 2011). The major function of platelets in atherosclerosis is the recruitment of leukocytes through direct receptor-ligand interactions or amplification of leukocyte recruitment through chemokine release. This bidirectional relationship is extremely important as platelets encourage leukocyte differentiation into a pro-adhesive and pro-migratory phenotype, and the leukocytes secrete mediators that reciprocally activate platelets.

Following atherosclerotic plaque rupture in severe CVD states, the exposure of thrombogenic substrates (collagen etc.) to circulating platelets instantly triggers platelet adhesion, activation and aggregation, forming a prothrombotic surface and subsequently encouraging thrombosis, vasoconstriction and vascular occlusion. Activated platelets expose phospholipids on their surface, which also promotes the coagulation cascade and subsequent fibrin production (Badimon *et al.*, 2012).

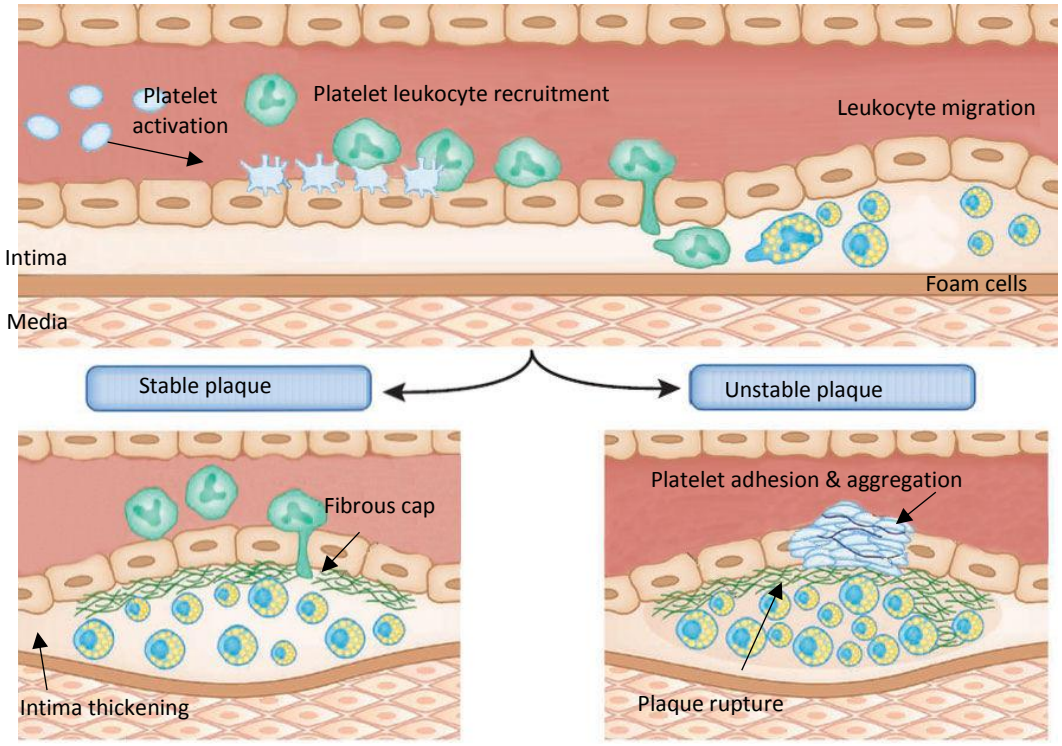


Figure 1.21: The role of platelets in atherosclerosis. Platelets participate in early stage disease initiation by adhesion to the dysfunctional endothelium. Secretion of platelet P-Selectin and CD40 ligand amongst other inflammatory molecules encourages inflammatory processes. Platelet recruitment of leukocytes through both receptor ligand interactions and chemokine release is a key step in the atherosclerotic process. *(Adapted from Jackson et al., 2011).*

Given the critical roles of platelets in the pathogenesis of atherosclerosis and the development of acute thrombotic events, antiplatelet therapy has been widely employed in the primary and secondary prevention of CVD. Some of the current anti-platelet therapy drugs include Aspirin, which irreversibly inhibits cyclooxygenase to subsequently decrease TxA_2 production and limit platelet aggregation. Clopidogrel and Prasugrel are examples of $P2Y_{12}$ receptor antagonists that inhibit the soluble agonist ADP, whilst Tirofiban and Abciximab block αIIbβ3-ligand interactions. Other anti-platelet therapies include thrombin inhibitors and phosphodiesterase inhibitors (block degradation of cyclic nucleotides) (Papp *et al.,* 2013; Metharom *et al.,* 2015).

1.6.2 Role of platelet miRNA in CVD

Given the impact of miRNA gene regulation, it is unsurprising that the dysregulation of miRNA is implicated in CVD. miRNA are central players in modulating gene expression of cells/platelets collectively involved in CVD, and mediate inflammation, lipid uptake and cell differentiation in atherosclerosis. Platelet miRNA signatures (miR-25-3p, miR-221-3p, and miR-374b-5) alter between patients with ST-segment elevation myocardial infarction (STEMI) and those with non-STEMI (Ward *et al.,* 2013) suggesting that levels of platelet miRNA could impact platelet thrombogenicity and type of infarction. Furthermore, circulating miRNAs associated with the risk of MI (miR-126, miR-150, miR-223 and miR-197) are abundantly expressed in platelets.

Platelet miRNA are implicated in premature CAD as two miRNAs in platelets are upregulated in patients compared to controls (miR-340* and miR-624*), although whether or not they are the cause or consequence is currently unknown (Sondermeijer *et al.,* 2011). Research concerning other CVDs showed that circulating levels of platelet enriched miR-328 were associated with atrial fibrillation (AF) in the Framingham heart study (Mc Manus *et al.,* 2014). Interestingly, this association was mitigated when antiplatelet therapy was incorporated in multivariable adjusted models.

Besides their roles as mediators and biomarkers of CVD, platelet miRNA act as novel surrogate measures of the responsiveness to antiplatelet therapies used in CVD (Zhang *et al.,* 2014). miR-223 levels are significantly down regulated in low responders to anti-platelet therapy (Chrychel *et al.,* 2015; Shi *et al.,* 2013). Furthermore, expression of platelet miR-26a has been linked with clopidogrel resistance during coronary stenting (Chen *et al.,* 2016). This theory is strengthened by research demonstrating how the switch from dual anti-platelet treatment with clopidogrel to ticagrelor is linked with significant changes in the level of platelet-specific circulating miRNAs, namely miR-223, miR-126 and miR-150 and miR-96 (Carina *et al.,* 2016). Other research investigating the effects of antiplatelet therapy on platelet miRNA levels showed that *in vitro* platelet activation resulted in transfer of miR-126 from platelets to plasma, whereas in aspirin-treated platelets, this process was not observed. *In vitro* aspirin intake resulted in platelet inhibition and lower circulating platelet-derived miR-126 levels than were seen in untreated subjects (De Boer *et al.,* 2013). Greater understanding of the meaning of platelet miRNA in CVD patients could aid in the diagnosis and treatment of these diseases.

1.6.3 CVD risk factors

Efforts in coping with CVD need further understanding of the science/risk factors behind it and developing strategies/therapeutics to control it. Epidemiological studies in adults have acknowledged a set of characteristics known as risk factors that predict the probability of a person developing clinical manifestations of disease (Dawber *et al.,* 1969; O'Donnell and Elosua, 2008). CVD risk factors can be classed as modifiable or non-modifiable. Non modifiable risk factors include age, ethnicity, gender and family history. Modifiable risk factors include hypertension, smoking, diabetes, unhealthy diet, cholesterol, physical inactivity (PI, sedentary lifestyle and low cardiorespiratory fitness) and overweight/obesity. Risk factors for CVD track from childhood into adulthood (Juhola *et al.,* 2011) and are strong predictors of subclinical atherosclerosis in early adulthood. The majority of CVD is caused by modifiable risk factors and up to 80% of CVD may be prevented if risk factors are avoided (Mc Neal *et al.,* 2010) (WHO, 2014). Physical inactivity and overweight are the risk factors focused on in this thesis.

1.7 Overweight and obesity

Overweight and obesity are major independent risk factors for CVD and are defined as excessive fat accumulation that adversely damages health (Hubert *et al.*, 1983; Aflague *et al.*, 2016). The fundamental cause of obesity and overweight is an imbalance in energy consumption in terms of calories consumed and calories expended. In general, this is due to an increase in both physical inactivity and intake in energy-dense, high fat foods. The global prevalence of obesity and overweight has more than doubled between 1980 and 2014 with a resulting 2.6 million deaths per year (WHO, 2014). In 2014, 40% of adults were overweight and 13% were obese. More alarmingly, childhood obesity is one of the most serious public health concerns of the 21st century. In 2013, more than 42 million children under 5 years old were overweight. This is of concern as overweight and obesity during adolescence are associated with a significantly increased risk for CVD later in life (May *et al.*, 2012).

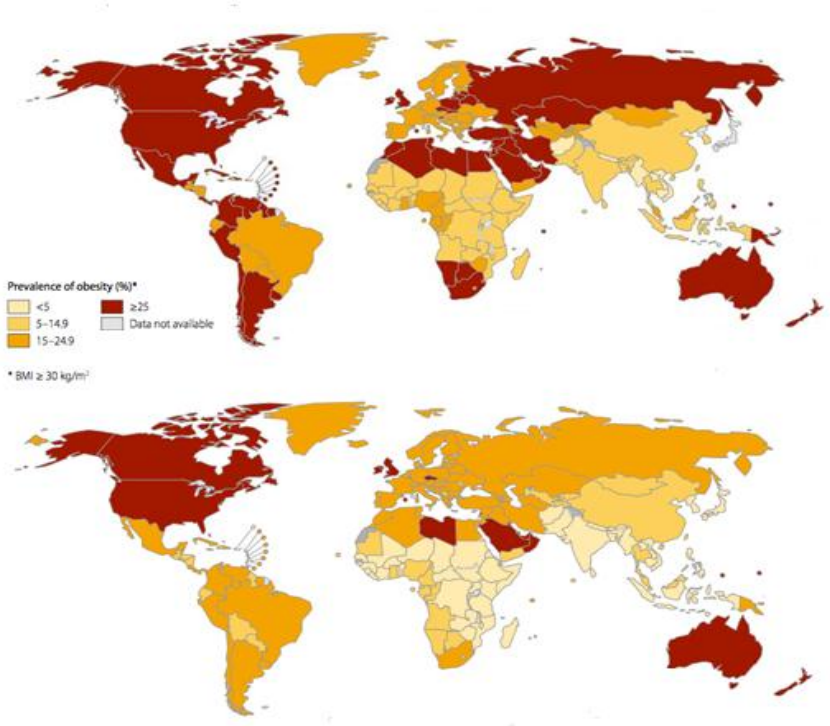


Figure 1.22: Global prevalence of obesity. Age-standardised prevalence of obesity in women (above) and men (below) aged 18 years and over (BMI > 30 KG/M2) in 2014. Ireland has a prevalence of over 25% obesity in both male and females. *(Image adapted from WHO, 2014).*

Nationally, estimations of global trends in obesity has put Ireland on course to become the most obese country in Europe within a decade, with Irish men already having the highest BMI in Europe (Figure 1.22) (Singh *et al.,* 2016). From self-reported data, the prevalence of overweight and obesity in Ireland is 36% and 14%, respectively (Kearns *et al.,* 2014). This is particularly prevalent in the male population and related with hypertension, elevated cholesterol and CVD. The extensive costs associated with overweight and obesity in Ireland is estimated at €1.13 billion (Safefood, 2012).

1.7.1 Effect of obesity on platelets

Obesity and overweight is a multifactorial condition involving a plethora of processes Alterations in lipid metabolism, insulin resistance, inflammation, endothelial dysfunction, adipokine imbalance, oxidative stress and platelet hyper-aggregability have all been postulated to trigger the relationship. Indeed, platelet activation markers are described as elevated in obesity, contributing to the inflammatory and prothrombotic state (Bordeaux *et al.,* 2011). Subjects with overweight and obesity display increased platelet activation markers urinary-11-deyhdro-TXB2 (Davi *et al.,* 2002), MPV (Coban *et al.,* 2005), and PLT (Samocha-Bonet *et al.,* 2008). Greater platelet activation (P-Selectin and PMP) is also linked to central arterial stiffness and carotid wall thickness amongst other atherosclerotic risk factors in overweight and obese subjects (Csongradi *et al.,* 2011; Cooper *et al.,* 2014).

The major mechanisms behind platelet function in obesity include a reduced sensitivity to insulin and resistance to their main inhibitory mediators PGI_2 and NO, elevated oxidative stress and an altered intracellular environment with increased cytosolic Ca^{2+} (Anfossi *et al.,* 2004). Platelets have insulin receptors which impact platelet function by regulating platelet response and sensitisation of platelets to inhibitory mechanism of PGI_2 and NO. In obese subjects, the anti-aggregating effect of insulin is diminished (Trovati *et al.,* 1998; Anfossi *et al.,* 2006).

Elevated oxidative stress also plays important roles in obesity-related platelet dysfunction. Oxidative stress results from an imbalance between the generation of free radicals and antioxidant enzymes (Otani *et al.,* 2011). High reactive oxygen species (ROS) generation by excess adipose tissue reduces NO bioavailability, enhancing surface expression of adhesion molecules, and enabling platelet activation and adhesion. Increased ROS also converts arachidonic acid into F_2-isoprostanes such as 8-iso-$PGF_{2\alpha}$ that can modulate platelet adhesive function (Patrono *et al.,* 2005). Activated platelets also produce ROS (Victor *et al.,* 2009),

amplifying their own aggregatory potential by increasing αIIbβ3 and CD40L expression (Begonja *et al.,* 2005; Pignatelli *et al.,* 2004) and stimulating intraplatelet F_2-isoprostanes production.

Both decreased NO synthesis and bioavailability from ECs and platelets contribute to the pathogenesis of obesity, likely promoting thrombosis. Research by Leite *et al.,* (2016) describes a decrease of Nitric Oxide synthase (NOS) activity and cGMP levels with simultaneous platelet hyperaggregability in obese subjects compared to healthy controls with impaired antioxidant responses as potential contributors. Anfossi *et al.,* (2004) showed that platelet sensitivity to antiaggregagtory effects of PGI_2 and NO is reduced in obesity. A summary of the mechanisms involved in increased platelet activation in overweight and obesity is shown in Figure 1.23.

Importantly, weight loss in obese subjects marks a reduction in platelet activation markers and can potentially reverse the platelet responsiveness to NO and prostacyclin (Russo *et al.,* 2010; Basili *et al.,* 2006). A 10% weight reduction in obese subjects resulted in significant reductions in BMI, endothelial dysfunction and platelet aggregation. The changes in platelet function were associated with improvement in insulin sensitivity, indicating a tight relationship between the two. Weight loss also resulted in reduction in lipid peroxidation markers (Vazanna *et al.,* 2012) and platelet P-Selectin expression in overweight CAD patients (Keating *et al.,* 2013).

Although an association between obesity and platelet activation is evident, the molecular mechanisms responsible have only begun to surface (Blokhin and Lentz, 2013). Platelet RNA is reflective of pathological disease states where inflammatory transcript profiles (e.g. INFG, IL1R1, IL6, and TLR2) correlate significantly with increasing BMI (Freedman *et al.,* 2010), supporting the hypothesis that surplus fat could unfavourably alter the inflammatory potential of platelets. However, obesity can also cause dysregulation of other factors that control haemostasis such as microRNA (miRNA). There is increasing evidence to show that miRNA is involved in the pathogenesis of obesity (Zampetaki *et al.,* 2010), where plasma levels of miR-223 are reduced in obese compared to lean subjects, suggesting that the miR-223/$P2Y_{12}$ alliance could signify a contributing mechanism of platelet activation in obesity (Bray *et al.,* 2013).

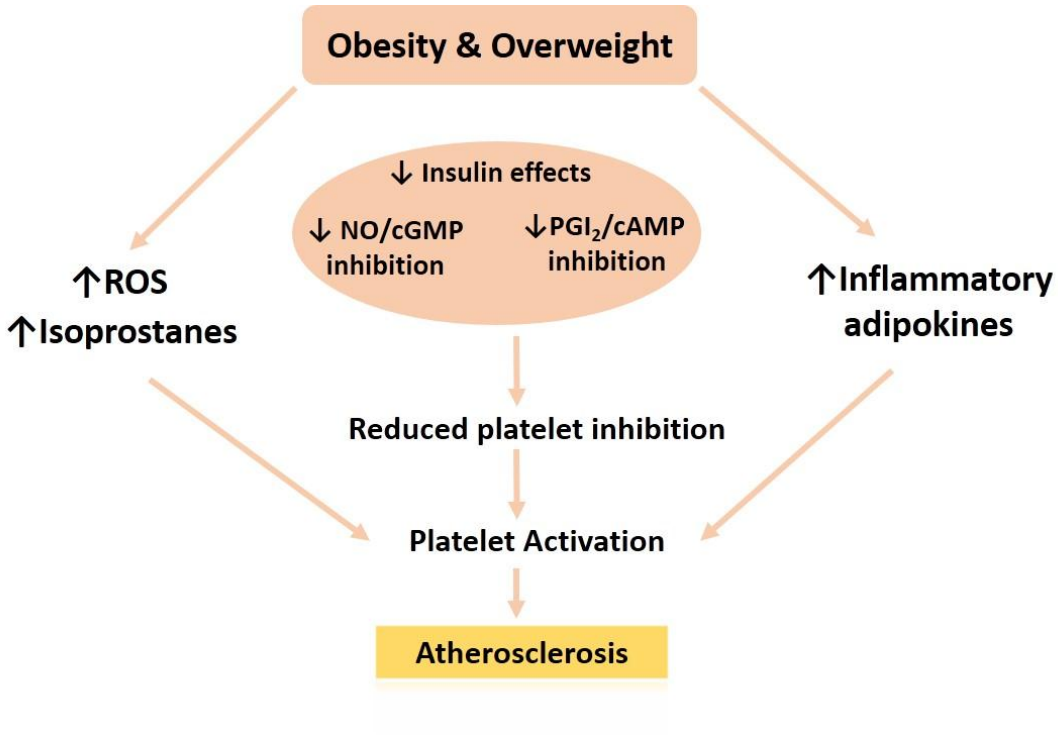


Figure 1.23: Summary of the mechanisms involved in the increased platelet activity in overweight and obesity. Chronic accumulation of excess fat in overweight and obesity lead to decreased sensitivity to physiological inhibitors NO and $PG1_2$. Concurrent with this is an increase in inflammatory mediators released from adipokines and an increase in ROS and isoprostane production, cumulatively resulting in elevated platelet activation and atherosclerosis. *(Adapted from Anfosso et al., 2009).*

1.8. Role of physical activity in cardiovascular health and CVD

Those who engage in regular physical activity or exercise have a reduced prevalence of CVD. PA has been extensively studied due to its beneficial effects on all-cause mortality. Evidence to support the inverse relationship between PA and either CVD, cancer, or depression continues to accumulate. With regard to CVD, regular PA/exercise, reduces blood pressure, serum triglycerides, total body fat and visceral fat, and LDL cholesterol (Li and Siegrist, 2012). Differences in these known factors have been demonstrated to explain a large proportion of the inverse relationship between physical activity and CVD risk (Mora *et al.*, 2007; Kwasniewska *et al.*, 2016). However, over 40% of the inverse association remains unexplained. Although the beneficial effects of regular exercise on blood lipids and blood pressure have been well documented, research focusing on platelet function has only recently gained greater attention. Since platelets play a key role in the pathogenesis of CVD, the protective effect of exercise against CVD may be partially due to alterations of platelet function (Kumar *et al.*, 2011).

Aerobic fitness is measured by maximal oxygen uptake (VO_2 max) during incremental exercise and is globally acknowledged as the best assessment of cardiovascular fitness (Shepard *et al.,* 1968). VO_2 max represents the maximal amount of oxygen that an individual can take in and use to produce energy. VO_2 max is a function of the ability of the cardiovascular system to deliver blood and oxygen to skeletal muscle, and the ability of skeletal muscle to extract this oxygen and use it to produce energy.

1.8.1 Effects of acute exercise on platelet function

Exercise effects on platelet function in both diseased and healthy populations has elicited profound interest in the last decade. The majority of research surrounding platelet function and physical activity/exercise has focused on acute (single bout) aerobic exercise. Potential effects of acute exercise on platelet function (mainly aggregation) have been investigated through various studies in adult subjects with varying intra- and inter-individual results, making interpretation problematic. Differences in population type (e.g. CVD versus healthy), methods employed to assess platelet function, and techniques to examine reactivity are the main reasons for discrepancies between research groups (Aurigemma *et al.,* 2007).

1.8.1.2 Effect of acute exercise on platelet adhesion

Different platelet adhesion experimental protocols have provided no definitive consensus on the platelet response to acute exercise in healthy adult subjects. Wang *et al.,* (1994) were at the forefront of platelet and exercise physiology, investigating the effects of strenuous aerobic exercise in healthy sedentary, healthy active and CVD subjects. Using a tapered parallel plate chamber, they observed elevated platelet adhesion in the three groups after strenuous exercise. Follow-on studies from this group used an assay developed to measure the resistance of adhered platelets against detachment by shear stress applied by a buffer flow (Wang 1995, Wang 1997, Wang and Chen 1999, Wang 2002). They reported increased platelet adhesion after strenuous exercise in both healthy subjects and CVD patients, suggesting a resilient platelet-surface collaboration post-exercise stimulus.

Other studies showed reduced platelet adhesion to fibrinogen in sedentary healthy males after acute exercise (Wang and Liao, 2004), however they employed submaximal testing (30 mins 60% VO_2). Cuzzolin *et al.,* (2000) showed that resting platelet adhesion (to fibrinogen), in addition to platelet adhesion in response to ADP and thrombin, did not change significantly after exercise (75% VO_2), but that both pre- and post-exercise inactive subjects had higher platelet adhesion levels than active subjects. Bonifazi *et al.,* (2004) suggest a role of exercise intensity-dependent cortisol levels in the response of platelet adhesion to acute exercise.

1.8.1.3 Effect of acute exercise on platelet aggregation

A number of studies have shown that acute exercise in either healthy individuals or CVD patients results in an increase in platelet function and aggregation (El Sayed, 2002; Chung *et al.,* 2008, Perneby *et al.,* 2007; Scalone *et al.,* 2009). These studies typically used *in vitro* platelet aggregation tests to assess platelet function. Interestingly, platelet activation elicited by exercise in both healthy subjects and CVD patients is not attenuated by simultaneous aspirin or clopidogrel use, suggesting that the COX/$P2Y_{12}$ pathway does not activate platelets during exercise. Aspirin could have a restricted anti-thrombotic impact during exercise (Hurlen *et al.,* 2000; Madsen *et al.,* 2009; El-Sayed *et al.,* 2005)

As previously mentioned, the integrin αIIbβ3 experiences conformational alterations upon platelet activation, resulting in a drastically heightened affinity for fibrinogen and therefore facilitating platelet aggregation. Accordingly, some studies have found an increase in αIIbβ3 activation post-exercise in trained and sedentary middle aged subjects (Peat *et al.,* 2010; Whittaker *et al.,* 2013). High levels of plasma fibrinogen after exercise result in elevated blood viscosity and this along with increased vWF binding, αIIbβ3 and P-Selectin expression all contribute to the heighted platelet aggregation after acute exercise (Wang *et al.,* 2004).

Measurement of platelet aggregation in whole blood has been performed with the PFA-100 in response to exercise. Acute strenuous exercise resulted in shorter PFA closure times in healthy sedentary males (Cadroy *et al.,* 2003; Madsen *et al.,* 2009). In the latter, almost all subjects showed increased platelet reactivity as measured by the PFA, however only minor differences were detected by LTA, emphasising the need to assess platelet function in whole blood. Whilst the majority of studies found increased platelet aggregation after exercise, some found no effect (Feng *et al.,* 1999) or even an inhibition (Knudsen *et al.,* 1982, El-

Sayed *et al.,* 2002, Aldemir and Cilic, 2005). However, these studies applied submaximal exercise protocols compared to the maximal protocols employed in the majority of studies.

In general, it appears that acute vigorous exercise induces a hyperreactive haemostatic state (Huskens *et al.,* 2016) and a transient increase in agonist-induced platelet adhesion and aggregation *in vitro* and *ex vivo*. Increased oxidative stress and excess ROS production after exercise can counteract the natural cellular antioxidant protection more prominently in sedentary subjects compared to physically active (Wang and Chen, 1999; Singh *et al.,* 2006). Alteration of cytosolic Ca^{2+} is an important factor in the activation of platelets, and the elevation of intracellular free calcium levels represents an initial factor in platelet activation and signalling (Wu, 2012). A rise in cytosolic Ca^{2+} in platelets has frequently been reported after exercise (Tozzi-Ciancarelli *et al.,* 2002; Wang *et al.,* 2002), contributing to platelet hyperreactivity. However, there is no definitive consensus regarding the short-term effects of exercise on platelet function.

1.8.2 Influence of cardiorespiratory fitness (CRF) and exercise intensity on platelet responses to acute exercise

1.8.2.1 Exercise intensity

Both exercise intensity and CRF have been suggested to impact the platelet response to acute exercise (Volf *et al.,* 2015). Most studies investigating acute exercise and platelet function define exercise intensity as a percentage of VO_2 max. Moderate aerobic exercise appears to suppress platelet function in healthy subjects, whilst strenuous aerobic exercise increases activation and aggregation (El-Sayed, 2002). For example, moderate exercising at both 50% and 60% of VO_2 was associated with reduced platelet adhesion and aggregation in sedentary subjects compared to exhaustive exercise, where there were increases in both (Wang *et al.,* 1994; Wang *et al.,* 2004). Cadroy *et al.,* (2002) compared the effect of 30-minute cycle at 50% and 70% VO_2 max on platelet function and observed shorter closure time using the PFA-100 in response to epinephrine and ADP at the higher intensity. P-Selectin expression and platelet-leukocyte formation were reduced at lower exercise intensities compared to higher (Wang *et al.,* 2006; Hilberg *et al.,* 2004; Hilberg *et al.,* 2008).

1.8.2.2 Cardiorespiratory fitness

Cardiorespiratory fitness (CRF) is the ability to perform large muscle, moderate to high intensity exercise for prolonged periods and depends on the respiratory, cardiovascular and skeletal systems. CRF represents the adaptation to long-term exercise. High CRF levels are also linked with reduced CVD risk factors such as hypertension, obesity in the general population, and CVD patients (Swift *et al.,* 2013; Myers *et al.,* 2015; Franklin *et al.,* 2013; De Fina *et al.,* 2015).

CRF was first postulated as a significant determinant for changes in platelet function in response to acute exercise after observations that acute strenuous exercise increased platelet activation in sedentary, but not physically active, subjects (Kestin *et al.,* 1993; Wang *et al.,* 1994). Consistent with these results, platelet activation markers (Platelet factor-4, β-thromboglobulin and P-Selectin) were more pronounced after incremental exercise in sedentary compared to physically active subjects (Coppola *et al.,* 2004). Furthermore, work by Sossdorf *et al.,* (2011) showed that 90 minutes of cycling at 80% individual anaerobic threshold (IAT) triggered a significant elevation of PMPs and their procoagulant activity in both sedentary and trained subjects. However, two hours later the concentration of PMPs remained elevated in the sedentary group only. The reduced PMP clearance in the sedentary subjects could result in increased circulating levels of proatherogenic elements. Additionally, inflamed endothelial cells with increased adhesion receptor expression could bind MPs, retaining high levels (Augustine *et al.,* 2014). Even more importantly, the exercise intensity of 80% IAT paralleled 60% of VO_2 max in the trained group but only 45% of VO_2 max in the sedentary group. Therefore, the trained group performed at a higher percentage of their VO_2 max, with an elevated blood flow and subjection of platelets to increased shear stress. As shear stress can activate platelets, this highlights the effect of CRF on platelet function (Chen *et al.,* 2013).

The actual relationship between CRF and platelet function has been referred to in a recent breakthrough study by Heber *et al.,* (2016), who investigated platelet function and CRF in 62 young women. Platelet function was assessed by determination of P-Selectin and CD40L expression and quantification of platelet ROS generation in platelet-rich plasma (PRP). Basal platelet activation (reflected by CD62P expression) and agonist-induced platelet activation (ROS, CD62P and CD40L) were higher in the LF compared to the MF and HF. The group found no difference between basal CD40L expression (non-agonist induced). Interestingly, basal platelet function in the MF and HF were almost equal, indicating a definite influence of CRF on platelet function.

1.8.3 Effect of exercise training on platelet function

A high CRF level is a result of exercise training and habitual physical activity. Therefore**,** research on the effects of longitudinal exercise training on platelet function has mainly shown that habitual exercise has favourable effects on platelet function. Eight weeks of exercise training (60% VO_2 max 5x/wk 30 min/day), reduced shear stress-induced platelet activation and ox-LDL-potentiated platelet function (Wang and Chow, 2004; Wang *et al.,* 2005). Importantly, after twelve weeks of deconditioning, the beneficial effects of exercise on platelets were non-existent and platelet function returned to its pre-training state.

De Meirelles *et al.,* (2009) reported that chronic physical activity had favourable effects on platelet activation in hypertensive patients at rest. Twelve weeks of regular exercise (75 - 85% VO_2 max 5x/week for 45-60 mins) reduced platelet aggregation in response to collagen. This response was attributed to elevated NO levels as NOS activity and cGMP levels were greater after training. Endothelial NOS (eNOS) and NO are increased after exercise training, thereby increasing platelet cGMP levels and potentially reducing platelet activation under shear stress ((Massimo *et al.,* 2004) Wang *et al.,* 2005)

Santilli *et al.,* (2012) investigated the effects of regular high intensity (60-75%) aerobic exercise for two months in low and intermediate CVD risk sedentary subjects. Exercise training was associated with reductions in TxA_2, plasma P-Selectin and platelet-derived CD40L, despite no reduction in CRP (representing systemic inflammation). Suggest a modulation in intermediate oxidative stress related determinants such as lipid peroxidation (8-iso-PGF_{2A}), the RAGE pathway and lipoproteins explaining the positive effect of exercise on the cardiovascular system.

Evidently, physical activity and exercise affects nearly all facets of platelet function (Volf *et al.,* 2015). Studies on the effects of acute exercise appear to heighten platelet reactivity. Regular exercise can improve this response, seems to have an anti-thrombotic effect on platelets and could represent a portion of the protective effects of exercise on CVD risk factors (Figure 1.24). Green *et al.,* (2012) suggest that vascular adaptation to habitual exercise in athletes having a possible influence on platelet function. Moreover, effects of exercise are not maintained with cessation of training. Of importance, all of these studies discussed were performed in adults and not adolescents when the CVD risk factors and atherosclerotic process has begun. Platelet function and exercise in children or adolescents is in its infancy, an area that requires urgent research (Ribieria *et al.*, 2007).

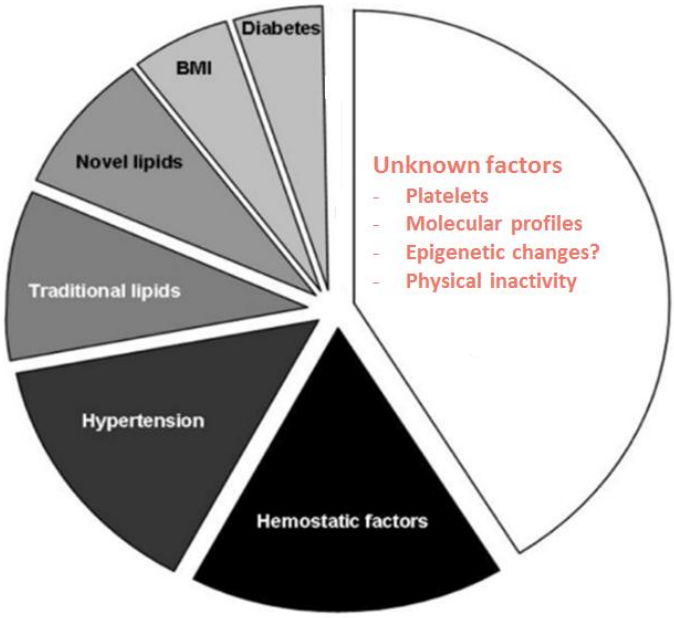


Figure 1.24: Impact of inactivity and exercise on the vasculature. Pie chart represents the allocation of reduction of CVD events associated with physical activity. Differences in the factors in black and grey (Diabetes, BMI, novel lipids, traditional lipids, hypertension and haemostatic factors) are thought to explain over 50% of the relative cardiovascular risk reduction associated with exercise. This suggests that at least 40% of the risk reduction associated with exercise cannot be explained by established risk factors. Platelets and their molecular profiles could be associated with the protective effect of exercise within this unexplained category. *(Figure adpated from Thissen et al., 2010).*

1.9 Physical inactivity and sedentary lifestyle

In contrast to physical activity, physical inactivity/sedentary behaviour is a universal leading cause of death and independent CVD risk factor (Blair, 2009, Khohl *et al.*, 2012). Sedentary behaviour refers to any waking activity characterized by an energy expenditure ≤ 1.5 metabolic equivalents in a sitting or reclining posture (Chevance *et al.*, 2016). In general, this means that any time a person is sitting or lying down, they are engaging in sedentary behaviour.

Alongside obesity and overweight, physical inactivity and sedentary behaviour patterns in Ireland have increased dramatically in the last 10 years. This is highlighted by the launch of Ireland's inaugural National Physical Activity Plan aiming to increase the number of Irish people participating in regular physical activity by 1% per year for the next decade (DH, 2013). Only 32% of Irish adults are meeting the national physical activity recommendations of at least 30 minutes a day of moderate intensity activity on 5 days a week. Worrying data from Woods *et al.,* (2010) indicate that 88% of Irish adolescents do not meet the current PA recommendations of 60 minutes of daily moderate-to-vigorous physical activity. Remarkably, sedentary behaviour and physical activity can co-exist in the same person – even though some people may be reaching the recommendations for regular physical activity, the detrimental effect of 8 hours sitting may be negating the positive effects of physical activity (Ekelund *et al.*, 2016).

However, in contrast to the evidence supporting the benefits of acute and chronic exercise, relatively little is understood about the mechanisms underlying the physiological, cellular and molecular responses to physical inactivity. Incomplete understanding of this relationship is a huge barrier to combating the development of CVD and its ancillary risk factors. Our knowledge of physical inactivity is somewhat indirect and is mainly based on the positive effects of exercise training on the sedentary population. As a sedentary lifestyle is often associated with obesity and overweight (Van Gaal *et al.*, 2006), some mechanisms involved in the pathogenesis of physical inactivity are similar to that of obesity such as insulin resistance (Hamburg *et al.*, 2007), hypertension, and increased inflammation (Nosova *et al.*, 2013). However, distinct factors associated with sedentary behaviour include reduced muscular activity of lower extremities, decreased blood flow, and reduction of shear stress, which increases oxidative stress, endothelial dysfunction (Thosar *et al.*, 2012) and arterial remodelling (Thissen *et al.*, 2012).

1.9.1 Models of experimental physical inactivity

Experimental models to mimic physical inactivity can be achieved through various techniques (Figure 1.25). Reducing ambulatory activity from > 10,000 steps to < 2000 steps per day has been previously employed (Krogh-Madsen *et al.*, 2010). However, compliance is problematic in this type of study. The European Space Agency (ESA) use ground-based models of microgravity to assess the effect of space flight on human physiology. Microgravity is the condition in which objects or people appear to be weightless, where g-forces are extremely low, but not quite zero (NASA). Ground-based models used include horizontal or head down bed rest and dry water immersion (DI). These models are unique and allow the study of human physiology in extremely controlled environments (Dignat-George *et al.*, 2007; Lee *et al.*, 2010; Treffel *et al.*, 2016). Comparative studies indicate that the unique DI is the best model of microgravity for short-term studies (Clement *et al.*, 2016) as, unlike bed rest, DI provides a means to study the physiological responses to the lack of a supporting structure – a phenomenon called 'supportlessness'.

While the DI method is used for the study of human physiology in space, it is also employed as a model to study the physiological adaptations associated with physical inactivity. While the level of physical inactivity experienced during DI and bed rest studies may appear to be extreme compared to that experienced in the normal ambulatory population, studies have shown that these physical activity levels are close to those measured in sedentary individuals (Bergouignan and Momken, 2010).

Figure 1.25: Models of physical inactivity. Models of experimental physical inactivity vary from a reduction of daily steps (left) to head down bed rest studies (middle) to the more recent method of dry immersion studies (right). In the dry immersion set up, subjects are immersed up to the neck and separated from the water with the unique waterproof fabric. *(Bottom right image from http://www.nasa.gov).*

1.9.2 Physiological responses to dry immersion

Weightlessness causes several physiological changes that impact the musculoskeletal, sensory, nervous and cardiovascular systems to name a few (Figure 1.26). Various factors act simultaneously on the body during immersion such as supportlessness, hydrostatic compression and extensive physical inactivity. In comparison to everyday standing and supine positions, where specific areas of the body (feet and back) carry weight, pressure forces in DI are almost equal around the whole body (Naviaslova *et al.,* 2011). The lack of supporting structures establishes a state called 'supportlessness' that is similar to weightlessness. The absence of perceptible signals from the feet results in a huge decrease in postural muscle activity (Koslovskaia *et al.,* 2008).

Hydrostatic pressure is the pressure applied by a fluid at equilibrium, owed to the force of gravity (Gunga *et al.,* 2016). Hydrostatic pressure intensifies with depth of immersion and therefore has a significant effect on lower body parts. During immersion, hydrostatic compression of blood vessels and superficial tissues reduces the peripheral vascular capacity, causing a reduction in vascular skin volume and an increase in transcapillary reabsorption (transfer of fluid from interstitial to intravascular space). This fluid shift is mainly due to changes in pressure gradient between the interstitial to intravascular space and results in rapid redistribution of blood volume/body fluid towards the thoracic-cephalic region (Miki *et al.,* 1989; Boussuges *et al.,* 2007; Noskov *et al.,* 2014). In blood circulation, both spaceflight and its proxies result in an initial decrease of plasma volume (approximately 10-15% on the first day), after which it remains stable (Navasiolava *et al.,* 2010; Larina *et al.,* 2008).

Hypokinesia and hypodynamia are the main characteristics of physical inactivity brought on by DI. Hypokinesia is a decrease in motor activity due to decreased bodily movement (Bhidayasiri *et al.,* 2016), whereas hypodynamia is a decrease in postural muscle load due to the absence of weight bearing. These mechanisms are accompanied by a substantial reduction in muscle tone and tension (Grigor'ev *et al.,* 2004) that cannot be attained with prolonged bed rest models.

The initial responses to DI are caused by the aforementioned immediate alterations in body fluid distribution and absence of support stimulation. Fluid-electrolyte, cardiovascular, muscle tone, and hemodynamic changes occur within 12 hours of DI. Congestion of the central vascular area is believed to be the main reason for water-electrolyte changes (Somody *et al.,* 1999). The redistribution of fluid results in relative central hypovolemia leading to cardiac output increase, suppression of the renin-angiotensin-aldosterone system (Epstein 2006), and reduction in vasomotor tone. Normally, muscle tone (the incessant partial muscle contractions that aid in posture maintenance without causing fatigue) is maintained. The rapid decrease in muscle tone in supportlessness is an acute response to DI (Grigorev *et al.,* 2004; Kozlovskaya *et al.,* 2007) and is manifested as a 40-50% decrease in postural muscle within the hours of exposure to DI.

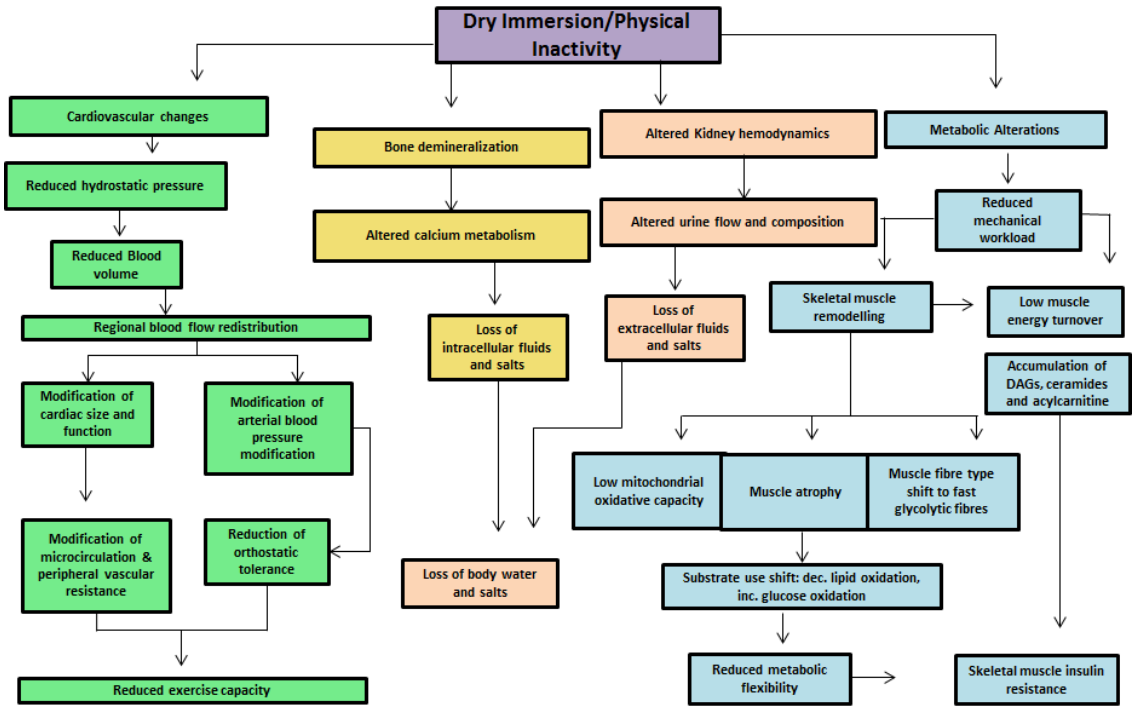


Figure 1.26: Flow chart summarising the physiological responses to dry immersion and physical inactivity. Alterations to various systems and processes occur during DI and physical inactivity – such as bone health changes, altered kidney dynamics and metabolic alterations. Cardiovascular modifications result in blood flow alterations and a reduced exercise capacity. *(Adapted from Vernikos et al., 1996; Bergouignan et al., 2011).*

1.9.2.1 Cardiovascular deconditioning

Changes induced by long-term exposure range from muscular deconditioning, bone health, sensorimotor alterations, hormonal changes and cardiovascular alterations, summarised in Figure 1.26. The cardiovascular changes induced by spaceflight, bed rest and DI are collectively termed 'cardiovascular deconditioning' – a state in which the CV system does not respond efficiently to challenge (Naviaslova *et al.*, 2011; Hackney *et al.*, 2015) This is symbolised by a reduced exercise capacity, a predisposition to orthostatic intolerance, and tachycardia (Coupe *et al.*, 2009). Other effects include a decrease in shear stress with a resultant disturbed flow, endothelial dysfunction, and vascular remodelling.

A major symptom of CV deconditioning is reduced aerobic capacity, as measured by maximal oxygen consumption (VO_2 max). A decline in the adaptive ability to exercise has been reported after just one day of DI (Beliaev 1981) and in 3-7 days of DI VO_2 max falls by 10-18% (Anashkin and Beliav, 1982; Sonkin *et al.,* 1996; Vinogradova *et al.,* 2002). Reduced aerobic capacity is well documented in bed rest and space flight. Additional factors that signify exercise capability include cardiac load, oxygen pulse, VO_2 peak, stroke volume, heart rate and oxygen ventilatory equivalent are also affected by DI.

Increases, decreases or no change of heart rate and blood pressure have all been described in DI. Most studies have not found significant changes in resting blood pressure after 3-7 days of DI (Eshmanova *et al.,* 2009; Iwase *et al.,* 2000, Bravyi *et al.,* 2008). Other authors have described 15-20% increases in diastolic BP after 7 days (Vil-Viliams and Shulzenko, 1980). In general, DI causes an initial increase in stroke volume and cardiac output that is followed by a substantial decline after 2-3 days (Atkov and Bdnenko 1992; Naviaslova *et al.,* 2011). In terms of cardiac morphology, one study suggested a slight reduction in left atrium diameter and increase in left ventricle size (Atkov and Bdnenko 1992), whilst another showed that DI induces changes in myocardial electrophysiological properties (Eshmanova *et al.,* 2009).

Other vascular responses involve decreases in blood velocity (Moreva *et al.,* 2008) and endothelial dysfunction characterised by reduced endothelial dependent vasodilation and elevations of endothelial MPs and diminished plasma VEGF levels suggesting a loss of anti-apoptotic tone (Navasiolava *et al.,* 2010). The reason for CV deconditioning is mainly owed to the absence of both the static G-force along the longitudinal body axis and the body's exertion against this G-force during movement. There have been no studies on the effect of DI on platelet function in humans to date.

1.9.3 Dry immersion applications

Undoubtedly, models of extreme physical inactivity such as DI are advantageous for understanding the underlying mechanisms involved in sedentary behaviour. Understanding the deleterious effects of sedentariness may help with optimal exercise prescription for cardiovascular health (Hughson and Shoemaker, 2015). The DI model enables research into the mechanism of microgravity-induced changes and subsequent assessment of various countermeasures to prevent deconditioning upon return to earth (Clement *et al.,* 2016). Such countermeasures include centrifugation (Clement *et al.,* 2004), antigravity suits, cycle ergometer training, (Eshmanova *et al.,* 2009), and pharmacological agents (Osbourne *et al.,* 2014). Apart from its excellent use as a research tool for microgravity and physical inactivity, DI also has significant healthcare applications. DI has been used in the recovery phase of children with central nervous system diseases (Kazanskaya *et al.,* 2008) in the treatment of oedema (Ivanov and Bogomazov 1998), and has been recommended for rehabilitation of neurological diseases (Berger *et al.,* 2001). Furthermore, DI has been suggested as a recovery method for elite athletes after strenuous training (Radzijewska and Radzijewski, 2007).

1.9.4 Physical activity/inactivity specific miRNA

The plasticity of platelets and other blood cells is vital for responding to environmental changes in response to physical (in) activity patterns. However, understanding of the molecular factors influencing platelet function/response/adaptation to physical (in)activity remain poorly understood. Recently identified miRNAs have gained attention as modulators of platelet function (Landry *et al.,* 2009).

Evidence for miRNA involvement in exercise-associated gene expression changes in a number of cell types including peripheral blood mononuclear cell, neutrophil, and skeletal muscle in non-trained and trained subjects has been illustrated (Radom-Azik *et al.,* 2012; Radom-Azik *et al.,* 2010; Nielson *et al.,* 2010). Work by Baggish *et al.,* (2011) showed altered expression of circulating miRNA (c-miRNA) in response to acute and chronic exercise in athletes. Eight c-miRNA involved in cellular processes related to exercise adaptation (muscle contractility, inflammation, and angiogenesis) were examined. They observed four distinctive signatures of c-miRNA; c-miRNA upregulated by acute exhaustive exercise pre- and post-exercise intervention, c-miRNA responsive to acute exercise pre- but

not post-intervention, c-miRNA only responsive to exercise intervention and non-responsive miRNA.

A number of cell types can release miRNA into plasma, in response to physiological or cellular stress. This could play a role in the adaptation to exercise and act as distinctive biomarkers of varying CRF levels (Nielson *et al.*, 2014). A global down regulation of plasma miRNA after acute exercise, followed by an up regulation after 1 and 3 hours of recovery was also observed (Nielson *et al.*, 2014). Another study revealed that low CRF is linked with high expression levels of three c-miRNAs (Bye *et al.*, 2013). As platelets contribute substantially to the circulating miRNA pool (Willeit *et al.*, 2013) their unique miRNA profile could be representative of adaptations to exercise.

Moreover, evidence of these physical activity specific microRNA signatures (Kangas *et al.*, 2013; Bye *et al.*, 2012 and Altana *et al.*, 2015) have ingrained concepts of physical inactivity-specific miRNA profiles. Epigenetic variation could therefore be a potential mechanism allowing for independent or synergistic effects of physical inactivity on platelet function. Hibler *et al.*, (2015) recently described indications for epigenetic variation (by miRNA expression) as a link between physical activity and sedentary lifestyle. An epigenetic adaptation to habitual exercise has been described (Ling *et al.*, 2014; Pareja-Galeano *et al.*, 2014). Similarly, an epigenetic adaptation to physical inactivity may exist.

1.10 Study background, hypothesis and objectives

Study background

CVD begins in childhood, progressing silently over a long preclinical stage before manifesting as a clinical event. Efforts in coping with CVD need further understanding of the risk factors behind it and development of strategies to control it. CVD risk factors can be classed as modifiable or non-modifiable. Modifiable risk factors included smoking, diabetes, unhealthy diet, cholesterol, physical inactivity (sedentary lifestyle and low cardiorespiratory fitness) and overweight and obesity. Risk factors for CVD track from childhood into adulthood and are strong predictors of subclinical disease in early adulthood (Juhola *et al.*, 2011). Up to 80% of CVD may be prevented if modifiable risk factors are evaded (Mc Neal *et al.,* 2010; WHO). This thesis focuses on the risk factors of physical inactivity and overweight/obesity.

Platelets are small, versatile, anucleate cells that circulate in blood. The small size of platelets and their proximity to the vessel wall facilitates their role as 'guardians of the vasculature', and potential to detect any vascular damage. (Zucker & Nachmias 1985; McFadyen and Kaplan, 2015). They travel as resting discoid fragments in circulation, however elaborate morphological properties allow shape changes to occur when they come in contact with an injured blood vessel. This enables platelets to perform their main physiological function to prevent blood loss in primary haemostasis by the formation of a 'platelet plug' through processes of adhesion, activation and aggregation (Cimmino and Golino 2013).

Platelets play a central role in CVD, both in the pathogenesis of atherosclerosis and in the development of acute thrombotic events (Maiwand *et al.,* 2015). It has been recognised that regular exercise may reduce the risk of major vascular thrombotic events and protect against CVD (Blair and Norris, 2009). Differences in known factors explain a large percentage of the inverse relationship between physical activity and CVD risk (Mora *et al.,* 2007; Kwasniewska *et al.,* 2016). However, over 40% of the inverse association remains unexplained. Although the beneficial effects of regular exercise on blood lipids and blood pressure have been well accepted, research focusing on platelet function has only recently gained greater attention.

In contrast to the accumulating evidence supporting the benefits of regular exercise, relatively little is acknowledged about the deleterious mechanisms underlying the physiological, cellular and molecular responses to physical inactivity, specifically with regard to platelet function. Small non-coding RNAs such miRNAs have been shown to play important post-transcriptional regulatory roles in cells. The role of microRNA (miRNA) in regulating platelet function in is a recent area of investigation (Landry *et al.*, 2009; Kaudevitz *et al.,* 2016). Platelets are highly reflective of physiological and lifestyle changes, making them extremely sensitive biomarkers of human health.

The purpose of this thesis is to build on previous knowledge surrounding the involvement of platelets in physiological health and overweight/obesity, their profile in physical activity and inactivity and the mechanisms that regulate their activity in response to such stimuli.

Overall hypothesis

This thesis hypothesises that physical inactivity, overweight/obesity and low cardiorespiratory fitness adversely impact platelet function. miRNA expression could influence and modulate the platelet function response to physical inactivity.

Objectives

The objectives for this project were to:

- Investigate associations between risk factors of CVD (such as obesity/overweight and physical inactivity) overall physiological health, and platelet function by performing a cross-sectional study.
- Determine the usefulness of platelet indices as biomarkers of health in the cross-sectional population.
- Investigate the effect of varying cardiorespiratory fitness levels on basal platelet function in adolescent and adult males
- Examine the effect of acute strenuous exercise on platelet indice markers and platelet function in adolescent and adult males of varying cardiorespiratory fitness levels
- Investigate the effects of 3 days of dry immersion, acting as a model of acute physical inactivity, on platelet function in 12 healthy males.
- Determine if the miRNA profile of platelets is altered with physical inactivity.

See Figure 1.27 below for a schematic depiction of the experimental approaches used in this thesis.

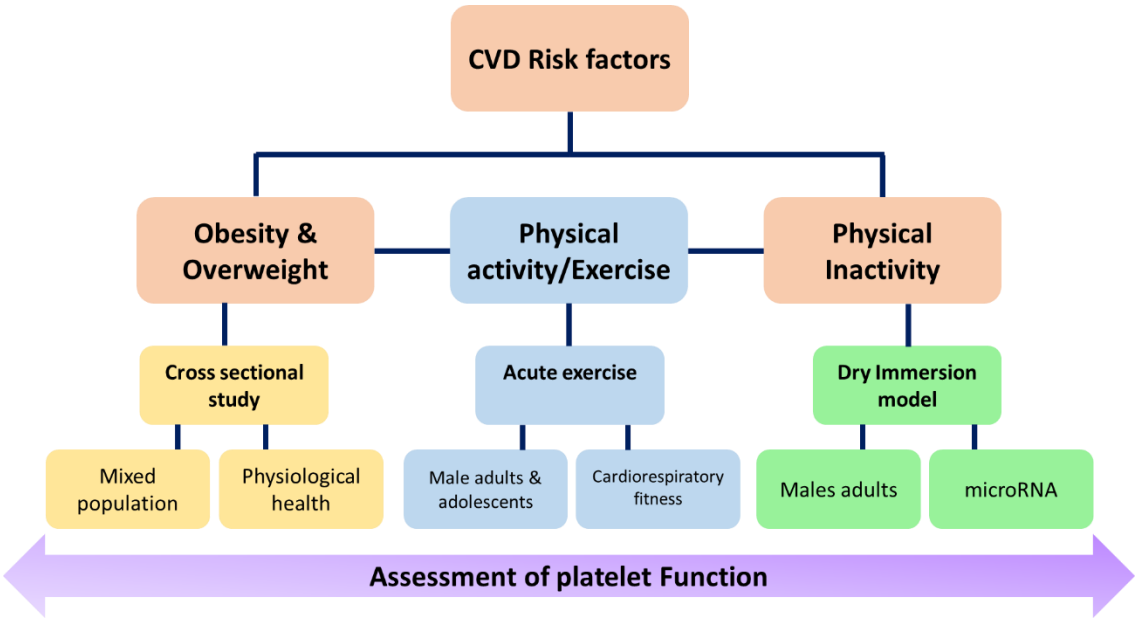


Figure 1.27: Schematic depiction of studies undertaken in this thesis. This PhD thesis consists of three interrelated studies that were designed to provide a more in depth understanding of the physiological, cellular and molecular responses to physical inactivity and physical activity. Study one involved a cross-sectional method approach. Study two examined the relationship between exercise and platelet function, and study three examined the impact of physical inactivity on platelet function.

Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Reagents and chemicals

Bio-Sciences (Dun Laoighaire, Dublin)

*mir*Vana® miRNA isolation Kit

Megaplex™ RT Primers Human Set (A & B) v2.0

Megaplex™ PreAmp Primers, Human Set (Pool A) v2.0

Megaplex™ PreAmp Primers, Human Set (Pool B) v3.0

TaqMan® Array Human MicroRNA A+B Cards set

High Capacity miRNA cDNA reverse transcription Kit

Rhodamine Phalloidin

TaqMan® Pre Amp Master Mix 2X

TaqMan® Array Human miRNA Cards Set v2.0 (A & B)

Taqman® Universal Master Mix II, no UNG

RNaseZap

DNAZap

Becton Dickinson (Oxford, UK)

anti-CD62 Mouse Monoclonal IgG_1 Antibody (PE)

anti-CD41a Mouse Monoclonal IgG Antibody (FITC)

anti-CD42b Mouse Monoclonal IgG_1 Antibody (APC)

FACS Clean™

FACS Rinse™ Solution

FACS Flow™ Sheath Fluid

BDH Chemicals (UK)

Tween-20

Bio-Rad, (Hercules, CA, USA)

Ammonium Persulfate

2-mercaptoethanol

10X TGS (Tris/Glycine/SDS)

TEMED

Biocytex (Marseille, France)
PLT VASP/P2Y12 Kit

DAKO (Glostrup, Denmark)
Fluorescent Mounting Media

Davidson and Hardy (Belfast, Northern Ireland)
Carl Zeiss High Performance Coverslips

Enzo Life Sciences (Exeter, UK)
Convulxin, lyophilised

GE-Healthcare (Buckinghamshire, UK)
Goat-anti-rabbit HRP-conjugated monoclonal secondary antibody
Goat-anti-mouse HRP-conjugated monoclonal secondary antibody

Lennox Laboratory Supplies LTD (Dublin, Ireland)
100% Industrial Methylated Spirits (IMS)

Merck Millipore (Nottingham, UK)
Anti-GAPDH rabbit monoclonal primary antibody
Anti-Drosha rabbit monoclonal primary antibody
Anti-Dicer1 mouse monoclonal primary antibody
Anti-Ago2 rabbit polyclonal primary antibody

EasyCheck™ Kit for HT Systems
ICF Cleaning Fluid for HT systems
Luminata™ Forte HRP Substrate
Fibrinogen, Human Plasma
HRP substrate

Roche Molecular Systems (Pleasanton, CA, USA)
Complete (EDTA-Free) Protease Inhibitor Cocktail Tablets
Real-time Ready Catalog Assays
Transcriptor First Strand cDNA synthesis Kit
Fast start essential DNA Green Master

Sigma Aldrich Chemical Company Ltd (Dorset, UK)

Acetone	Acrylamide/BIS acrylamide solution
Agarose	Ammonium Persulfate
Bovine Serum Albumin	Bromophenol blue
Chloroform	Dextrose
Dimethyl sulfoxide	EDTA 0.5M
Ethanol	Fetal Bovine Serum
Formaldehyde	Glycerol
Glycine	HEPES salt
Human Collagen Type 1	Magnesium Chloride
Magnesium Sulphate	May Grunwald Stain solution
Methanol	Paraformaldehyde
PGE_1, lyophilised	Phosphate Buffered Saline Tablets
PIPES Salt	Ponceau S Solution
Potassium chloride	Sepharose 2B
Sodium Chloride	Sodium Deoxycholate
Sodium Dodecyl Sulfate	Sodium Fluoride
Sodium Orthovanadate	Sodium Phosphate
Sodium Phosphate Dibasic	Sucrose
Thrombin (from human plasma)	TritonX- 100
Trizma Base	Sterile filtered water

StemCell Technologies (Vancouver, British Columbia, Canada)

EasySep Human Whole Blood CD45 Depletion Kit

Thermo Fisher Scientific (Leicestershire, UK)

Hydrochloric Acid

Methanol

10x PBS Solution

Isopropanol

BCA Protein Assay Reagent

Buffer Solution pH4 (Phthalate) J/2825/15

Buffer Solution pH7 (Phosphate) J/2855/15

Buffer Solution pH10 (Borate)

PageRuler Plus Prestained Protein Ladder

Spectra Multicolour High Range Protein Ladder

2.1.2 Consumables and plastics

Becton Dickinson (Oxford, UK)

1, 10, 20, 30, 60 ml Syringes

19 g, 21 g and 22 g Needles

21 g Butterfly Needles

Vacutainer™ Stretch Tourniquet

BD™ Alcohol Swabs

BD Vacutainer® Sodium Citrate

BD Gauze Pads

Round-bottomed FACS Tubes (5 ml & 15 ml)

Eppendorf (Cambridge, UK)

Safe-lock 2 ml PCR Clean Eppendorf tubes

1.5 ml sterile Eppendorf Tubes

0.2/0.5 ml Eppendorf tubes

Fannin Healthcare (Ireland)

Econo-Columns

GE-Healthcare (Buckinghamshire, UK)

Whatman Filter Paper

Matis Medical (Beersel, Belgium)

Impact R Cones and Plates

Pall Life Science (NY, USA)

Nitrocellulose Transfer Membrane

Roche Diagnostics (Pleasanton, CA, USA)

xCELLigence E-Plate 16

LightCycler PCR Strips

LightCycler 96 well PCR plates

LightCycler Sealing Foil

Sarstedt AG & Co (Numbrecht, Germany)

Individually wrapped 2, 5, 10 and 25 ml Serological Pipettes

Aspiration blow-out Pipette

1.5ml Transfer Pipettes

15ml polypropylene reagent and centrifuge tubes

50ml polypropylene reagent and centrifuge tubes

Cell scrapers

Weigh boats

6 well plates

96 well plates

StarLab (Hamburg, Germany)

1000 µl Graduated Filter Tips

200 µl Graduated Filter Tips

20 µl Graduated Filter Tips

10 µl Graduated Filter Tips

0.2 µm syringe filters

Vygon (Wiltshire, UK)

Red Universal Plugs

2.1.3 Instrumentation

Accoson (Harlow, Essex)

Dekamet Sphygmanometer

Classic II 3M Littman Stethoscope

Life Technologies (Carlsbad, CA, USA)

7900HT Fast Real-Time PCR System with Sequence Detection Software (SDS)

QuantStudio™ 12K Flex Real-Time PCR System

QuantStudio™ 12K Flex OpenArray® Plates

Beckman Coulter (Brea, CA, USA)

Beckman Coulter CBC Ac. Diff Analyser

Becton Dickinson (Oxford, UK)

BD FACS Aria™

Accuri C6™

Bennett Scientific Limited (Devon, UK)

Clifton Duo Water Bath

Bio-Rad (Hercules, CA, USA)

MJ-Mini Gradient Thermocycler

Mini-PROTEAN Tetra Cell System (4 x electrode assembly, electrophoresis powerpak® basic, companion running module, tank, lid with power cables, mini cell buffer dam, gel casting stands, 10 x well combs, gel casting frames, 5 x short plates, 5 x spacer plates and transfer electrode rig)

BioTEK (Winooski, VT, USA)

Microplate Reader ELX800

Carl Zeiss (Oberkochen, Germany)

Zeiss 710 Confocal Microscope

Eppendorf (Cambridge, UK)

Centrifuge 5702, 5403R, 5810R, 5415D

Matis Medical (Beersel, Belgium)

Impact R Cone & Plate Analyser

Fluidigm Corporation (South San Francisco, CA, USA)

Biomark™ HD PCR System

Dynamic Array™ IFC

Heraeus (Frankfurt, Germany)

HERA Safe Laminar Air Cabinet

ThermoFisher Scientific (Leicestershire, UK)

Jouan GR412 Centrifuge

Labtech (East Sussex, UK)

Nikon® Eclipse TS100 Phase-Contrast Microscope

Medical Supply Company (Dublin, Ireland)
Horizontal Agarose Gel Electrophoresis Rig
Liebherr 4 °C Refrigerator
Liebherr Profiline -20 °C Freezer

Malvern Instruments Ltd. (Malvern, UK)
NanoSight NS300
NanoSight Syringe Pump

Mason Technology (Dublin, Ireland)
Nanodrop 1000 ™ Spectrophotometer
S220 pH Seven Compact

Memmert (Schwabach, Germany)
Cell Culture Incubator INC 246

Merck Millipore (Nottingham, UK)
Guava® Easy Cyte™ 8HT Bench Top Flow Cytometer

Nalgene (Rochester, NY, USA)
Cryo-Freezing Container

Roche Molecular Systems (Pleasanton, CA, USA)
LightCycler® 96 Real Time PCR System

SensorMedics Corp (Yorba Linda, CA, USA)
Vmax 229 System
Treadmill 2000

Stuart Scientific Ltd (Staffordshire, UK)
Block Heater SBH130D
Orbital Shaker SSM1
Rotator PTR30
See-Saw Rocker SS24
Vortex SA8

StemCell Technologies (Vancouver, British Columbia, Canada)
EasySep™ Magnet

Syngene (Cambridge, UK)
G-Box Chemi-Luminescence Analysis System

Sysmex Ltd (Milton Keynes, UK)
XS-1000i Automated Haematology Analyser
Sysmex XN-3000^{TM} Automated Haematology Analyser

Taylor-Wharton (Theodore, AL, USA)
Liquid Nitrogen Cryo-Freezer Unit (VHC-35 ®)

Tanita (Amsterdam, The Netherlands)
Tanita SC-331S Body Composition Analyser

Polar Electro (Kempele, Finland)
Polar Heart Rate Monitors

ThermoFisher Scientific (Leicestershire, UK)
Holten LaminAir Laminar Flow Cabinet
-80 °C Freezer
HERA Cell 150 Cell Culture Incubator

2.1.4 Preparation of stock solutions and buffers

Physiological buffers

ACD anticoagulant

Sodium Citrate	85 mM
Dextrose	111 mM
Citric Acid	71 mM

Make up to 500 mls with distilled water

JNL Physiological Buffer (Ph 7.4)

JNL A

Dextrose	60 mM

JNL B

Sodium Chloride	1.3 M
Sodium Bicarbonate	90 mM
Sodium Citrate	100 mM
Tris-Base	100 mM
Potassium Chloride	30 mM

JNL D

Monopotassium Phosphate	8.1 mM

JNL E

Magnesium Chloride Hexahydrate 90 mM

JNL A, B, D & E were stored at 4 °C. For combined JNL, 10 mls of JNL A/B/D and 1 ml of JNL E were added to and made up to 70 mls with dH_20, then pH to 7.35 with ACD. Buffer was allowed to stand at RT for 5 minutes and made up to a further 100 ml with distilled water.

2.1.4.1 Immuno-blotting

RIPA Cell Lysis Buffer Stock (1.28X) 500 ml

HEPES, pH7.5	64 mM
Sodium Chloride	192 mM
Triton X-100	1.28% (v/v)
Sodium Deoxycholate	0.64% (v/v)
SDS	0.128% (w/v)

dH_2O to final volume – 500 ml

RIPA Cell Lysis Buffer (1x)

1.28X RIPA Stock	1x
Sodium Fluoride	10 mM
EDTA, pH8.0	5 mM
Sodium Phosphate	10 mM
Sodium Orthovanadate	1 mM
Protease Inhibitors	1x

Sample Solubilisation Buffer (4x)

Tris-HCl, pH6.8	250 mM
SDS	8% (w/v)
Glycerol	40% (v/v)
β-Mercaptoethanol	4% (v/v)
Bromophenol Blue	0.008% (w/v)

Filter the Sample Solubilisation Buffer using a 0.25 μm filter

Transfer Buffer (1x)

Tris-HCl	25 mM
Glycine	192 mM
SDS	0.1% (w/v)
Methanol	20%

Running Buffer (1x)

Tris	25 mM
Glycine	192 mM
SDS	0.1% (w/v)

Tris Buffered Saline (10x)

Tris	2.4% (w/v)
Sodium Chloride	8.8% (w/v)

Coomassie Stain

Coomassie Brillant Blue R250	0.2%
Methanol	45%
Glacial Acetic acid	10%
dH_2O	44.8%

Filter the Coomassie stain using a 0.25 μm filter.

Coomassie Destain Solution

Methanol	20%
Glacial Acetic acid	10%
dH_2O	70%

2.1.4.2 Molecular biology buffers

PFE Buffer

PBS	1X
FBS	100%
EDTA	0.5 M

TE Buffer

Tris	10 mM
EDTA (pH 8.0)	1 mM

2.2 Methods

2.2.1 Preparation of platelet suspensions

2.2.1.1 Blood draw

Before all blood draws, ethics were sought and subjects were questioned on use of anti-platelet drugs and anti-inflammatory drugs (e.g. NSAIDS, Aspirin) during the previous 14 days. Blood was drawn from the antecubital forearm vein in a relaxed atmosphere using a 21 g needle into sodium citrate vacutainer or plastic syringes of varying volumes (10-60 mls) depending on the experimental procedure. Care was taken to ensure a steady blood flow was achieved to avoid haemolysis or contamination by tissue factor and the tourniquet was released quickly. Blood samples were maintained at room temperature and gently inverted every 10 minutes until further use.

Anticoagulants of choice were sodium citrate (for functional studies) or acid citric-acid-dextrose (ACD) for washed platelets (low pH 4.5). For blood draws using ACD, the volume of anticoagulant used was 1/10 the volume of the blood sample drawn. When drawn into a syringe, blood was aliquoted into 15 ml tubes using a transfer pipette.

2.2.1.2 Platelet indices and blood counts

Full blood counts were performed on every sample immediately after the blood draw using semi-automated haematology analysers requiring minute sample volumes. Haematological and platelet indices values were obtained this way. Semi-automated counters employ electrical impedance to measure the volume of particles (cell types) as they individually pass through an aperture of defined size. Particles are suspended in a dilute conducting liquid and electrodes are introduced on both sides of the aperture. As particles are pulled by a vacuum through the aperture, they displace a volume of conductive liquid equal to their size. This generates measureable pulses which can be analysed with advanced electronics and results in simultaneous analysis of particle concentration and size (see Figure 2.1 for analysers used in this thesis). The Sysmex XS-1000i aspirated 20 µl of blood and was used for the cross-sectional study. The Beckman Coulter aspirated 12 µl and was used for the exercise studies. The Sysmex XN-3000^{TM} aspirated 12 µl and was employed for the inactivity study.

Figure 2.1: Automated counters used for blood cell measurements. Left: Sysmex XN-3000™ Haematology System; Middle: Sysmex XS-1000i Haematology System; Right: Beckman Coulter CBC Ac. Diff Analyser *(www.beckmancoulter.com; www.sysmex.co.uk)*

2.2.1.3 Preparation of washed platelets

The initial stage of platelet preparation is the preparation of platelet rich plasma (based on the method of Watson and Authi (2002). After blood draw, vacutainers/syringes were gently mixed by inversion. When working with large quantities of blood (20-60 mls), blood was aliquoted into 15 ml tubes, 5 ml of blood in each tube. Blood was centrifuged (with the brake off to reduce platelet activation) in an Eppendorf centrifuge at 150 xg for 10 minutes at RT resulting in three layers as shown in Figure 2.2.

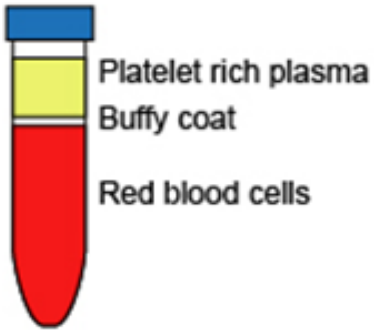


Figure 2.2: Layers produced after centrifugation of blood. Red - contains packed red cells, minimal white blood cells and minimal platelets. Buffy coat – middle layer containing an interphase of white cells. Platelet Rich Plasma (PRP) - upper pale yellow layer containing platelets. *(www.abcam.com).*

PRP was removed with a transfer pipette and placed into a fresh 50 ml tube. Care was taken not to disturb the buffy coat or packed red cells. Platelets were isolated from PRP by a second centrifugation step, involving pelleting platelets and subsequent resuspension in a suitable buffer (a degree of activation can occur during this step and therefore a platelet activation inhibitor was used to minimize this – PGE_1). Samples with greater than 10 ml per tube were split into two tubes to ensure leukocytes pelleted.

The pH of PRP was brought to 6.5 using ACD to aid platelets from becoming activated. Prostaglandin E_1 (PGE_1), a platelet aggregation inhibitor, was added (1 µl per 1 ml of PRP). This helped to prevent platelet activation by raising cyclic AMP (Samuelsson *et al.*, 1978). PRP was centrifuged at 2000 xg for 12 minutes with the brake on to pellet the platelets. The supernatant (PFP) was carefully removed and discarded, ensuring no disruption to the platelet pellet.

The platelet pellet was carefully resuspended in 1 ml of buffer (either PBS/PFE/JNL) by gently pipetting up and down with the opening of the pipette tip angled just above the pellet to dislodge it. The sample was pipetted up and down without fully emptying the tip or fully aspirating the liquid from the tube to completely resuspend the pellet. Care was taken not to introduce bubbles and pipetting continued until there were no visible clumps of platelets remaining and a cloudy suspension was visible.

A further 1 ml of buffer was added. 2 µl of PGE_1 was added and the washing step was repeated twice, adding PGE_1 before each wash. The platelet pellet was resuspended in buffer to the required concentration and platelets were allowed to sit at room temperature for 45 minutes to allow the PGE_1 to dissipate. Resuspended platelets were transferred to a fresh tube for further analysis. Platelets were then ready for use – stimulated or resting. Calcium chloride ($CaCl_2$) was added prior to aggregation or stimulation studies to reactivate platelets.

2.2.1.4 Gel filtered platelets

For a very pure platelet population, gel filtered platelets were prepared. This is a gentler method of isolating platelets whereby they are passed through a gel filtration column of sepharose 2B (Watson and Authi, 2002) Platelets are generally more quiescent, however it is impossible to concentrate the platelets using this technique.

A 20 ml plastic column (15cm x 1.5cm) was prepared as depicted in Figure 2.3. The Sepharose 2B was well shaken before pouring to ensure an even distribution of the gel and diluted with 50 mls of dH_20 in a beaker. Depending on the volume of blood taken at the initial draw, the column was packed with sepharose 2B accordingly:

- 30 ml original blood volume: 5-6 ml packed column
- 40 ml original blood volume: 6-7 ml packed column

The diluted sepharose was added to the column using a transfer pipette. The contents were allowed to settle before adding more sepharose in order to get an accurate measurement of the column. The packed column was washed with 10 times its volume of dH_20 and then with 4 times its volume with the buffer of choice (JNL/PBS). This equilibrated the column to pH 7.4 (the natural environment of platelets). As the last few drops of buffer had passed through the gel, PRP was applied directly onto the top of the column and eluted using the JNL/PBS buffer. Platelets were eluted as a white cloudy fraction which were collected in a 15 ml tube and kept at RT for use.

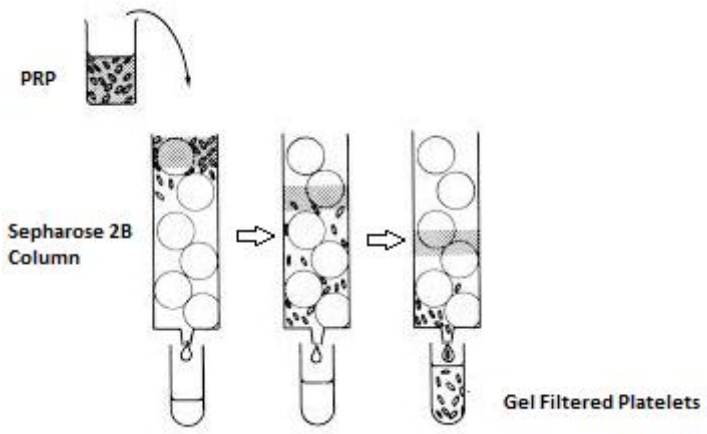


Figure 2.3: Isolating gel filtered platelets. PRP was placed on top of the sepharose 2B packed column. Buffer was applied to encourage the PRP to move fown the column. Gel filtered Platelets were eluted as a white cloudy fraction into a 15 ml tube *(Image adapted from Tangen et al., 1978).*

2.2.1.5 Leukocyte (CD45) depletion of platelets

Washing platelets removes plasma contaminants, effectively diluting out plasma proteins and therefore leaving a pure platelet sample. Where a highly pure platelet population is required, further purification was necessary. For RNA analysis, the lesser RNA content of platelets may be contaminated by a relatively small number of white blood cells (WBCs), relative to platelet number (as each WBC can potentially contain over 10,000 times the RNA mass of platelets (Fink *et al.*, 2003)).

To prepare CD45-depleted platelets for RNA analysis, EasySep™ magnetic technology was used, as outlined in Figure 2.4. This combines the specificity of monoclonal antibodies with the simplicity of a column-free magnetic system for rapid isolation of highly pure platelets ready for downstream applications. Cells are cross linked to EasySep™ magnetic particles using the Tetrameric Antibody Complex (TAC) technology and easily separated from unwanted message rich leukocytes (and CD45+ cells – a leukocyte specific marker) using the magnet.

PFE Buffer (PBS + FBS + EDTA) was fresh each day RNA was prepared. For 19.5 ml of PFE buffer, 0.4 ml of FBS, 40 µl of 0.5 M EDTA and 19.5 ml of PBS were prepared in a 50 ml tube. This assay included the preparation of PRP as previously described. Platelets were suspended in 1 ml of PFE buffer after the second centrifugation step in a FACS tube. After a cloudy suspension of platelets was visible, a further 1 ml of PFE buffer was added and mixed by gentle pipetting and platelets were counted using semi-automated counters as described in section 2.2.1.6.

The EasySep™ whole blood depletion cocktail was then added to washed platelets depending on starting blood volume (200 µl for 40 mls and 50 µl for 10 mls, diluted accordingly for lower blood volumes). This sample was mixed gently by pipetting up and down and incubated for 15 minutes at RT. The EasySep™ magnetic nanoparticles were mixed vigorously by pipetting to ensure they were in a uniform suspension and 200 µl was added to the sample mixture (or reduced equivalent for smaller blood volumes).

The sample was mixed by pipetting and incubated at room temperature for 10 minutes. After the incubation, PFE buffer was added to a total volume of 5 mls and the sample was mixed gently. The tube was placed into the magnet and incubated without the cap for 10 minutes at RT. The magnet was picked up and inverted in one continuous motion, pouring off the supernatant into a 15 ml RNAse-free tube. The magnet and tube were held inverted for 2-3

seconds and then returned to the upright position. Platelets in the fresh tube were CD45-depleted platelets. 5 µl PGE_1 was added to the CD45-depleted platelets and mixed by inversion. The tube was centrifuged at 2000 xg for 10 minutes at RT with the brake on to pellet the platelets. The plasma supernatant was removed and the pellet was ready for RNA extraction (lysed with *mir*Vana® lysis buffer at this point).

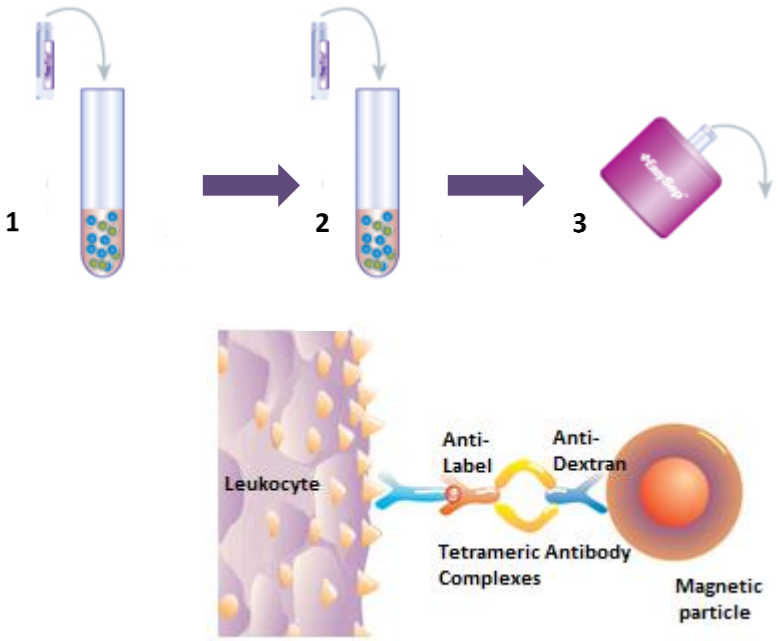


Figure 2.4: Isolating leukocyte-depleted platelets. 1 - EasySep™ CD45 depletion cocktail is added to the platelet suspension. 2 - EasySep™ magnetic particles are added and incubated and 3 – The suspension is incubated in the magnet and CD45-depleted platelets are poured off. *(www.stemcell.com).*

2.2.1.6 Counting platelet suspensions

Different experimental procedures required enumeration of platelet count in the final suspension. This was achieved using semi-automated counters (as explained previously for full blood counts). If the original blood draw was large, the platelet suspension was diluted 1:1000 in the buffer. Appropriate dilution factors were then applied to calculate the final platelet count.

2.2.1.7 Platelet-free plasma isolation

Platelet free plasma (PFP) was isolated for microvesicle work. To generate PFP, a double centrifugation method was employed. Blood was drawn using a 21G needle into a sodium citrate vacutainer (0.32% v/v final concentration). The first 3-5 mls of blood was discarded to avoid contamination from cell fragments or tissue factor from venepuncture being collected. The blood sample was mixed by gentle inversions to ensure even distribution of the anticoagulant. Within 15 minutes of collection, it was centrifuged at 1550xg for 20 minutes at room temperature (20-22ºC) to pellet the cells. The supernatant PFP containing the microvesicles (MVs) was carefully aspirated leaving a layer of approximately 0.5 cm undisturbed on top of the cells. The collected PFP was placed in a sterile 1.5 ml microcentrifuge tube and centrifuged again at 13,000xg for 2 minutes to remove any contaminating cells or debris. The PFP was then collected, leaving 20% of the sample at the bottom of the tube to be discarded. The PFP was separated in 250 µl aliquots in Sarstedt screw cap tubes, snap frozen in liquid nitrogen and then stored at -80°C until further analysis, at which point it was thawed on ice.

2.2.2 Immuno-detection techniques

2.2.2.1 Western blotting

2.2.2.1.1 Preparation of platelet lysates

For western blot assays, platelets were harvested in the following manner. Platelet pellets to be lysed were placed on ice and the buffer was removed. Platelets were lysed with 1x radio immunoprecipitation assay (RIPA) buffer (30 µl per well of a 6 well plate/ 60 µl per pellet) and collected using a cell scraper or pipetted to mix the sample. The lysate was collected in a sterile 1.5 ml micro-centrifuge tube and rotated at 4 °C for 45 minutes to ensure full lysis of platelets. The lysate was then centrifuged at 10,000 xg for 20 minutes at 4 °C to ensure the sedimentation of triton insoluble material to a compact pellet. The protein containing supernatant was then carefully lifted off into 20 µl aliquots and frozen at -80 °C. Alternatively the lysate was used straight away in a BCA assay and ultimately a western blot.

2.2.2.1.2 Bicinchoninic acid assay (BCA assay)

To ensure that samples are in the proper range for detection of the assay, and so they can be compared on an equivalent basis, it is important to know the concentration of total protein in each sample. This was achieved using a BCA assay (Smith., 1985). The assay depends on two reactions, firstly the ability of peptide bonds to reduce Copper (Cu^{2+}) ions to Cu^{+} and secondly, the ability of bicinchoninic acid (green) to chelate with each Cu^{+} to form a complex (purple) that strongly absorbs light at 562 nm.

A sterile 96-well plate was used for this assay. 10µl aliquots of the protein samples, BSA protein standards (concentration range 0-2 mg/ml) and 1x RIPA (buffer used to lyse platelets) were added in triplicate to the plate. The BCA kit reagents came as two solutions; (A) an alkaline bicarbonate solution and (B) a copper sulphate solution. These were mixed in a ratio of 50 parts A to 1-part B so that 200 µl of this mixture was added to each well to be assayed on the 96-well plate. The plate was immediately covered to protect from light and incubated for 30 minutes at 37 °C. The absorbance was then read at 562 nm on an ELx800 Microplate Reader and the unknown concentrations calculated using standard curve analysis.

2.2.2.1.3 Preparing samples

Samples were prepared in a solution of 4x sample solubilisation buffer (SSB), (recipe outlined in section 2.1.4) which contained glycerol to enable the sample to sink to the bottom of the well, and bromophenol blue which allowed separation to be tracked as the sample migrated through the gel. SDS and β-mercaptoethanol were added to fully reduce and denature the protein and remove any higher order structure. Samples were then heated to 95 °C for 5 minutes to further aid in denaturing.

2.2.2.1.4 Polyacrylamide gel electrophoresis (SDS-PAGE)

After the samples were prepared, they were separated by size using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the protocol of Laemmli (Laemmli, 1970). 10x100 mm glass plates (one short one long containing 1 mm spacers) were cleaned with 70% v/v (ethanol) and dried completely. They were assembled and sealed tight using the casting frame and stand. Resolving gels of 7.5% and 10% were prepared as described in Table 2.1. 7-8 ml of the resolving gel was poured between the glass plates, avoiding air bubbles, this was then topped with a layer of 70% v/v ethanol to remove any

surface air bubbles and give an even surface to the gel. The gel was allowed polymerise for 30-40 minutes at room temperature. Following this the ethanol was carefully poured off and the stacking gel was prepared as described in Table 2.2. The stacking gel was added on top of the polymerized resolving gel and a 1.0 mm comb was gently inserted to create the loading wells. A 4% v/v polyacrylamide stacking gel was used throughout all experiments.

Table 2.1: Recipe for SDS-PAGE resolving gel.

Solutions	**Gel (%)**		
	7.5%	**10%**	**12%**
Distilled Water	4.85 ml	4.00 ml	3.35 ml
1.5 M Tris-HCL, pH 8.8	2.5 ml	2.5 ml	2.5 ml
Bis Acrylamide (30%)	2.5 ml	3.33 ml	4.0 ml
10% SDS	100 µl	100 µl	100 µl
10% Ammonium persulfate	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl
Total Volume	10 ml	10 ml	10 ml

Table 2.2: Recipe for SDS-PAGE stacking gel.

Solutions	**4%**
Distilled Water	6.1 ml
0.5M Tris –HCL, Ph6.8	2.5 ml
Bis- Acrylamide (30%)	1.3 ml
10% SDS	100 µl
10% Ammonium Persulfate	100 µl
TEMED	5 µl
Total Volume	10 ml

Upon polymerization, the comb was carefully removed and the gel was inserted into the cassette and loaded into the electrophoresis tank. The chamber between the gel and buffer dam was filled with running buffer and any loose acrylamide flushed from the wells using a pipette. The tank was filled to the mark with the remaining running buffer. The wells were then loaded with 30 µl of prepared sample in SSB buffer in addition to 5 µl of the molecular weight marker in specific lanes. The samples and marker then underwent electrophoresis at 80 V for 20 minutes, after which the voltage was increased to 100 V for 80 minutes.

2.2.2.1.5 Electrophoretic transfer

Electrophoretic transfer is the transfer of proteins from a gel to a blotting membrane while simultaneously preserving their relative position. The wet transfer technique was employed in this thesis and the membrane of choice was nitrocellulose. The nitrocellulose membrane and transfer components were soaked in ice cold transfer buffer for 10 minutes prior to use. Following electrophoresis, the gel was removed from the glass plates encasing it and the stacking gel was removed. The gel and blotting membrane were assembled into a sandwich outlined in Figure 2.5.

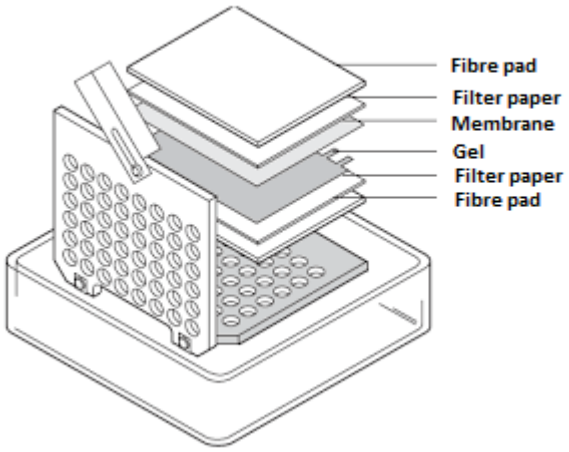


Figure 2.5: Assembly of a wet tank transfer cassette. The gel and membrane are securely placed between a sandwich of filter papers and fibre pads**.** *(www.bio-rad.com).*

The sandwich was then pressed with a roller wetted in transfer buffer to eliminate possible air bubbles. The gel/membrane sandwich was then placed in the transfer cassette. The cassette was submersed in the electrophoresis tank filled with ice cold transfer buffer and an ice pack subjected to an electrical field. The cassette was organised so that the membrane sat between the gel surface and the positive electrode so that the negatively charged proteins migrate from the gel to the membrane (Figure 2.6). Transfers were performed overnight at 4 °C in a cold room. The transfer buffer was agitated using a magnetic stirring bar and voltage set to 50V.

2.2.2.1.6 Blocking

To reduce non-specific binding of the antibody, the membrane was blocked by incubating with either 5% non-fat dried milk or BSA in 0.05% Tween PBS at room temperature for 40 minutes on a see-saw rocker. Post-block the membrane was washed 3 times for 5 minutes on a see-saw rocker with 0.05% Tween PBS.

2.2.2.1.7 Antibody incubation

After blocking and washing, the blot was incubated, usually overnight at 4 °C with gentle rocking, with the appropriate primary antibody. The antibody was made up in a solution of 1-5% BSA in 0.05% PBS Tween or TBS Tween. See Table 2.3 for antibodies used in this thesis.

Table 2.3: List of antibodies used for immunodetection.

1° Antibody	Block	Tween %	Species	Dilution Factor	2° Antibody	Species
Argonaute2	PBS/BSA	0.05	Rabbit	2 µg/ml	1:3000	Rabbit
Dicer	PBS/BSA	0.05	Mouse	1:200	1:3000	Mouse
GAPDH	PBS/BSA	0.05	Rabbit	1:3000	1:5000	Rabbit
Drosha	PBS/BSA	0.05	Rabbit	1:1000	1:3000	Rabbit

After incubation with the primary antibody, the blot was washed 3 times with 0.05% TBS/PBS Tween for 5 minutes with gentle rocking. The blot was then incubated with the appropriate species of secondary antibody conjugated with HRP (horse radish peroxidase) in 1-5% BSA 0.05% TBS/PBS Tween for 2 hours at room temperature. HRP is a small stable enzyme with specificity and rapid turnover. See Table 2.3 for appropriate secondary species and the dilution factor for each protein. Finally, the membrane was washed for 3 x 5 minutes in 0.05% TBS/PBS Tween with gentle rocking.

2.2.2.1.8 Blot development

Detection of target proteins was possible once they were specifically tagged with the appropriately labelled antibody. This was achieved through use of either Millipore Luminata® Enhanced Chemiluminescent (ECL) substrate or Thermo Scientific SuperSignal® West Pico Chemiluminescent Substrate. When probing for an abundant cellular protein with a reliable antibody, the less sensitive SuperSignal® West Pico Chemiluminescent Substrate was used. The peroxide solution and luminal enhancer solution were mixed in equal parts and approximately 1 ml of this mixture was added for an entire 10 well blot. When probing for minimally expressed proteins or using weak antibodies the Millipore, Luminata® was used, capable of detecting picogram (pg) amounts. Approximately 500 µl was added to a full 10 well membrane. In both cases, detection imaging was carried out immediately. The light emission was captured using a Syngene G-Box. Exposure times ranged from 30 seconds to 10 minutes depending on the signal strength.

2.2.2.1.9 Ponceau-S membrane staining

Ponceau S is a red stain applied in an acidic solution which reversibly stains all protein present on the membrane prior to any immuno-blotting. This was used to confirm the transfer and help to show equal transfer of protein from the gel. The stain was subsequently removed by washing in distilled water.

2.2.2.1.10 Coomassie gel staining

Coomassie gel staining was used to visualise proteins on the SDS-PAGE gel, which was useful to ensure an efficient transfer by showing protein left behind. In brief, the SDS-PAGE gel was covered with filtered coomassie solution and agitated by gentle rocking for up to 4 hours. The solution was then poured off and the gel de-stained using a mixture of methanol and acetic acid (50:50) until the protein bands appeared clear with no background.

2.2.2.1.11 Membrane stripping

To allow for re-probing of the membrane with different antibodies or to optimize antibody concentration, the membrane was stripped with RESTORE® stripping buffer. This was used as per the manufacturer's instruction. In brief, the blot was washed in 0.05% TBS/PBS Tween to remove any previous ECL substrate then covered with stripping buffer and agitated by rocking gently for 15 minutes. Removal of both primary and secondary antibody was confirmed by first incubating the membrane with the appropriate secondary and subsequent detection with ECL substrate. If no bands were detected the blot had been successfully stripped. It was then washed again to remove ECL substrate and reprobed with alternative antibodies as previously described.

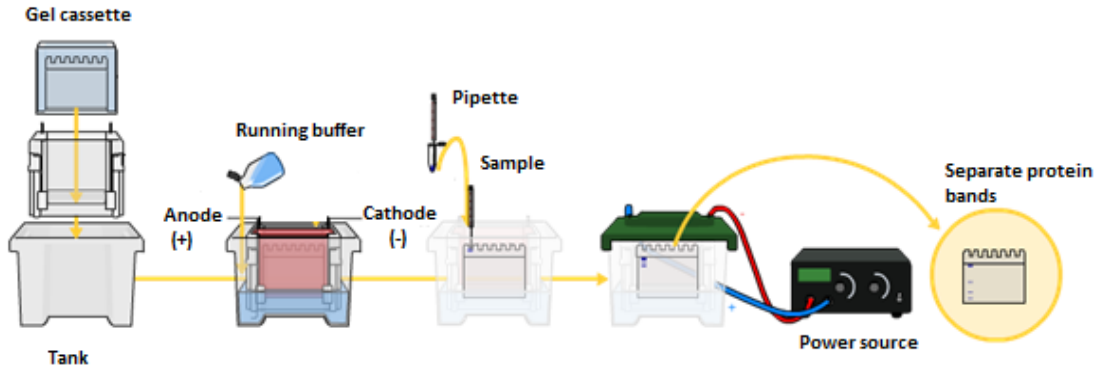


Figure 2.6: Apparatus used to run an SDS-PAGE gel. *(www.bio-rad.com).*

2.2.2.2 Human protein biomarker assay - Proseek multiplex immunoassay

(as performed in collaboration with Olink, Sweden)*

Proseek® multiplex CVD II^{96x96} & Proseek® multiplex inflammation I^{96x96} are high-throughput multiplex immunoassays, each enabling analysis of 92 CVD- or inflammation-related protein biomarkers using 1 µl of sample and across samples simultaneously. This high level of multiplexing is achieved by proximity extension assay (PEA) technology (Figure 2.8). A comparison of Proseek probes against conventional immunoassay is depicted in Figure 2.7.

A pair of oligonucleotide-labelled antibodies (Proseek probes) specific for each biomarker are allowed to pair-wise bind to each target protein in the sample. When two Proseek probes are in close proximity, a new PCR target sequence is created by a proximity-depended DNA polymerization reaction. Each oligonucleotide pair holds a unique DNA sequence enabling hybridisation only to each other. This sequence can then be detected by real time PCR and measured.

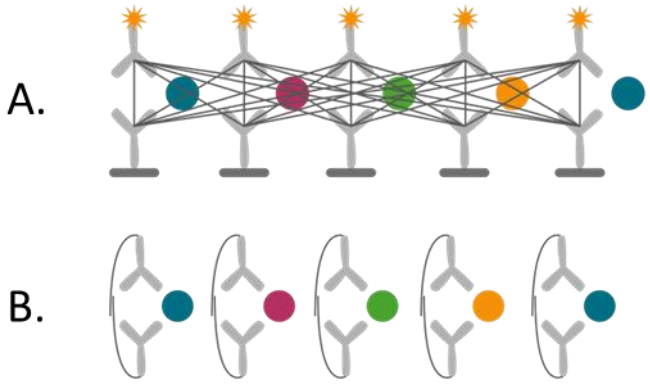


Figure 2.7: Proseek multiplex probes. A – represents conventional immunoassays where cross reactivity occurs due to unspecific binding of antibodies, thereby limiting the degree of multiplexing. B - unique DNA oligo sequences report only matched DNA-pairs (e.g. 1A+1B) and cross reactive events are not identified. *(www.olink.com)*

2.2.2.2.1 Sample preparation and run

Citrated PFP samples were thawed, centrifuged briefly and 20 µl of each sample was aliquoted into a well on a 96 well plate and shipped to Olink Bioscience on dry ice. Proseek assays were performed by Olink Bioscience (Upsala, Sweden) to evaluate the expression of two panels of potential CVD and inflammatory biomarkers (see appendix C for full biomarker lists). Overlap between panels resulted in the total measurement of 152 biomarkers in the PFP samples.

Briefly, 1 µl of each sample or negative control was incubated with the conjugated antibodies at 4 °C overnight (day 1). On day 2, the PEA mixture was added and the products were extended and pre-amplified using PCR (ABI 2720 Thermal cycler, Life Technologies). The detection reagent was added to 2.8 µl of the extended and pre-amplified product, mixed and then loaded into the Fluidigm Gene Expression 96x96 Dynamic arrays (Fluidigm Corporation) on one side and the Primer plate with specific primers on the other side of the chip. The chip was primed using Fluidigm IFC controller HX and afterwards loaded into a Fluidigm Biomarker system.

Detection and sample analysis was performed by high-throughput real-time PCR analysis using the Fluidigm® BioMark™ HD System. This PCR platform enables simultaneous detection of 96 analytes in 96 samples creating 9,216 data points from a single run. The BioMark HD platform uses microfluidic distribution of sample and assays requiring only 7 nl reactions. The BioMark HD platform consists of a real-time PCR instrument, 96x96 Dynamic Array™ IFC for multiplexing the samples and biomarkers, and an IFC loader to load the 96x96 Dynamic Array IFC.

Raw data was analysed using Fluidigm PCR software. The Proseek assay generated Cq values for each biomarker and data was normalized using the extension control and a background value. The data used for statistical analysis was expressed on a log2 scale, where a high value corresponded to a high protein expression and vice versa with a low value. The limit of detection (mean negative control plus 3 x standard deviation) was determined for each biomarker for each sample. The data was normalised and analysed using GenEx software (MultiD, Gothenburg, Sweden). All statistical analyses (dynamic principal component analysis and one-way ANOVA) were performed on normalized data.

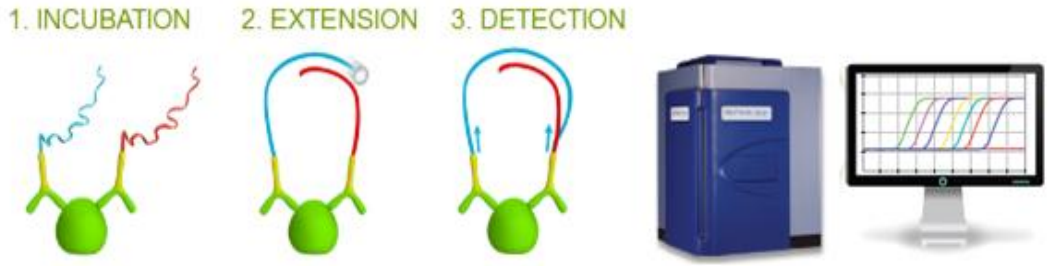


Figure 2.8: The main steps in the Proseek assay. Part 1- Two oligonucleotide labelled antibodies called proseek probes, bind to the target protein in the sample. Part 2 – The close proximity of the probes results in the formation of a new PCR target sequence by a proximity-dependent DNA polymerisation event. In part 3 – the resulting sequence is then detected and quantified using standard real time PCR. *(www.olink.com)*

2.2.3 Molecular techniques

As RNA is easily damaged and degraded by ubiquitous ribonuclease (RNase), all RNA work was carried out according to the protocol outlined by Sambrook (Evans, 1990). To prevent genomic DNA contamination, prior to RNA work, surfaces and equipment were cleaned down with DNA Zap (Invitrogen). This consists of two solutions that are innocuous when used alone, but become a potent nucleic acid degrading solution when mixed. This mixture is able to instantaneously degrade high levels of contaminating DNA and RNA from surfaces. Gloves were used and changed frequently throughout the procedure.

2.2.3.1 RNA isolation

The *mir*Vana® RNA extraction kit was used for isolation and purification of total RNA from platelet samples and plasma samples. This was found to obtain an increased RNA yield and superior purity compared to other methods including Trizol-based extraction. The *mir*Vana® kit uses an organic extraction method followed by immobilisation of RNA on glass-fibre filters to purify total RNA.

Platelets were prepared as previously outlined and pelleted by centrifugation at 2000xg for 12 minutes at RT. 400 µl of total lysis binding solution was added to the cells (for adhesion assays, in the case of a 6 well plate, 100 µl into each well). The cells were then scraped and collected, using a rubber cell scraper, into a 1.5 ml RNase-free micro centrifuge tube). The sample was pipetted for 30-60 seconds to obtain a homogenous lysate and vortexed briefly. The volume of the lysate was recorded and 1/10 of this volume of miRNA homogenate additive was added to the lysate. This solution was well mixed by vortexing for 30-60 seconds. The solution was then incubated on ice for ten minutes after which the volume prior to addition of the miRNA homogenate additive was the volume of acid phenol chloroform added (ensured it was taken from the bottom phase of the acid phenol chloroform). The lysate solution was again vortexed for 30-60 seconds before being centrifuged for 5 minutes at 10,000 xg at room temperature to separate the aqueous and organic phases.

Following centrifugation, the lysate solution was checked for a compact interphase and if this was not evident the centrifugation step was repeated. The upper aqueous phase was transferred to a fresh tube taking care not to disturb or carry over any of the bottom organic phase. The volume of upper aqueous phase recovered was noted and 1.25x of this volume of room temperature 100% high grade ethanol was added to the fresh tube. A maximum of

700 µl of this lysate/ethanol mixture was pipetted onto a glass-fibre filter cartridge, which was placed in a fresh tube. This was centrifuged at 10,000xg for 15 seconds to pass the mixture through the filter. This step was repeated until all of the lysate/ethanol had been passed through the filter and the flow-through was discarded each time. 700 µl of wash buffer 1 was then added to the filter column and passed through by centrifugation at 10,000 xg for 15 seconds. This was repeated using 500 µl of wash buffer 2/3 again with the flow-through being discarded each time. After the third wash the filter column was centrifuged at 10,000xg for 1 minute to dry off the filter column and prevent ethanol carry over to the new tube in which filter column was placed. The total RNA was then eluted into a fresh collection tube by centrifugation at 10,000 xg for 30 seconds using 100 µl of elution buffer, which was pre-heated to 95 °C. The RNA was quantified and qualified on a NanoDrop® Spectrophotometer. The sample was stored at -80 °C.

2.2.3.2 RNA/DNA quantification

The NanoDrop® ND-1000 Spectrophotometer was used to determine nucleic acid sample concentrations and integrity (Figure 2.9). An undiluted 1.2 µl sample was pipetted onto the end of a fibre optic cable (the receiving fibre). A second fibre optic cable (the source fibre) was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fibre optic ends. A pulsed xenon flash lamp provides the light source and a spectrometer is used to analyse the light after passing through the sample. The instrument is controlled through use of specific PC-based software.

The NanoDrop® automatically calculates the purity of the nucleic acid samples by reading the absorbance at 260 nm and the absorbance at 280 nm and then determining the ratio between the two (Abs@260/Abs@280). Pure DNA, which has no protein impurities has a ratio of 1.8, whereas pure RNA has a ratio of 2.0. Lower ratios indicate the presence of protein; higher ratios imply the presence of organic reagents.

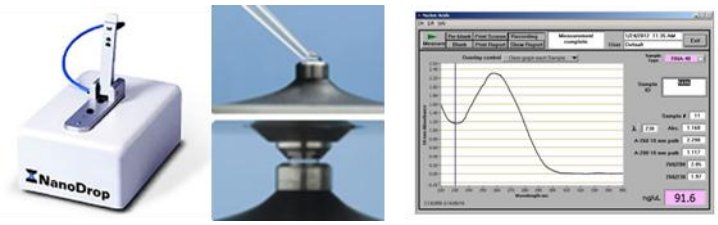


Figure 2.9: The NanoDrop instrument used to determine nucleic acid sample concentration and integrity. Left: NanoDrop Instrument, middle: NanoDrop technology, right: Typical output. (www.nanodrop.com).

2.2.3.3 Polymerase Chain Reaction (PCR)

PCR is a process used to amplify specific sequences of DNA at an exponential rate for use in a variety of applications. Standard ("bench top") PCR was used for creating complementary DNA (cDNA) and as a cost effective method before the samples were subjected to quantitative real time PCR (qPCR).

2.2.3.4 Quantitative RealTime PCR (qPCR)

qPCR reactions follow the standardized principles of PCR. It is used to amplify target DNA with a view to simultaneously quantifying the targeted DNA sequence as it accumulates during the reaction. This technique was used for both microRNA profiling and gene expression work and was facilitated by the use of TaqMan hydrolysis probes shown in Figure 2.10. This chemistry, also known as "fluorogenic 5' nuclease chemistry uses a fluorogenic labelled 5' probe. This relies on the nuclease activity of Taq DNA polymerase. An oligonucleotide probe is constructed containing a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end.

While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET). If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of *Taq* DNA polymerase as this primer is extended. This cleavage of the probe:

- Separates the reporter dye from the quencher dye, increasing the reporter dye signal.
- Removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process.

Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of PCR product produced.

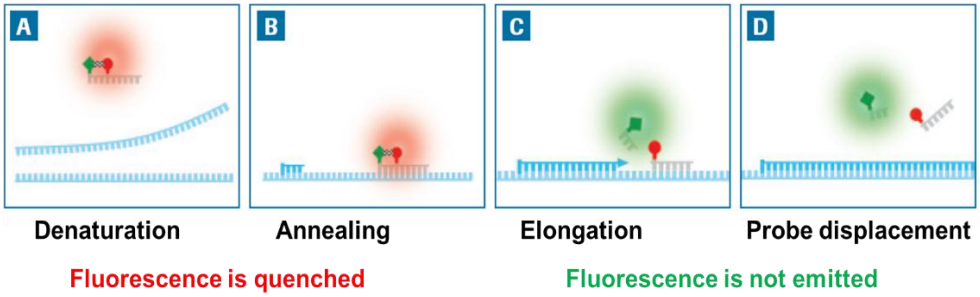


Figure 2.10: TaqMan Probe chemistry. Images A-D highlight the behaviour of taqman probes during PCR. Taqman probes carry two fluorescent dyes in close proximity, one of which (quencher) quenches the fluorescence from the other (reporter) while the probe is intact. In the denaturation phase (A) DNA strands separate as temperature increases. During the annealing phase (B), the primers and probes specifically anneal to the target sequence. As DNA polymerase extends the primers, the 5' nuclease activity of the enzyme cleaves the probe (C), allowing the reporter dye to emit green fluorescence. The probe fragments are then displaced from the target and polymerisation of the new amplicon continues (D). Accumulation of PCR products is detected directly by monitoring the increase in green fluorescence from the reporter dye.

2.2.3.5 MicroRNA (miRNA) profiling

2.2.3.5.1 miRNA isolation and amplification

miRNA profiling was carried out using the Applied Biosystems® TaqMan® Low Density Array (TLDA) Human miRNA A (v2.0) and B (v3.0) cards set. For analysis on the TLDA cards, total RNA was firstly extracted from platelets using the Ambion™ miRVANA miRNA® isolation kit.

2.2.3.5.2 Reverse transcription of RNA for TaqMan® array analysis

Single stranded cDNA was synthesised from total platelet RNA using the Applied Biosystems TaqMan® miRNA Reverse Transcription (RT) Kit. For a full miRNA profile two RT reactions were needed incorporating primers for both pool A and B miRNA panels. This kit uses specific stem-looped RT primers (Figure 2.11) to lengthen the target cDNA (Kramer, 2011), as the short length of miRNA make it difficult for traditional primers to anneal for the RT reaction. The RT reaction had a final volume of 7.5 µl and contained: 3 µl (1-350 ng) total RNA and 4.5 µl of RT master mix. The master mix was made up as shown in Table 2.4 in a 2 ml RNAse-free tube.

Table 2.4: Recipe for reverse transcription of miRNA to cDNA (pool A and B).

RT reaction mix components	**Volume for one samples (µl)**	**Volume for ten samples (µl) ***
Megaplex RT primers (Pool A & B) 10x	0.8	9.00
dNTPs (100 mM)	0.2	2.25
Multiscribe reverse transcriptase (50 U/µL)	1.5	16.88
10 X RT Buffer	0.8	9.00
MgCl	0.9	10.12
RNase Inhibitor (20 U/µL)	0.1	1.12
Nuclease free water	0.2	2.25
Total	4.5	50.62

* volume includes 12.5% excess for volume loss from pipetting.

The samples were mixed by gentle pipetting and then incubated on ice for 5 minutes. The samples were then run on the bench top PCR thermo cycler under the conditions found in Table 2.5.

Table 2.5: Thermocycler conditions for miRNA specific reverse transcription.

Stage	Temp °C	Time
Cycle (40 cycles)	16	2 min
	42	1 min
	50	1 min
Hold	85	5 min
Hold	4	∞

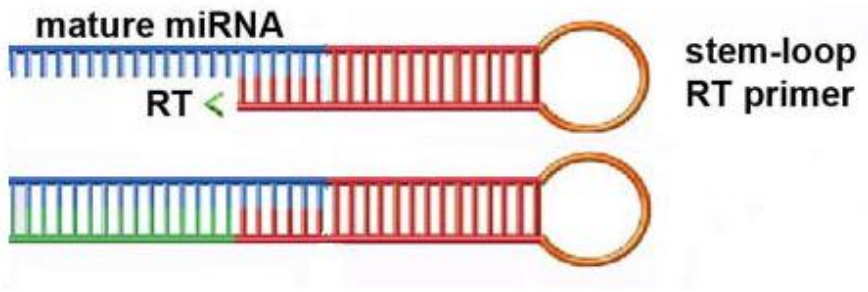


Figure 2.11: Reverse transcription of microRNA using stem-looped primers. A RT binding site is created using a stem loop specific primer set binding to the 3' end of the miRNA. The stem-looped primer is highly specific for miRNA and can differentiate between mature and pre miRNA. *(Image adapted from Kramer, 2011).*

2.2.3.5.3 Pre-amplification of cDNA

As the total RNA yield from platelets is generally lower than nucleated cells (less than 350 ng), a preamplification step was carried out prior to committing the cDNA to the TaqMan miRNA arrays. This uniformly pre-amplifies desired cDNA prior to quantification with the TLDA cards. The preamplification reaction had a final volume of 25 µl containing 2.5 µl RT product and 22.5 µl Pre-amp reaction mix The master mix was made up as shown in Table 2.6 in a 2 ml RNAse-free tube. The sample was then mixed by gentle pipetting and run on a bench top PCR thermocycler under the conditions found in Table 2.7.

Table 2.6: Recipe for pre-amplification master mix.

PreAmp reaction mix components	Volume for one sample (µl)	Volume for ten samples (µl)
TaqMan PreAmp master mix (2x)	12.5	140.62
Megaplex PreAmp primers (10x)	2.5	28.13
Nuclease Free Water	7.5	84.37
Total	22.5	253.12

Table 2.7: Thermocycler conditions for miRNA cDNA pre-amplification.

Stage	Temp °C	Time
Hold	95	10 min
Hold	55	2 min
Hold	72	2 min
Cycle (12 cycles)	95	15 secs
	60	4 min
Hold (for enzyme inactivation)	99.9	10 min
Hold	4	∞

Following PCR, the sample was diluted with 75 µl of 0.1x TE buffer (pH 8) and used immediately for array analysis or stored for up to one week at -20 °C/ -80 °C for future use.

2.2.3.5.4 Running the TaqMan® microRNA array

A 7900HT PCR system was used for initial miRNA profiling and a QuantStudio™ 12K Flex Real-Time PCR system was used for large scale profiling. DNA polymerase from the TaqMan® Universal PCR Master Mix amplifies the target cDNA using sequence-specific primers and a probe on the TaqMan microRNA array. The presence of the target is detected in real time through cleavage of the TaqMan probe by the polymerase 5'-3' activity. The master mix for the cards was made up as shown in Table 2.8 for pre-amplified cDNA.

Table 2.8: Recipe for miRNA array sample when using a pre-amplified product.

Component	Volume for One Array (µl)
TaqMan Universal PCR master mix	450
Diluted Pre-Amp Product	9
Nuclease-Free Water	441
Total	900

The sample was mixed by gentle pipetting and centrifuged briefly. The arrays were allowed to come to room temperature after which, 100 µl of the master mix was dispensed into each chamber of the array. The card was centrifuged twice for 1 minute at 1000 x g to fill each of the 384 wells of the card in an Eppendorf 5810R centrifuge. The card was then sealed and the loading chambers were cut off. The card was run on the Applied Biosystems® 7900 HT thermocycler using the parameters contained within the SDS setup file on the supplied CD as shown in Table 2.9.

Table 2.9: Real time thermocycler parameters for the miRNA array.

Temperature(°C)	Time (min)	Cycles
50	2	40
94.5	10	40
97	30 (seconds)	40
59.7	1	40

OpenArray® plate technology on the QuantStudio™ 12K Flex Real-Time System (Paris, France) was used for large scale miRNA profiling to achieve high sample throughput. One OpenArray plate can hold the equivalent of eight traditional 384-well cards and three open array plates can run simultaneously. This technology uses a microscope slide sized plate (PCR chip) with 3,072 pores. Each pore is 300 µm in diameter and 300 µm deep and is pre-treated with hydrophilic and hydrophobic coatings. Reagents are then retained in the through holes/pores by surface tension. Each through-hole accepts 33±1 nl of sample and master mix.

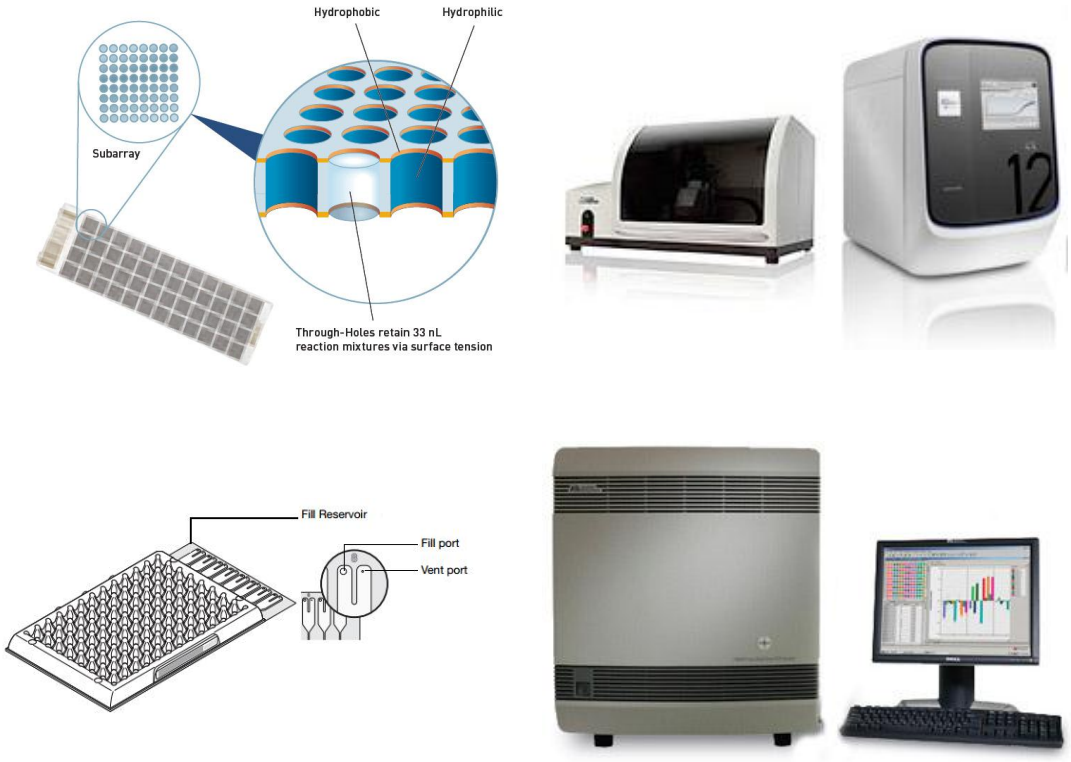


Figure 2.12: PCR systems used for miRNA quantification. Image above shows the OpenArray plate technology (left) and QuantStudio™ 12K Flex Real-Time System to the right. The image below (left) shows the 384 well miRNA taqman card and the 7900HT PCR system it is used with (right).

2.2.3.4 Gene expression work

2.2.3.4.1 Reverse transcription of platelet RNA

Reverse transcription was performed using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics). The kit is designed to reverse transcribe RNA for gene expression studies via two-step RT-PCR using qPCR on the LightCycler®96 instrument. cDNA synthesis for a two-step RT-PCR was performed using a combination of Anchored Oligo(dT) priming and random hexamer priming which are explained in Table 2.10. This was the method of choice used to increase sensitivity. The enzyme used - Transcriptor reverse transcriptase, is a recombinant reverse transcriptase expressed in *E coli* and has an RNA-directed DNA polymerase activity, unwinding activity and RNase H activity that degrades RNA in RNA: DNA hybrids. Thus, there is no need to perform an additional RNase H incubation step after reverse transcription, shortening the reaction time.

Table 2.10: Overview of priming methods used for cDNA synthesis.

Type of RT primer	Binding site	Advantages
Anchored Oligo(dT)$_{18}$	Very beginning of the poly (A) tail	• Prevents priming from internal sites of the poly(A) tail. • Generates full-length cDNA. • Preferred priming method for most two-step RT-PCR.
Random hexamer	Many sites throughout the length of an RNA	• Provides uniform representation of all RNA sequences in mRNA. • Can prime cDNA transcription from RNAs that do not carry a poly(A) tail. • The ratio of random primers to RNA in the RT reaction determines the average length of cDNA's generated. • Short cDNA transcripts may help to over-come difficulties caused by RNA secondary structures.

2.2.3.4.2 Preparing the reverse transcription mix

All frozen reagents were thawed before use, centrifuged briefly and kept on ice while preparing the reaction. The template-primer mixture was prepared in a nuclease-free PCR tube on ice, by adding the components in Table 2.11.

Table 2.11: Template-primer mix for cDNA synthesis.

Template-primer mix (for one 20µl reaction)		
Component	**Volume**	**Final concentration**
Total RNA	10 µl	10 ng- 5 µg total RNA or
Anchored Oligo(dT)$_{18}$ primer (50 pmol/µl)	1 µl	2.5 µM
Random hexamer primer (600 pmol/ µl)	2 µl	60 µM
PCR grade water	Variable	To make total volume = 13 µl
Total volume	13 µl	

The template-primer mix was heated for 10 minutes at 65 °C in a thermal block cycler with a heated lid (minimises evaporation). This ensured denaturation of RNA secondary structures if present. The tube was immediately cooled on ice. The remaining components of the RT mix were then added to the tube containing the template-primer mix, in the order listed in Table 2.12.

Table 2.12: Reverse transcription mix.

Component	**Volume**	**Final concentration**
Transcriptor Reverse Transcriptase Reaction Buffer (5X concentration)	4 µl	1x (8 mM $MgCl_2$)
Protector RNase Inhibitor (40 U/ µl)	0.5 µl	20 U
Deoxynucleotide Mix, 10 mM each	2 µl	1 mM each
Transcriptor Reverse Transcriptase (20U/ µl)	0.5 µl	10 U
Final volume	20 µl	

The reagents were mixed carefully and centrifuged briefly to pull the sample to the bottom of the tube. The tube was then placed in the thermal cycler block. Depending on the length of the target mRNA, the RT reaction was ran using according to manufacturer's instructions (10 mins at 25 °C, 60 mins at 50 °C and 5 mins at 85 °C). The reaction was stopped by placing the tube on ice and the sample was stored at -20 °C for further analysis.

2.2.3.4.3 Running the qPCR assay

The assay was performed using RealTime Ready (RTR) function tested custom qPCR assays (Roche Diagnostics) to detect different targets. Each RTR assay contained gene specific primers for the target gene and a Universal Probe Library (UPL) probe, a short FAM-labelled hydrolysis probe with locked nucleic acid. PCR mixes were prepared in PCR tubes on ice. PCR reaction mix was arranged by adding the components listed in the order in Table 2.13. 15 µl of the PCR mix was placed into each well of the LightCycler®96 multi-well plates. 5 µl of the template (cDNA of varying concentration depending on the experiment) was added to the PCR mix (to make a 20 µl reaction) and the plate was sealed with sealing foil. It was centrifuged for 2 minutes at 1500 xg in a swing bucket and placed into the LightCyler®96. Using the PCR program described according to manufacturer's instructions, the reaction was performed. The LightCyler®96 software was used to analyse relative quantification of target genes (Figure 2.13).

Table 2.13: PCR mix for RTR assays.

Component	Concentration	Volume
PCR grade water	-	4 µl
Probes master	2x conc	10 µl
Real time ready Assay	20x conc	1 µl
Total volume	-	15 µl

Figure 2.13: LightCycler® 96PCR application software and system *(www.lifescience.roche.com).*

2.2.4 Platelet function assays

2.2.4.1 Impact-R cone and plate analyser

The Impact-R device tests platelet function in anticoagulated whole blood under near physiological conditions. The test involves the application of laminar shear stress on a blood sample placed on a well, using a rotating cone. This results in platelet adhesion and aggregation on the bottom of the well. An image analyser measures the adhered platelets and results are expressed as a percentage of the well surface covered by aggregates (SC %) as an index of platelet adhesion, and average size of the aggregates (AS μm^2) as an index of aggregation. Figure 2.15 shows the system, components and workflow for testing samples. For this assay, blood samples drawn into sodium citrate vacutainers were analysed 1-hour post-draw (manufacturers recommend that whole blood samples should be tested not earlier than 45 minutes and no later than 3 hours after collection).

2.2.4.1.2 Sample incubation and activation of platelets by applied shear force

At 1 hour, blood samples were mixed for 1 minute at 10 rpm prior to loading on the apparatus. The well, cone and bell housing were set up on the instrument, and the bell was then removed by lifting from the side to ensure the cone was attached and placed beside the well. A 130 µl aliquot of the blood sample was taken from the midpoint of the blood tube and the sample was applied to the centre of the polystyrene well with appropriate care to ensure the tip did not contact the bottom of the well and that no bubbles were introduced (damages formation of aggregates). Immediately after the blood was placed onto the well, the bell housing with cone attached was placed on top. The selected programme was started (arterial shear rate of 1800 per second for 2 minutes was the programme used in all studies – This included a 15 second static time to allow sample to spread evenly across well. A maximum of two wells were ran at any given time.

2.2.4.1.3 Washing and staining

Once the run was complete, the bell housing with the cone attached was removed and the collet pin was pulled to release the cone into a waste container. Excess red cells/white cells/plasma mix were pipetted off ensuring that the tip did not touch the well sides or surface. The well was then gently washed with a Pasteur pipette (wide orifice) and deionised water. The water was directed on the inner sidewall of the well in a circular motion. Excess water was then pipetted off when the well was cleaned. 500 µl of May Grunwald stain solution was added to the well to stain adherent platelets and incubated for 1 minute. The stain solution was pipetted off and the well left to dry at room temperature for 3-4 minutes. See Figure 2.15 for the workflow for Impact R analysis.

2.2.4.1.4 Image analysis

The well was inserted into the sample tray and the first image was captured. It was then rotated manually 6 times with an image captured at each stop point. Seven images were captured in total. The software analysed the captured images by eliminating the four least readable images and calculating the average of the remaining three. Results were expressed as SC and AS with a visual and graphical result provided for each test.

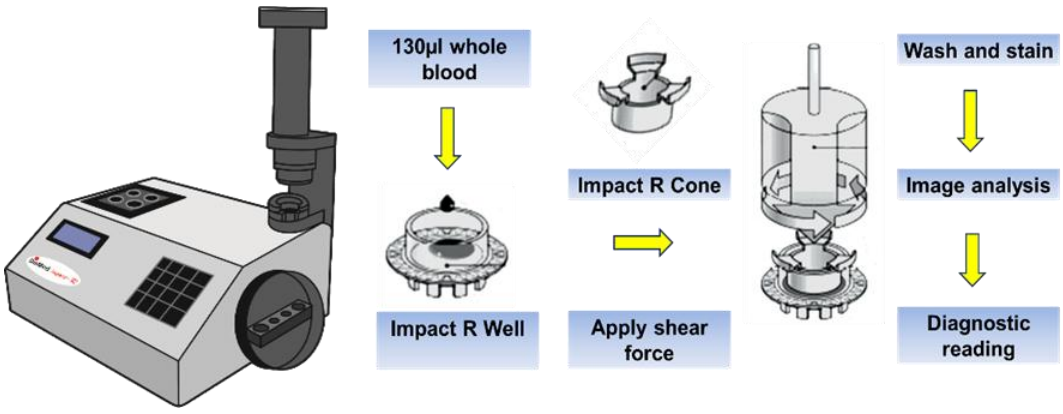


Figure 2.15: The Impact-R cone and plate device. Left: Impact R Cone and Plate Device with attached microscope. Right: Workflow for sample analysis using the Impact-R.

2.2.4.2 Flow cytometry

Flow cytometry is a sensitive technology which can give a quantitative and qualitative measurement on the size, complexity and condition of an individual cell. As the name suggests, it involves flowing single cells through a capillary past a point of detection. Detection is typically achieved through use of lasers and detectors. The technology has been increasingly used in platelet studies enables studies of a multitude of cell functions such as apoptosis, cell surface markers, platelet function and mitochondrial assays. Forward scatter is a measure of the size of the cell, whereas side scatter refers to the granularity of the cell. Fluorescence is the property of a molecule to absorb light and re-emit it at a different wavelength. This is depicted in Figure 2.16.

Flow cytometry experiments in this thesis were performed on either the FACS Aria or the Accuri C6 (Physical inactivity and dry immersion study). To ensure day-to-day sample reproducibility, all cytometers were calibrated daily.

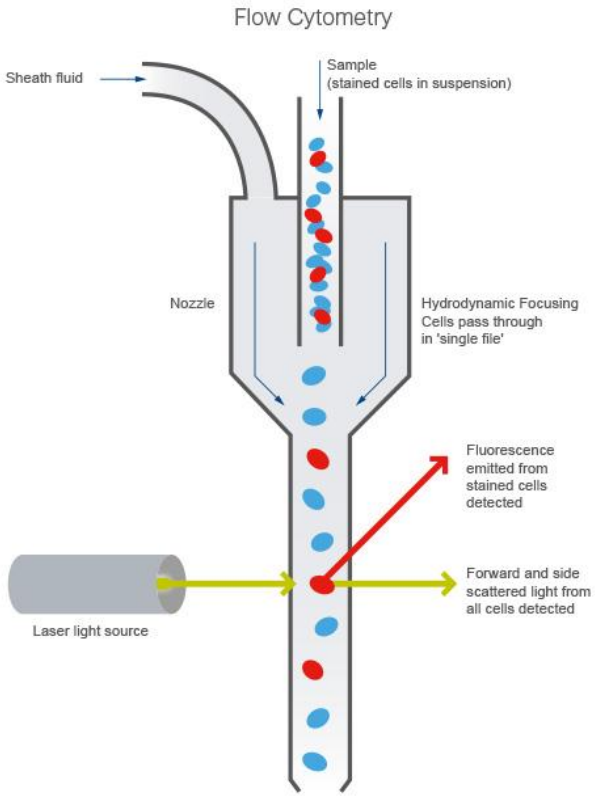


Figure 2.16: The principle of flow cytometry. Single cells flow through a capillary past laser detector, emitting light scatter and fluorescence. *(www.abcam.com).*

2.2.4.2.1 VASP/P2Y$_{12}$ phosphorylation assay

VASP (vasodilator stimulated phosphoprotein) is an intracellular actin-binding protein expressed at high levels in platelets and negatively regulates platelet secretory and adhesive functions which play a role in platelet aggregation. It is non- phosphorylated at basal resting state. VASP phosphorylation is regulated by cAMP (cyclic AMP). PGE$_1$ (prostaglandin E1) activates this cascade whereas ADP inhibits it through P2Y$_{12}$ receptors (Figure 2.17).

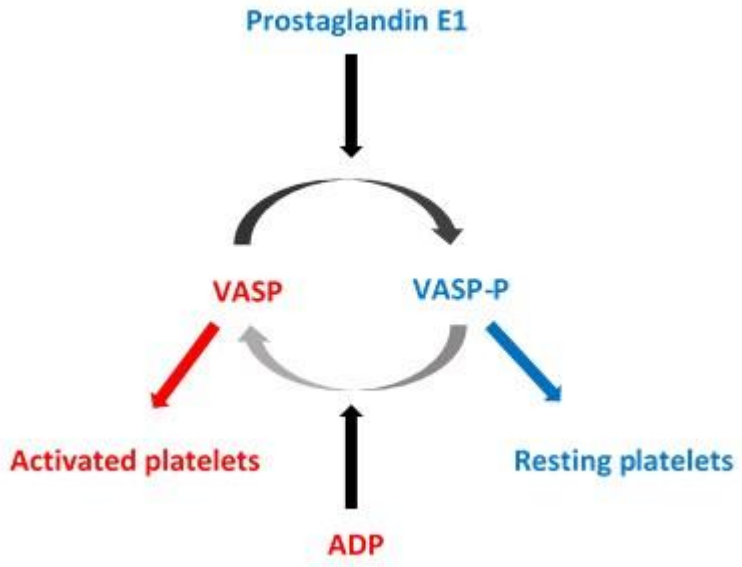


Figure 2.17: Regulation of platelet adhesion by VASP phosphorylation.

To determine the VASP phosphorylation state of whole blood, a standardized flow cytometric assay (BioCytex, France) was used. This was an adaptation of the method of (Schwarz *et al.,* 1999). The advantage of this kit was the use of small volumes of whole blood, allowing platelets to be analysed in their natural environment.

Reagents were brought to room temperature before use and all procedures were performed at room temperature. The VASP-P analysis was performed within 4 h after blood collection. Blood samples were incubated with PGE1 alone or PGE1+ADP. After cellular permeabilisation, phosphorylated VASP was labelled by an indirect no wash immunofluorescence using a specific monoclonal antibody. Dual colour flow cytometry analysis allows a comparison of the two conditions and evaluation of each samples capacity of ADP to inhibit phosphorylation.

2.2.4.2.1.2 Sample processing

Blood samples for VASP analysis were collected in 3.8% sodium citrate tubes (BD, Austria). 10 µl of whole blood was added to three separate sterile 1.5 ml Eppendorf tubes. 5 µl of prostaglandin E1 (PGE_1) was added to tube 1, while 5 µl of ADP&PGE_1 was added to tube 2 and tube 3. Samples were vortexed and incubated for 10 mins before fixation. 5 µl of fixative was added to the three tubes which were lightly vortexed and incubated for 5 minutes. Platelets were permeabilised and labelled with a primary antibody against serine 239-phosphorylated VASP by adding 5 µl of anti VASP-P mouse monoclonal antibody and permeabilisation agent to tubes 1 and 2 and 5 µl negative isotype control (mouse monoclonal antibody) + permeabilisation agent to tube 3. Tubes were vortexed and incubated for 5 minutes. Fluorescent staining and platelet counter staining was performed by adding 5µl of polyclonal antibody anti-mouse IgG-FITC, platelet counter-staining reagent-PE (anti CD61-PE) and permeabilisation agent to each tube. Tubes were vortexed and incubated for 5 min. 500 µl of diluent was added to each tubes, which were then vortexed and stored at 4 °C until flow cytometric analysis.

2.2.4.2.3 Flow cytometric analysis

Tubes were vortexed briefly before analysis to ensure even sample distribution.
The platelet population was identified by its forward and side scatter distribution and by expression of platelet specific antibody (CD61-PE) and 10,000 platelet events were gated. The median and geometric mean fluorescence intensity (MFI) of VASP-P was determined. When performing the flow cytometric analysis using the BD Accuri C6^{TM}, the background threshold was reduced from 80,000 events to 20,000 in order to ensure all platelet events were identified. A threshold of 5000 was set on the BD FACS Aria for the same purpose.

2.2.4.2.1 Results analysis

The value of the "corrected" MFI (MFIc) for each tube was obtained. MFIc was obtained after subtraction of the negative control tube (T3) from the value obtained for VASP-P (tubes one and two). The results from this test are labelled by the manufacturer as the "platelet reactivity index" (PRI) expressed as a percentage change in VASP fluorescence intensity between resting (+PGE_1) and activated (+ADP stimulated) platelets.

The PRI % was calculated from the median MFI using the calculation;

$$PRI\ \% = (MFIPgE1 - MFIPgE1{+}ADP\ /\ MFIPgE1) \times 100$$

For comparisons, the PRI was also calculated from the mean fluorescence intensities. The working range of this assay was between 0-100%.

2.2.4.3 Microvesicle quantification

The NanoSight NS300 and Syringe Pump (Figure 2.18) were used to quantify microvesicles (both exosomes and microparticles) in PFP samples. Nanoparticle tracking analysis technology (NTA) used in this device combines the properties of light scattering and Brownian motion to attain measurements including concentration and size distribution of particles in a liquid suspension. A finely focused laser beam is introduced to the sample (particles in dilute suspension) through a glass prism and illuminates the particles in the sample chamber. The particles in the beam path scatter light and are visualised by 20x magnification microscope fitted with a video camera. The camera functions at 30 frames per second and captures a video file of the particles moving under Brownian motion. The software tracks particles individually and uses the Stokes-Einstein equation to calculate their hydrodynamic diameters and particle size;

$$Dt = \frac{K_B T}{6\pi n r_h}$$

Where Kb is Boltzmann's constant, T is temperature, n is solvent viscosity, and Dt is particle diffusion coefficient (hence sphere equivalent).

Figure 2.18: The Nanosight NS300 and syringe pump. *(www.malvern.com).*

2.2.4.3.1 Microvesicle analysis

The syringe was loaded with 1ml of 10% EtoH to rinse the chamber slowly (10 second run) and repeated with 1ml of PBS. The cleanliness of the chamber was confirmed by checking for particles visible on the NTA screen and considered clean if < 3 particles were visible. This procedure was performed between each sample. If sample particles persisted in the image after rinse through cleaning, the flow-cell was cleaned manually. A 1:2500 dilution of PFP and high pure PBS was used after serial dilution optimisation (dilution 1= 4 µl of sample and 996 µl of PBS to make a 1:200 dilution, dilution 2= 100 µl of dilution 1 and 900 µl of high pure PBS). Diluted samples were kept on ice until ran on the Nanosight.

Video capture (camera gain and shutter speed (1206), syringe pump speed (50)) and analysis (background subtraction, minimum track length) setting were adjusted, allowing optimal particle identification for platelet poor plasma. The camera level was set to obtain an image that had sufficient contrast to clearly identified particles while minimising background noise. The sample was loaded into a fresh 1 ml syringe which was loaded into the chamber. Continuous pressure was applied to the syringe pump to ensure a smooth flow. Upon image identification on the capture screen, the focus was fine-tuned to give a sharp image of particles. The number and duration of captures was set to 15 x 60 second captures, providing 15 replicates of the same sample. After the 15 videos for each sample were taken, the NTA software tracked the Brownian motion of individual vesicles by automatically locating and following the centre of each and every particle, measuring the average distance it moved per frame. NTA converted the distances moved into a particle size and plots accumulated results in real time as a particle size distribution profile. NTA analysed the raw data, and calculated size and concentration and displayed different particle parameters (size versus relative intensity versus number) against each other.

2.2.4.3.2 Results analysis

Settings were optimised and maintained between samples. For each 60 second video, the concentration and size of the particles (from 0-1000 nm) were recorded. Each video provided the mean vesicle size together with an estimate of the total concentration. An experiment summary file was automatically generated, displaying the concentration of the sample at each vesicle size. The sum of the concentrations at each size were calculated and the average taken.

2.2.5 Human physiological tests

2.2.5.1 Maximal oxygen uptake (VO_2 max)

VO_2 max is the gold standard for measuring aerobic fitness and is the maximum rate of oxygen consumption as measured during incremental exercise. This test was employed as a cardiovascular stressor and to measure aerobic fitness.

Vmax machines were used to test VO_2 max and were calibrated before each test. The flow sensor was calibrated initially where different speed strokes of the syringe were used to simulate different breathing rates. Before every test the oxygen and carbon dioxide analysers were calibrated. This ensured that all variables measured were correct and reliable. During the calibration three known quantities of gases are delivered to the machine.

VO_2 max was determined using an incremental treadmill protocol with open circuit spirometry. The treadmill protocol was dependent on the fitness level of the subjects. For the adolescent's acute exercise study, the treadmill protocol was determined by the results of their MSST. For the adults, a physical activity questionnaire gave an indication of their fitness levels and protocols were chosen accordingly.

2.2.5.1.2 Rating of perceived exertion (RPE)

RPE was obtained using the 15 point Borg category RPE scale (Borg, 1990). Prior to the test, subjects read a set standard set of perceptual scaling instructions. Low and high "perceptual anchors" were established during the test, which involved asking the subjects to assign a rating of 6 (low anchor) to the lowest exercise intensity and 20 (high anchor) to the highest exercise intensity. Subjects were instructed to make their own subjective assessment of perceived exertion relative to these minimum and maximum standards (perceptual anchors).

2.2.5.1.3 Preparing the subject

Prior to each VO_2 max test, the testing procedures were explained to the subjects. They signed a consent form and completed a medical history questionnaire. The RPE scale was explained and their height, weight, resting heart rate and blood pressure were recorded. Subjects wore a Polar heart rate monitor (Polar Team^2 Pro, Polar Electro Inc., NY, USA) for the entirety of the test to record heart rate. This was clipped to a belt and worn on the centre of the chest. The subject was connected to the Vmax machine with the flow sensor and line

attached while standing on the treadmill. The head-gear was placed on the subject's head and adjusted for a tight but comfortable fit (Figure 2.19). The subject placed the mouthpiece in their mouth ensuring a tight seal and a nose clip was placed on their nose. Subjects were then familiarized with the treadmill and safety instructions issued. They were instructed to signal a thumb down to end the test at any stage. A baseline measurement of O_2 and CO_2 were taken for two minutes at rest.

Figure 2.19: VO_2 max test using an open circuit spirometry during an incremental exercise test.

2.2.5.1.4 Running the VO_2 max test

All VO_2 max exercise tests took place under standard laboratory conditions: 19-21 °C, 40-55% relative humidity and with a physician in close proximity. Each protocol was tailored to elicit a VO_2 max measurement within 8 – 12 minutes. The subject was instructed to straddle the treadmill until it reached starting speed, at which point they eased themselves onto the treadmill using the bars. There was a warm up of two minutes at 0% gradient and (for the adolescent's acute exercise study, 6-8 km/h and 9 km/h for the low/moderate fit and high fit groups respectively), (for the adult acute study: warm up 8 km/h and speed increased by 2 km/h until reaching 12 km/h and at this point the gradient increased). This warm up was followed by the exercise stage where treadmill speed increased by 1 km/h every two minutes until maximum speed was achieved. The maximum speed for the low/moderate group and high fit groups were 10-11 km/h and 12-13 km/h respectively (.2% every 12 seconds). If necessary, a stage was added or removed prior to incline to tailor for the estimated VO_2 max.

RPE was taken during the last ten seconds of each stage. Systolic and diastolic blood pressure were measured by the physician during the last minute of each stage of exercise. Heart rate was recorded in the last 20 seconds of each stage.

Expired oxygen, carbon dioxide, ventilatory volume, respiratory exchange ratios and VO_2 max were determined by indirect calorimetry using the Vmax systems. Subjects exercised until reaching volitional fatigue. The test was terminated by volitional exhaustion or if contraindications arose. Subjects were verbally encouraged throughout the test to encourage maximal effort. Oxygen uptake was deemed to have peaked if two or more of the following criteria were satisfied (I) plateau of oxygen consumption with increasing power output (increase of less than 2 ml/kg/min), (ii) heart rate within 10 beats of the subjects' age predicted maximum heart rate (220bpm – age in years) and (iii) respiratory exchange ratio >1.10. O2max was determined as the highest minute average recorded for oxygen uptake during the test.

2.2.5.2 Vital signs

2.2.5.2.1 Blood pressure and heart rate

Subjects were required to sit upright for ten minutes after which systolic and diastolic blood pressure was recorded using either a fully automated arm blood pressure monitor (Omron M2 blood pressure monitor) (Cross-sectional study) or manually using a mercurial sphygmomanometer (Dekamet Accoson Sphygmomanometers, Harlow Essex) and stethoscope (Classic II 3M Littmann, St. Paul, MN) (Acute exercise study) Heart rate was measured using automated HR monitors which were placed around subjects chests.

2.2.5.3 Anthropometric measurements

2.2.5.3.1 Height and weight

Height (m) was measured to the nearest centimetre and weight (kg) was measured to the nearest 0.1 kg. Both were measured in light clothing without shoes.

2.2.5.3.2 Body composition

Body composition is a key component of health in both individuals and populations. Different methods were applied in order to obtain body composition measurements. Circumferences, bioelectric impedance analysis (BIA) and BMI measurements were performed to estimate body composition.

2.2.5.3.3 Body mass index (BMI)

BMI was used to measure weight relative to height and was calculated by dividing body weight in kilograms by height in meters squared ($kg \cdot m^{-2}$). It was determined by the following formula:

$$BMI = Weight\ (kg)/Height\ (m^2)$$

2.2.5.3.4 Circumferences

The pattern of body fat distribution is recognized as an important predictor of the health risks of obesity. Android or visceral obesity which is characterized by more abdominal fat is linked with an increased risk of hypertension, metabolic syndrome, coronary artery disease etc., compared with those who show fat distributed in the hip and thigh (gynoid or gynaecoid obesity). Waist circumference was used as another measure of abdominal obesity. Subjects stood upright with their feet together. Waist circumference was measured with the subject standing arms at side, feet together and abdomen relaxed and a horizontal measure taken at the narrowest part of the torso.

2.2.5.3.5 Bioelectrical impedance analysis (BIA)

Techniques of BIA are used for general health fitness testing and body composition monitoring. The Tanita SC-331S Body Composition Analyser (Figure 2.20) is a medical device that uses Bioelectrical Impedance Analysis (BIA) technology with a scales and control panel. Subjects removed their shoes and socks and stood on the scales for the measurement. An electrical signal (50 kHz, 90µA) was sent through the body via 8 pressure contact electrodes, positioned to ensure that the electric current was supplied from the electrodes on the tips of the toes of both feet, and voltage was measured on the heels of both feet. The current then flowed into the lower limbs.

Fat allows little electricity to pass through, whereas water found in muscles allows electricity to pass through easily. Electrical resistance is the degree of difficulty with which electricity passes through a substance and the percentage of fat and other body constituents can be calculated from this measurement of resistance. The machine provided a result sheet in 20 seconds with readings for the following parameters of physiological health; weight, impedance, body fat percentage, fat mass, fat free mass, total body water percentage, total body water mass, muscle mass, basal metabolic rate (BMR), metabolic age, bone mass, visceral fat rating and BMI.

Figure 2.20. Tanita SC-331S body composition analyser and height measurement apparatus. *(www.tanita.com).*

2.2.5.4 Physical inactivity model - dry immersion method

A European Space Agency (ESA) funded Dry Immersion (DI) study was used to investigate physical inactivity. DI studies, along with bed rest studies are utilised as an important platform to prepare for future human exploration in space. Additionally, they provide a unique experimental design to investigate human physiology in the setting of extreme physical inactivity. This DI study took place from Jan 2015 – Feb 2015 in the Medes Clinic, Toulouse, France. All subjects provided informed consent in compliance with the Helsinki Declaration. The experimental protocol was approved by the local ethics committee (CPP Sud-Ouest Outre-Mer I, France) and the French Health Authorities. The study was conducted and organised by the Institute for Space Medicine and Physiology (MEDES) Toulouse, France.

2.2.5.4.1 Subject recruitment

Subjects were recruited by internet advertisement on the MEDES website (http://www.medes.fr/en/index.html) and by media advertisements.

2.2.5.4.2 Subject selection

This section was carried out in two phases. The preliminary selection was based on applications files comprised of two questionnaires on 1) the subject's way of life, education and professional experience; and 2) the subject's medical background, both personal and on his family. 24 volunteers were pre-selected based on their applications, to have a final selection of 14 volunteers (12+2 backup). The second selection phase consisted of a visit to the Medes Space Clinic where subjects underwent clinical and paramedical examinations.

This included the following measurements;

- Medical and surgical history including habits, alcohol, caffeine intake, smoking status, previous/current medication
- A complete clinical examination including an electrocardiogram test (ECG) systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) performed in both a supine position and a standing position after both 3 and 10 minutes (stand test). These tests checked for the absence of orthostatic hypotension.
- A measurement of maximal oxygen consumption (VO_2 max test)
- A DEXA measurement of bone density
- An alcohol breath test
- A biological screening comprising of biochemistry, urine drug screen haematology, molecular screening (research for the factor V Leiden mutation and prothrombin gene. A positive result for phlebitis markers or the presence of one of these mutations was considered as an exclusion criteria) serology, urine drug screen, urinalysis, and vitamin and mineral status.

The full Dry Immersion Inclusion and Exclusion Criteria is listed in Appendix C.

2.2.5.4.3 Hospitalisation periods

When the final 12 subjects were certified healthy by the comprehensive medical assessment, they began their participation in the study. Each subject attended the clinic twice. A first hospitalization of one day (7 am to 7 pm) was required to perform a muscle biopsy and to take blood samples. One/two weeks later, the main hospitalization period of 8 days began. This phase was organised as follows; each 3-day immersion began with a four-day ambulatory control period (BDC: Baseline data collection). Subjects arrived on BDC-4 and during this period, subjects were allowed to be ambulatory during the day while remaining in the clinic for baseline evaluations. This was followed by a 3 day DI plus two recovery days.

2.2.5.4.4 Verification of selection criteria

The subjects completed an interview and complete clinical examination on their day of arrival to check the permanence of the selection criteria. A second biological screening was performed during the baseline data collection (BDC) period to ensure that all parameters were still within the normal range.

2.2.5.4.5 Daily medical supervision

Adherence to all study rules were controlled by the study nurse manager. Additionally, compliance with the study requirements were monitored with 24-hour video surveillance using random real-time control. During each hospitalisation phase, the daily medical supervision included the following measurements;

- A clinical assessment
- Arterial BP measurements and HR measured twice daily at 6.30am and 6.30pm
- Weight was recorded three times daily using a scales (weighing trolley) designed to weigh subjects in the bed rest position
- Body temperature was measured with a tympanic thermometer twice daily
- Hydric intake and 24-hour dieresis
- Body temperature monitoring

2.2.5.4.6 Dry immersion method

DI involves immersing a subject in thermoneutral water, covered with a waterproof fabric (Figure 2.21). Consequently, the immersed person, who is freely suspended in the water, remains dry. For a relatively short duration, the model can realistically reproduce the physiological effects of microgravity, hypokinesia and therefore, act as model of physical inactivity.

The experiment was performed in a specially designed bath filled with tap water. The bath was 2.2 m long, 1.1 m wide and 0.85 m deep. A unique highly elastic waterproof fabric was attached to a metal rim around the external margin of the bath. The area of the fabric greatly exceeded the area of the water surface (if the bath was empty, the fabric would reach the bottom). The bath contained a built in lift for lowering and raising the subject. Subjects were dressed in under garments and placed on the waterproof fabric (after the fabric was first covered by a cotton sheet for hygiene reasons). Subjects were slowly lowered into the water on the lift and their bodies were gradually covered with the folds of fabric, together with the water they contained. The fabric is thin and of sufficient area to allow the subject to appear to be freely suspended in the water mass, under conditions that are similar to a complete lack of structural support. The subjects were allowed to put their hands out to eat, drink, work with a computer and perform experimental tasks.

The water temperature was regulated automatically and was initially set to 32-24.5 °C (thermoneutral) and adjusted for comfort within those limits at the subject's request. The air temperature was approximately 24 °C to maintain the heat balance when subjects were raised from the bath. The subjects remained under constant medical observation 24 hours a day. For physical tests, the folds of fabric could be moved apart without changing the experimental conditions substantially. Subjects followed a “normal” day/night cycle and were woken at 6.30 am and light was turned off at 11 pm. There was no artificial light.

Figure 2.21: The Dry immersion (DI) method. Subjects were immersed up to the neck and separated from the water with the unique waterproof fabric. *(Right figure from Navisaslova et al. 2011).*

2.2.5.4.7 Nutritional intake

To reduce confounding factors on those being investigated, nutritional intake was quantified and monitored during the study. Food was selected and supplied in order to characterise its contents through standard nutritional databases. Dietary intake was quantified by measuring the weight of each dish before and after each meal and by calculating the intake for each nutrient. The calculation of the food content was done using a database specifically built from validated European databases. Subjects received three main meals and one snack per day. On the first hospitalisation day, subjects BMR was calculated in kcal/day using the WHO equation;

$$Age<30y: BMR=15.3 \times body\ weight\ (kg) + 679$$
$$Age>30y: BMR=11.6 \times body\ weight\ (kg) + 879$$

The reference weight was the weight measured on the morning on BDC-3, before breakfast and after the first void. During the pre-immersion period and the recovery ambulatory period, caloric intake was equal to 160% of BMR. During the immersion period, caloric intake was equal to 130% BMR. The measured BMR was used for diet planning instead of the calculated value whenever it was feasible to do so. Macronutrient intake was controlled to: protein 1-1.2 g/KGBW/day, total fat 35-38% TEE and carbohydrates: remaining.

Electrolyte intake was controlled to; sodium: 2000 – 4500 mg/day, potassium: 3000 – 5000 mg/day, calcium: 900-1200 mg/day chloride: 750 - 4500 mg/day. Liquid intake was controlled; between 35 and 50ml/kg/day (total water taking into account beverages and food). No coffee, tea or alcohol were allowed. As part of the study rules, there was a requirement to finish all meals.

2.2.5.4.8 Leisure time

During the entire experiment, subjects had to perform extensive scientific tests. There was free time between these and subjects were permitted various leisure activities including reading and computer activities. No nap was allowed during the pre and post immersion periods. The subjects were allowed to make phone calls to family after scientific tests and between 6 pm – 11 pm each day.

2.2.5.4.9 Blood sampling and assays

A total of 45 ml of blood was taken at the selection phase and a total of 420 mls of blood was taken throughout the study, with an allocation of 13.5 mls of blood for platelet function analysis at selected time points.

2.2.5.4.10 Medical check-up post experiment

At the end of the second hospitalisation period, subjects underwent a final clinical examination including blood pressure and heart rate measurements in supine and after two and five minutes in the upright position to re check orthostatic tolerance. Furthermore, recommendations for physical training to allow for full recuperation was advised to each subject.

2.2.5.4.11 Visual analogue Scale

The VAS scale is an ease of use measurement of pain intensity (Hawker *et al.*, 2011) including a horizontal line, 10cm in length with a verbal descriptor at each end to highlight symptom limits (Figure 2.22). The subjects completed the scale each day during the DI. Using a ruler, the subject marked a point on the line to best represent their pain intensity. The distance from "pain free" to their mark is measured in millimetres (mm) and provides their VAS score (1-100).

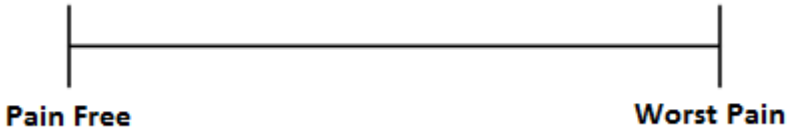


Figure 2.22: The visual analogue scale. *(Image from Hawker et al., 2011).*

2.2.5.5 Acute exercise methods

2.2.5.5.1 Adolescent population

In conjunction with an ongoing research study in the school of health and human performance school, male adolescents were recruited for testing procedures. The experimental protocol was approved by the Dublin City University Ethics committee.

2.2.5.5.1.1 Research design

Contact was made with principals of post primary schools within the greater Dublin area to provide a brief outline of the study. Cluster sampling was used to recruit male, consensual adolescents of varying physical fitness and in transition year, from schoolsclose to DCU. All subjects were recruited based on fitness level, proximity to DCU and their willingness to participate. Ethical approval was obtained from the Dublin City University Research Ethics Committee.

Exclusion criteria:

- Failure to present an informed consent form signed by a parent/guardian
- current smokers,
- Medical condition which prevented them from engaging in exercise
- Systolic BP >180 mmHg and/or diastolic BP >100 mmHg.

The first school visit provided detail on the study, physical activity readiness questionnaire (PAR-Q), informed consent forms to be completed by the student and parent/guardian. Each student received forms on the study and testing procedures.

The second school visit included a 20 m multi stage shuttle test (MSST) during a physical education class. The MSST required students to run back and forth between two lines 20m apart, while keeping time to a series of audio signals or "bleeps" from a CD. Initial running speed was 8.0 km/hr and increased every minute by .14 $m.s^{-1}$. A test was ended if the student voluntarily dropped out or was unable to maintain the set pace and failed to reach the line for two consecutive shuttles. Subjects wore heart rate monitors to provide data on maximum age predicted heart rate during the MSST to ensure that they were not dropping out for reasons other than fatigue.

The scores from the 20m MSST were used to provisionally categorise subjects into groups of fitness (Low/moderately fit or high fit) categories using percentile data proposed by FITNESSGRAM and used on data collection during a 20 m MSST as part of the HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence) Study (Moreno *et al.,* 2014), illustrated in Table 2.14. This was subsequently used to determine the VO_2 max treadmill protocol that subjects used during their visit to the health and human performance (HHP) labs in DCU.

Table 2.14: Percentile norms for low, moderate and high fit adolescent males aged 15-17 years.

Gender	Age	Low Fit	Moderately Fit	High Fit
Male	15	3.2-4.3	5.9-7.3	8.9-13.3
Male	16	3.3-4.3	5.8-7.2	8.7-12.8
Male	17	3.4-4.5	6.1-7.5	9.1-13.2

2.2.5.5.1.2 Visit to DCU HHP labs

On completion of the MSST and consent, the selected subjects came to DCU at 9am following an overnight fast. Anthropometrics and body composition was assessed by standard procedures as explained previously. Resting BP and electrocardiogram (ECG) were recorded to ensure no contraindications to exercise were present. Blood samples were drawn at selected time points. Fitness categories used to separate groups was based on VO_2 max (ml/kg/min) values using the percentile data from a study undertaken on youths aged 12-19 years illustrated in table below: The percentile cut off for the high fit group was determined as >53.0 and <48.0 for the moderate/low fit.

Table 2.15: Deciles of estimated VO_2 max for adolescents by sex and age group. *(Adapted from Eisenmann et al., 2011).*

Mean VO2 max (ml/kg/min)									
Age	**Percentile**								
	10th	20th	30th	40th	50th	60th	70th	80th	90th
14-15 years	38.1 (36.8-39.4)	40.0 (39.5 – 41.)	41.9 (40.3-43.6)	43.8 (42.6-45)	45.8 (44.2-48.1)	48.2 (46.6-49.3)	50.2 (48.5-51.5)	52.5 (51.3-54.4)	58.8 (54.9-61.7)
16-17 years	36.4 (35.0-37.8)	38.9 (38.2-41/0)	42 (41.0-43.5)	44.4 (42.8-45.9)	46.2 (45.1-47.3)	47.9 (46.5-49.4)	50.2 (48.8-51.9)	53.8 (51.5-55.4)	58.3 (56.8-60.5)

2.2.5.5.2 Adult population

Healthy males were recruited from the School of Health and Human performance. Subjects were informed of testing protocols and procedures prior to consenting. All subjects adhered to the same pre testing protocol; no strenuous exercise 24 hrs pretesting, no consumption of alcohol 48 hrs prior and free of NSAID consumption. Prior to testing all subjects complete a general questionnaire to ensure suitability for testing and signed consent forms. This testing procedure followed the same principle as the adolescent population. Subjects arrived in the HHP at 9am fasting and lifestyle data was collected. Anthropometric, body composition measurement, resting heart rate (RHR) pressure (BP) were taken at different time points. Blood samples were drawn at selected time points.

2.2.6 Statistical analysis

Results are expressed as mean ± SD. Statistical comparisons were performed using a variety of tests, depending on the experimental procedure. Main tests included independent t-tests, paired samples t-tests, repeated measures analysis of variance (ANOVA) and one-way ANOVA. Pearson product coefficient and partial correlations were used to examine relationships between variables. Statistical significance was set at a level of 0.05. SPSS v19 statistical package was used to analyse results.

To control for potential covariates which could act as a confounder (a variable whose presence affects the variables being analysed) such as age, analysis of covariance (ANCOVA) was used in place of ANOVA and partial correlations were used in place of Pearson product coefficient variables. For example, to assess the relationship between platelet count and BMI, a Pearson product coefficient was used. However, if it was suspected that a third variable may influence this relationship (e.g. age), a partial correlation was used. In this procedure, SPSS controls for the effect of the third variable, also known as a covariate using a unique algorithm. Similarly, analysis of variance (ANOVA) was the statistical model used to identify differences between the means of three or more groups. Analysis of covariance (ANCOVA) is a more complex form of ANOVA, which is used to observe the difference between the means of three groups, while controlling for the variability of other variables. This statistical procedure was used when a covariate (such as BMI, VO_2 max) were suspected to influence the means. SPSS uses a specific algorithm to compute this data (Green and Salkind, 2010).

Chapter Three

Outputs:

- High platelet counts and plateletcrit were associated with measures of overweight
- Platelet adhesion was significantly increased in both males and females with an overweight BMI
- BMI and body fat percent were the most sensitive adiposity measures in reflecting the changes in platelet parameters, whereas waist circumference was specific for alterations in females only
- There were variances in platelet indices and function according to fitness levels. As fitness levels decreased, platelet indice values increased. This was particularly relevant to males

Contributions from others:

- Sysmex Ireland kindly provided the Sysmex XS-1000i automated haematology analyser which was used throughout this study
- Science Gallery, Trinity College Dublin facilitated the exhibition “Fat” providing the platform for a large population to be recruited for the study

Chapter Three: A Cross-sectional Study of Platelet Function and Lifestyle Factors

3.1 Introduction

The pathogenesis of atherosclerosis in CVD is multifactorial and evidently, lifestyle factors including physical activity, smoking, diet and obesity exert significant influences on CVD. Sedentary behaviour and concordantly, obesity, represent a growing problem worldwide whose prevalence has more than doubled between 1980 and 2014. Subsequently, it is one of the biggest health burdens in the world and Ireland. Metabolic syndrome (metS), a condition epitomising a group of cardiometabolic risk factors including central obesity, hypertension and insulin resistance, is also an increasing concern (Depres and Lemieux, 2006; Esser *et al.,* 2014).

Platelets are versatile cells that have a pivotal role in atherosclerosis, not only due to their influence in haemostasis but also as a result of their cytokine, chemokine, growth factor and proinflammatory mediator release (Lievens *et al.,* 2011). Therefore, they are intricately involved in inflammation, one of the most important pathogenic mechanisms of atherosclerosis. Importantly, some CVD risk factors are found to be elevated years before manifestation of acute events and cardiovascular dysfunction is implicated in early stages of disease.

Most studies demonstrate that platelet function is altered in CVD or in obese individuals displaying risk factors for CVD (Santilli *et al.,* 2011). Whilst it is known that platelet indices and markers of platelet health are expressed differently in diseased populations (Goshal, 2014), only a minority of studies have solely examined the relationship between overall physiological health and platelet function in healthy subjects. Platelet indices have been recently investigated as possible biomarkers for a range of diseases including inflammatory bowel disease (Ozturk *et al.,* 2013), severe endometriosis (Avioglu *et al.,* 2014), malaria (Leal-Santos *et al.,* 2013), CVD in arthritic patients (Jurcut *et al.,* 2010), Alzheimer's disease (Wang *et al.,* 2013) and as early predictors of growing risk of cardiovascular complications in Diabetic and Non-Diabetic subjects (Ashraf *et al.,* 2015). ***This study explored the feasibility of platelet indices and whole blood platelet function measurements, as useful, non-invasive initial biomarkers of early/subclinical CVD risk and unhealthy lifestyle.***

3.1.1 Chapter aims and experimental approach

This cross-sectional study was carried out to investigate associations between risk factors of CVD (including obesity/overweight and physical (in) activity), overall physiological health, and platelet function**.**

Hypothesis:

Platelet function and platelet indice markers will be elevated in subjects with unhealthy lifestyle habits, in particular with regard to those who are overweight and physically inactive.

Main aims:

- The primary aim of the study was to elucidate if platelet function is related to overall physiological health, and to investigate the relationship between platelet function and lifestyle factors
- A secondary aim was to establish if the Impact-R cone and plate device is a reliable device to measure platelet function across a heterogeneous population.
- To determine if platelet indices could be employed as biomarkers of health in a disease-free population

3.1.2 Study design

Platelet function was profiled in apparently healthy adults (n=155) between 18 and 90 years to determine the relationship between platelet function, platelet count, and various parameters of physiological health, reflective of lifestyle, including but not limited to BMI, waist circumference, body fat percentage, visceral fat and physical (in) activity levels.

Subject recruitment

This study took place from May-June, 2014 in the Science Gallery, Trinity College Dublin, in conjunction with an exhibition "Fat" which explored all aspects of obesity. It was a completely randomised selection of subjects. Subjects visited the gallery and were informed about the various tests by different researchers (see https://dublin.sciencegallery.com/fat) for more information. There was a seven-day reflection period before consenting to various tests, in which subjects were provided with information and returned one week later for voluntary testing. All subjects gave informed consent. This study received ethical approval from the Faculty of Trinity Health Science and Dublin City University ethics boards. Each subject provided a citrated blood sample for analysis.

Assessment of covariates

Participants provided information on demographic characteristics and lifestyle habits by completing questionnaires (Appendix A). Information on race was categorized as Irish, any other white background, Asian or Indian. Smoking status was defined as current, past, or never smoker. Information was obtained on individuals' perception of their own physical fitness/physical activity levels. This was measured using a scale from 1-5, 1 being unfit and 5 being very fit. Participants self-reported any medication use, or NSAID intake in the previous 10 days.

Blood sample

A single blood sample was taken from each subject in a resting position. Blood was obtained by standard venepuncture from the antecubital forearm vein and collected into vacutainer tubes containing 3.2% sodium citrate. Platelet, Erythrocyte and Leukocyte indices were measured by the Sysmex XS-3000i.

Platelet function

Two parameters of platelet function were evaluated using the Impact R: surface coverage (SC, %) representing platelet adhesion, and the average size (AS, μm^2) of the polystyrene bound platelet clusters/aggregates representing platelet aggregation.

3.2 Results

3.2.1 Characteristics of the study population

In total, 155 subjects volunteered to partake in this study. The age range of the subjects was 18-90 years (Figure 3.1). Except for BMI, there was a significant difference between anthropometric and body composition characteristics between males and females, shown in Table 3.1 below. Medication, Cardiovascular and Demographic characteristics are shown in Tables 3.2, 3.3 and 3.4 respectively. Haematological characteristics are displayed in Table 3.5.

Table 3.1: Anthropometric and body composition characteristics of the subjects. Values are mean ± SD. BMI - Body mass index, VAT - Visceral adipose tissue, WC - Waist circumference, TBW - Total body water, BMR - Basal metabolic rate. *P<0.05. Independent *t*-test. See appendix A for reference ranges for these parameters.

	Gender		
	Male n=68	**Female n=87**	**P value**
Age (years)	37.4 ± 17.47	39.4 ± 17.66	.850
Height (cm)	176.6 ± 7.98	166.3 ± 669	*
Weight (Kg)	76.8 ± 10.44	66.9 ± 9.79	*
BMI (kg/m^2)	24.8 ± 4.48	24.3 ± 4.17	0.298
Body fat (%)	18.63 ± 5.56	28.88 ± 7.69	*
Visceral adipose tissue	6.1 ± 5.77	4.4 ± 3.12	*
WC (in)	34.9 ± 3.45	33.05 ± 3.75	*
Fat mass	14.1 ± 5.27	20.8 ± 7.32	*
Fat free mass	63.5 ± 6.34	46.1 ± 3.88	*
Muscle mass	60.4 ± 6.05	43.9 ± 3.65	*
TBW (%)	56.7 ± 4.03	49.7 ± 5.09	*
Bone mass	3.19 ± 0.68	2.14 ± 0.88	*
BMR (kcal)	7802 ± 885	5859 ± 524	*

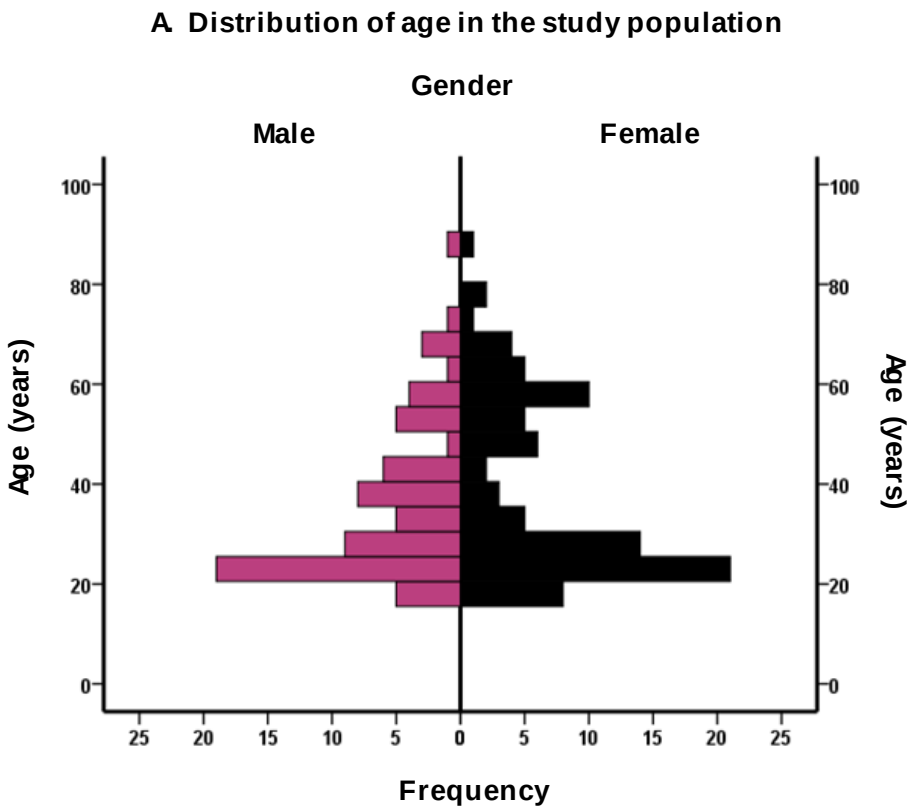


Figure 3.1: Distribution of age in the study population. The youngest subject was 18 years and oldest aged 90, with the average age falling between 20-35.

Table 3.2: Medication characteristics of the subjects. Values are numbers of participants taking medication. NSAIDS – Non-steroidal anti-inflammatory drugs.

	Gender	
	Male	**Female**
Statins	2	3
Blood pressure	3	3
NSAIDS	0	3
Contraceptive pill	0	45
Insulin	0	1
Antiplatelet	1	1
Anaemia	0	2

Table 3.3: Blood pressure and resting heart rate characteristics of the subjects. Values are mean ± SD. SBP - Systolic blood pressure, DBP - Diastolic blood pressure, RHR - Resting heart rate. *P<0.05. Independent *t*-test and one-way analysis of covariance (ANCOVA) adjusting for age.

	Gender		
	Male	**Female**	**P value**
SBP (mmHg)	121 ± 16.53	116 ± 14.93	0.082
DBP (mmHg)	73 ± 9.22	72 ± 11.33	0.486
RHR (bpm)	70 ± 11.36	76 ± 13.14	*

Table 3.4: Demographic and lifestyle characteristics of the subjects. Values are percentage of subjects in each category. AOWB – Any other white background, including Asian and Indian.

		Gender	
		Male	**Female**
Physical activity levels			
	Unfit	15%	13%
	Fairly fit	54%	42%
	Fit – very fit	30%	46%
Smoking status			
	Non-Smoker	83%	79%
	Smoker	8.5%	8%
	Past smoker	8.5%	13%
Ethnicity			
	Irish	65%	79%
	AOWB	29%	15%
	Other	6%	6%

Table 3.5: Haematological characteristics of the subjects. Values are mean ± SD. WBC - White blood cell count, RBC - Red blood cell count, HGB - Hemoglobin, HCT - Haematocrit, MCV - Mean corpuscular volume, RDW - Red cell distribution width. *P<0.05. One-way ANCOVA adjusting for age.

		Gender		
	Reference range	**Male**	**Female**	**P value**
WBC (10^6/µl)	3.98 – 10.04	6.17 ± 1.64	6.25 ± 1.51	0.750
RBC (10^6/µl)	3.9 - 6.1	4.50 ± 0.65	4.05 ± 0.43	*
HGB (g/dL)	11.2 – 17.5	13.85 ± 1.58	11.96 ± 1.26	*
HCT (%)	34.1 – 51.0	40.07 ± 4.25	35.76 ± 3.30	*
MCV (fL)	79.0 – 94.1	88.61 ± 3.51	89.74 ± 5.42	0.090
RDW (fL)	35.0 – 46.3	40.26 ± 4.22	41.22 ± 2.53	*

3.2.2 Comparison of platelet parameters according to gender

In order to assess the variance in platelet indices and function according to gender, comparisons between male and female subjects were performed. As shown in Table 3.6, although all platelet indices were elevated in females, there was no statistical difference between gender. Table 3.7 compares platelet function values between gender and showed no statistically significant difference between platelet adhesion/aggregation in male and female subjects.

Table 3.6: Platelet indice characteristics of the subjects. Values are mean ± SD. PLT - Platelet count, PDW - Platelet distribution width, MPV- Mean platelet volume, PLCR - Platelet large cell ratio, PCT - Plateletcrit. One-way analysis of covariance (ANCOVA) adjusting for age.

		Gender		
	Reference range	**Male**	**Female**	**P value**
PLT (10^3/µl)	160.0 – 400	192 ± 40.88	200 ± 42.05	0.285
PDW (fL)	9.3 – 16.0	11.29 ± 1.77	11.47 ± 1.87	0.317
MPV(fL)	8.8 – 12.50	10.11 ± 0.81	10.26 ± 0.89	0.269
PLCR (%)	1-2	25.6 ± 6.52	26.8 ± 6.11	0.236
PCT (%)	19 – 40	19.2 ± 4.01	20.4 ± 4.23	0.080

Table 3.7: Platelet function characteristics of the subjects. Values are mean ± SD. SC - Surface coverage, AS- Average size. One-way ANCOVA adjusting for age.

	Gender		
	Male	**Female**	**P value**
SC (%)	12.27 ± 3.02	11.84 ± 3.25	0.471
AS (μm^2)	42.7 ± 21.23	43.9 ± 20.54	0.432

3.2.3 Body composition measurements of obesity/overweight

To assess the relationship between platelet parameters and adiposity, several body composition measurements were performed including BMI, Waist circumference and bioelectrical impedance analysis. Correlation analysis and comparisons between subjects of varying body composition allowed us to examine variances in platelet parameters.

3.2.3.1 Body Mass Index (BMI)

136 subjects chose to have their BMI measured. To explore the relationship between BMI and platelet parameters, correlations analysis was performed (Table 3.8). The most pertinent finding was the significant positive correlation between BMI and PLT, BMI and PCT and BMI and SC in females as shown in Figure 3.2. The WHO (World Health Organisation) scale for BMI was utilised to categorise subjects based on BMI, illustrated in Table 3.9. Comparisons were then performed to examine the variance in platelet indices according to BMI categories highlighted in Figure 3.3, with significant increases in platelet adhesion in the overweight group in both genders.

Table 3.8: Correlation between BMI and platelet parameters. PLT - Platelet count, PDW - Platelet distribution width, MPV - Mean platelet volume, PLCR - Platelet large cell ratio, PCT – Plateletcrit, SC – surface coverage, AS – Aggregate size. *P<0.05. Partial correlation adjusting for age.

		Platelet parameter						
		PLT	**PDW**	**MPV**	**PLCR**	**PCT**	**SC**	**AS**
BMI	**Male**	.251	-.254	-.259	-.271	.132	.277	.060
	Female	.319*	-.018	-.088	-.053	.351*	.332*	.124

Table 3.9: BMI categories. Values are the number and percentage of participants in each category.

	BMI category and reference range		
	Healthy 18-24.9 kg/m^2	**Overweight 25-29.9 kg/m^2**	**Obese 30+ kg/m^2**
Male	46% (32)	33% (29)	8% (5)
Female	63% (55)	22% (20)	9% (8)

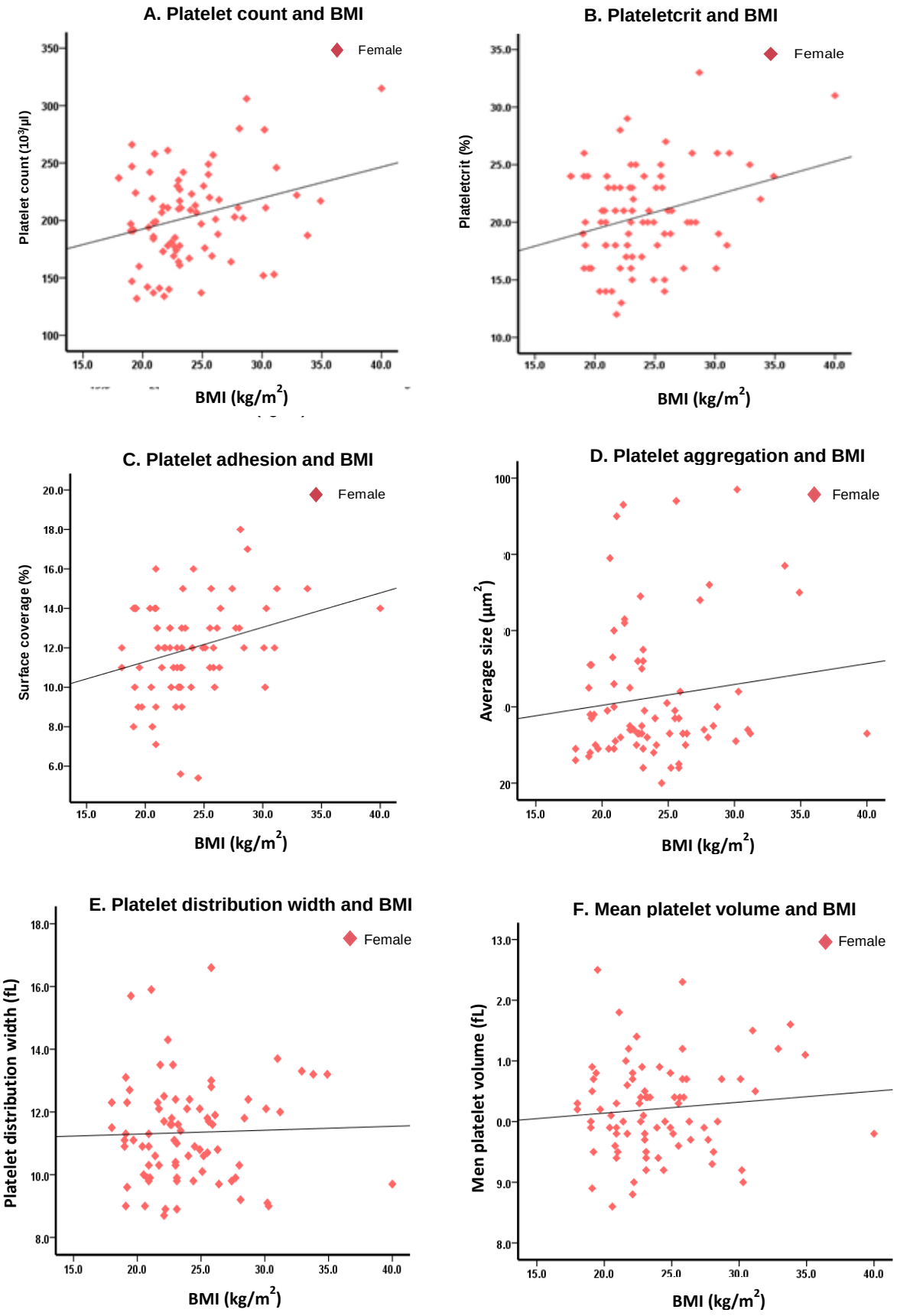


Figure 3.2: Correlation between BMI and platelet parameters in females. A - Platelet count and BMI, B - Plateletcrit and BMI, C - Platelet adhesion and BMI, D - Platelet aggregation and BMI, E - Platelet distribution width and BMI, F – Mean platelet volume and BMI.

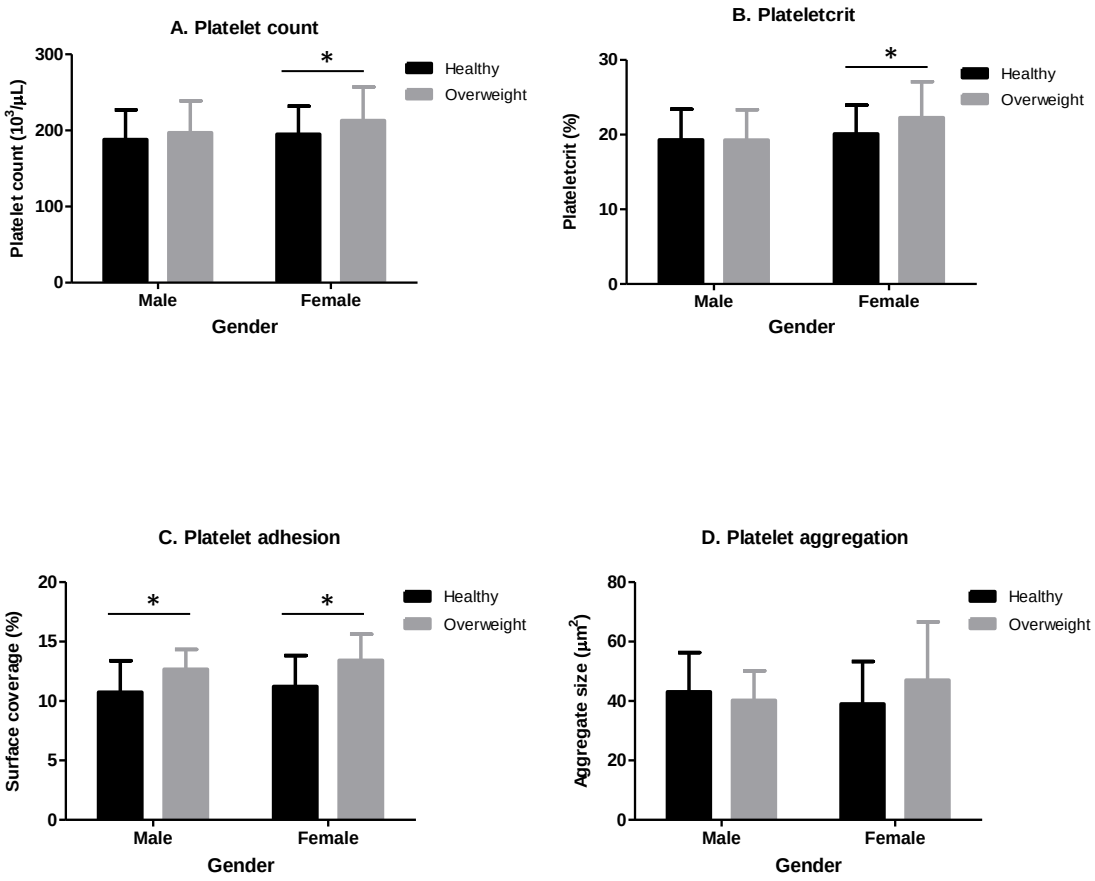


Figure 3.3: Comparison of platelet parameters between healthy and overweight subjects according to BMI. Graphs represent mean ± SD variance in A – Platelet count, B – Plateletcrit, C - Platelet adhesion and D – Platelet aggregation. *P<0.05. One-way ANCOVA adjusting for age.

3.2.3.2 Waist circumference (WC)

Most of the study population (n=120) consented to have WC measurements taken and therefore were included in this analysis. To examine the association between WC and platelet parameters, correlations between variables were performed and are shown in Table 3.10. The main findings include a significant relationship between PLT and WC, plateletcrit and WC and platelet adhesion and WC in female subjects only, shown in Figure 3.4. To compare variances in WC, subjects were categorised using the disease risk criteria for waist circumference in adults (Bray GA 2004) outlined in Table 3.11. When stratified by WC, results show a significant difference in PLT, PCT and platelet adhesion between low and high risk groups in females (Figure 3.5).

Table 3.10: Correlation between waist circumference and platelet parameters. PLT - Platelet count, PDW - Platelet distribution width, MPV - Mean platelet volume, PLCR - Platelet large cell ratio, PCT – Plateletcrit, SC – surface coverage, AS – Aggregate size. *P<0.05. Partial correlation adjusting for age.

		Platelet parameter						
		PLT	**PDW**	**MPV**	**PLCR**	**PCT**	**SC**	**AS**
WC	**Male**	.174	-.054	-.055	-. 067	.001	.275	.071
	Female	.305*	.015	.060	.044	.295*	.310*	.121

Table 3.11: Proportion of subjects in each waist circumference category. Values are percentages and numbers of subjects in each category.

Waist circumference (in) and reference ranges				
Risk category	**Female**		**Male**	
Very low	< 28.5	0	< 31.5	0
Low	28.5 - 35.0	75% (51)	31.5 - 39.0	82% (42)
High	35.5 - 43.0	23.5% (16)	39.5 - 47.0	16% (8)
Very high	> 43.5	2.9% (2)	> 47.0	2% (1)

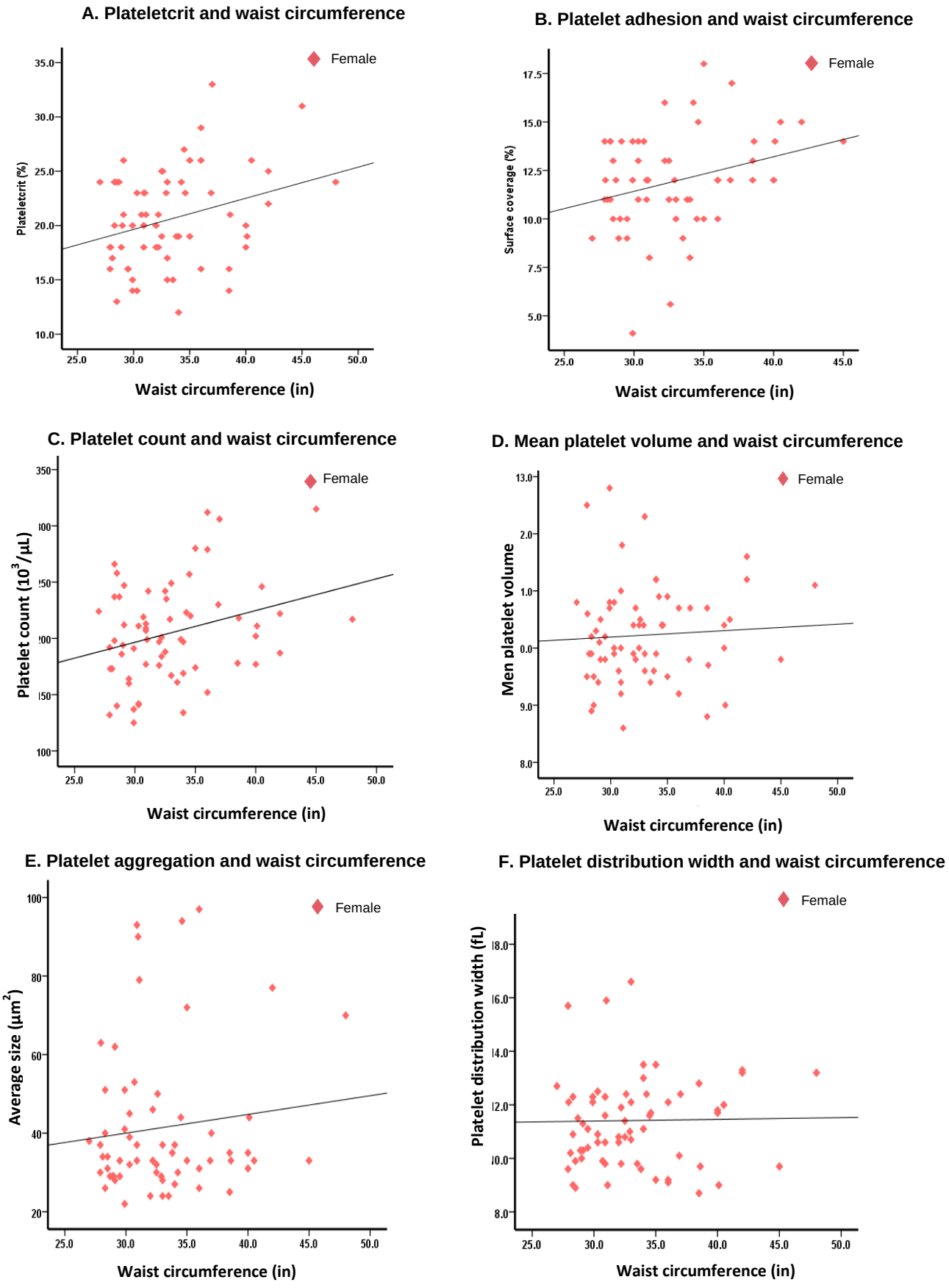


Figure 3.4: Correlation between waist circumference and platelet parameters in females. Scatterplot A - relationship between PCT and WC, B - relationship between platelet adhesion and WC, C- PLT and WC, D - MPV and WC, E – Aggregation and WC and F – PDW and WC.

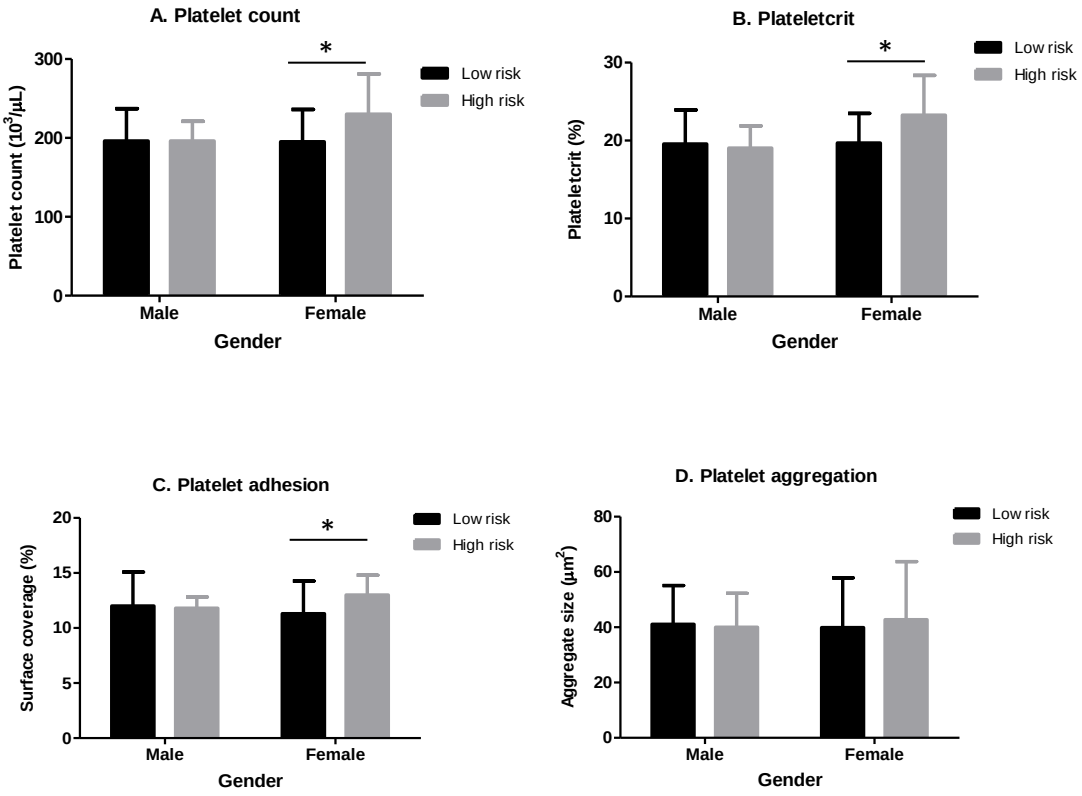


Figure 3.5: Comparison of platelet parameters between low and high risk waist circumference groups. Graphs represent mean ± SD. A - Platelet count, B – Plateletcrit, C - Platelet adhesion, D - Platelet aggregation. *P<0.05. One-way ANCOVA adjusting for age.

3.2.3.3 Bioelectric impedance analysis (BIA)

Bioelectric impedance analysis was utilised to measure multiple components of body composition including weight, body fat %, fat mass, fat free mass, total body water %, muscle mass, basal metabolic rate, bone mass and a unique indicator called visceral adipose tissue (VAT) which measures visceral fat. The Tanita SC-331S body composition analyser provided healthy range indicators which compares several measurements to their respective healthy range. To assess the associations between the BIA measures and platelet parameters, correlation analysis was performed. Table 3.12 displays correlations between platelet indices and BIA measurements. Figure 3.6 shows scatterplots of selected associations. The platelet volume indices MPV, PDW and PLCR were inversely correlated with BF, VAT, FM and positively correlated with MM, TBW in males. In females there was no correlation between the platelet volume indices and BIA measurements.

Table 3.12: Correlation between Tanita body composition measurements and platelet parameters. PLT- Platelet count, PCT – Plateletcrit, SC - Surface coverage, AS - Aggregate size, BF % - body fat percent, VAT - Visceral adipose tissue, FM - Fat mass, FFM - Fat free mass, MM - Muscle mass, TBW % - Total body water percent, P<0.05. Partial correlation adjusting for age.

	BIA Body Composition Measurement					
Male	**BF**	**VAT**	**FM**	**FFM**	**MM**	**TBW**
PLT	.295*	.262	.245	.034	.007	-.289*
PCT	.178	.137	.245	-.044	.053	-.206
SC	.345*	.065	.313*	-.244	-.244	.136.
AS	.106	.160	.067	-.253	-.254	.304*
Female	**BF**	**VAT**	**FM**	**FFM**	**MM**	**TBW**
PLT	.265*	.301*	.216*	-.045	-.016	-.194
PCT	.226*	.293*	.282*	.037	.026	-.110
SC	.288*	.262*	.292*	.247	.211	-.181
AS	.071	.058	.184	.017	.062	-.074

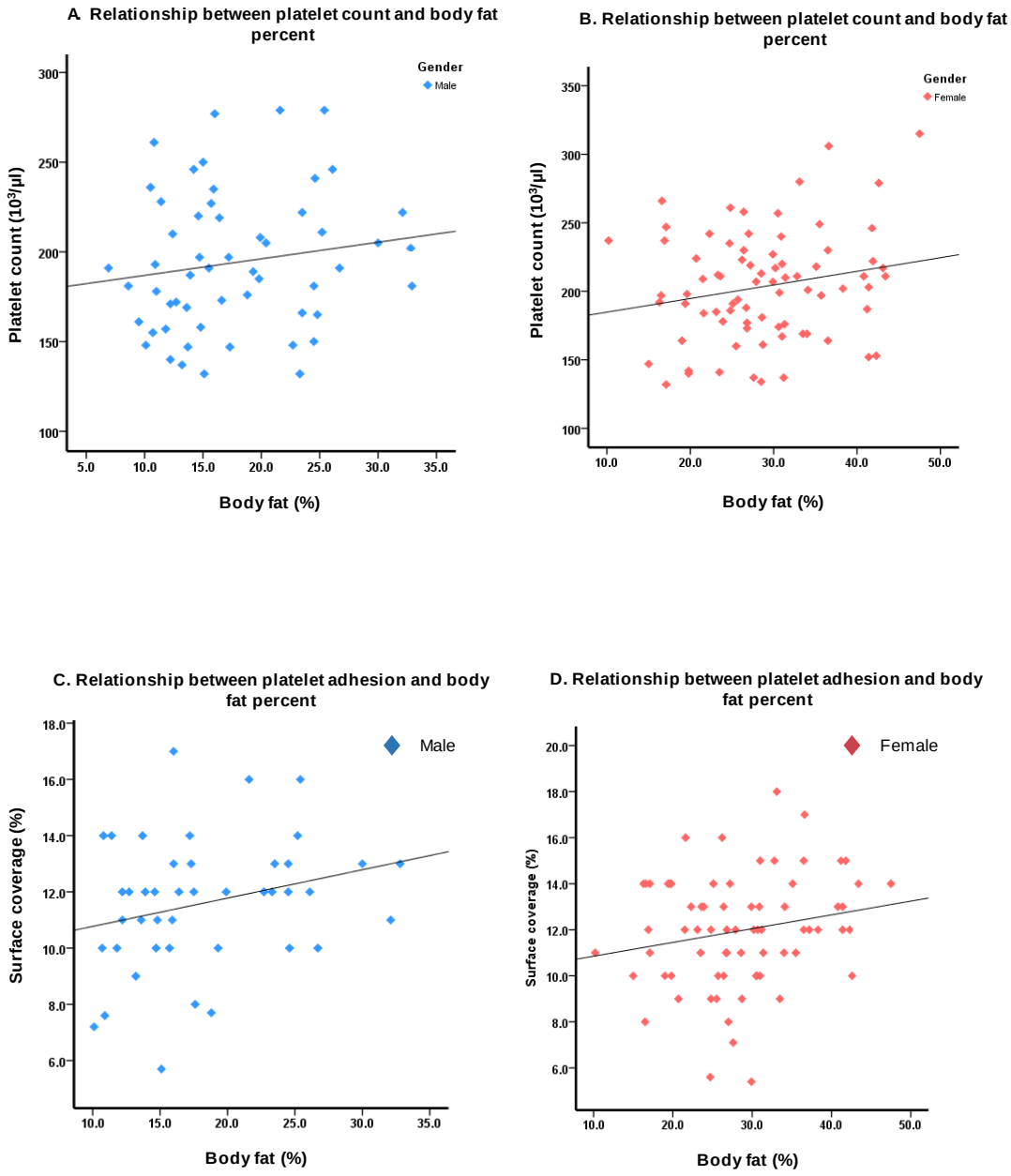


Figure 3.6: Correlations between selected BIA measurements and platelet parameters. A – Relationship between body fat % and platelet count in males. B – Relationship between body fat % and platelet count in females. C – Relationship between body fat % and platelet adhesion in males, and D – Relationship between body fat % and platelet adhesion in females.

3.2.3.3.1 Body fat percentage (BF %)

As BMI or WC cannot distinguish body fat from muscle mass, BIA analysis enabled us to examine these variables separately. In order to examine variability in platelet parameters according to body fat, subjects were split into subgroups according to percentile norms for body fat % for age and gender (Thompson *et al.*, 2008), shown in Table 3.13. Differences in the means of platelet parameters according to the subgroups are shown in Figure 3.7.

Table 3.13: Body fat percent categories. Values represent the number of subjects in each category. Good – body fat percent between the 60th-90th percentile, Fair - body fat percent between the 40th-55th percentile and Poor - body fat percent between the 15-35th percentile.

	Body fat percent category		
	Good	**Fair**	**Poor**
Male	31	16	9
Female	21	13	44

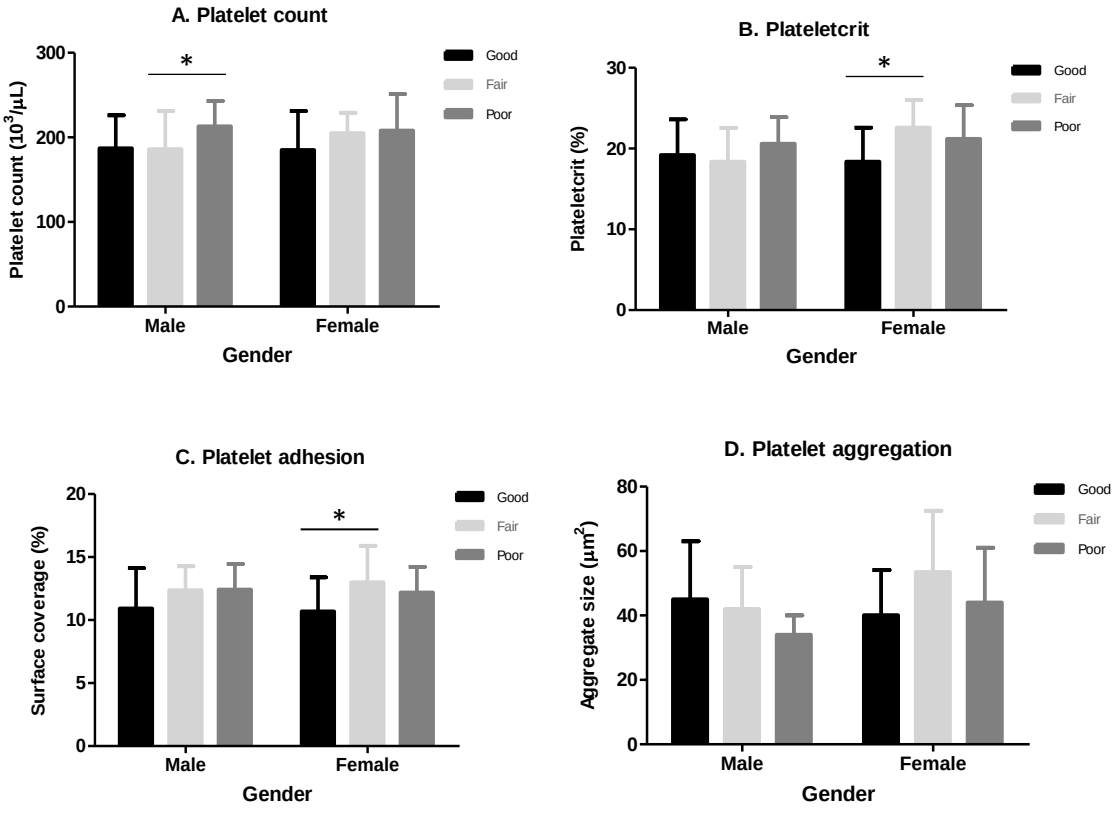


Figure 3.7: Difference in platelet parameters between body fat percent categories. Graphs represent mean ± SD. A – variance in PLT, B – variance in PCT and C – variance in SC. One-way ANCOVA adjusting for age. *P < 0.05.

3.2.3.3.2 Fat mass (FM)

Fat mass was measured by BIA. The Tanita scales provided a dimensionless number for fat mass, with 1 = lowest possible fat mass and higher values corresponding to an increasing fat mass. Fat mass was significantly correlated with PLT and PCT and SC, shown in Table 3.12. Subjects were categorised as having very low, low, moderate or high fat mass to compare platelet parameters. The distribution of subjects in each fat mass category is shown in the Table 3.14. As shown in Figure 3.8, there was a significant increase in platelet adhesion in the High group for females compared to the moderate group. PLT and PCT were highest in the High groups for both male and females.

Table: 3.14: Fat mass categories. Values represent the number of subjects in each category.

	Fat mass category and reference range			
	Very low 4-10	**Low 11-19**	**Moderate 20-29**	**High 30+**
Male	22	24	12	0
Female	10	36	19	15

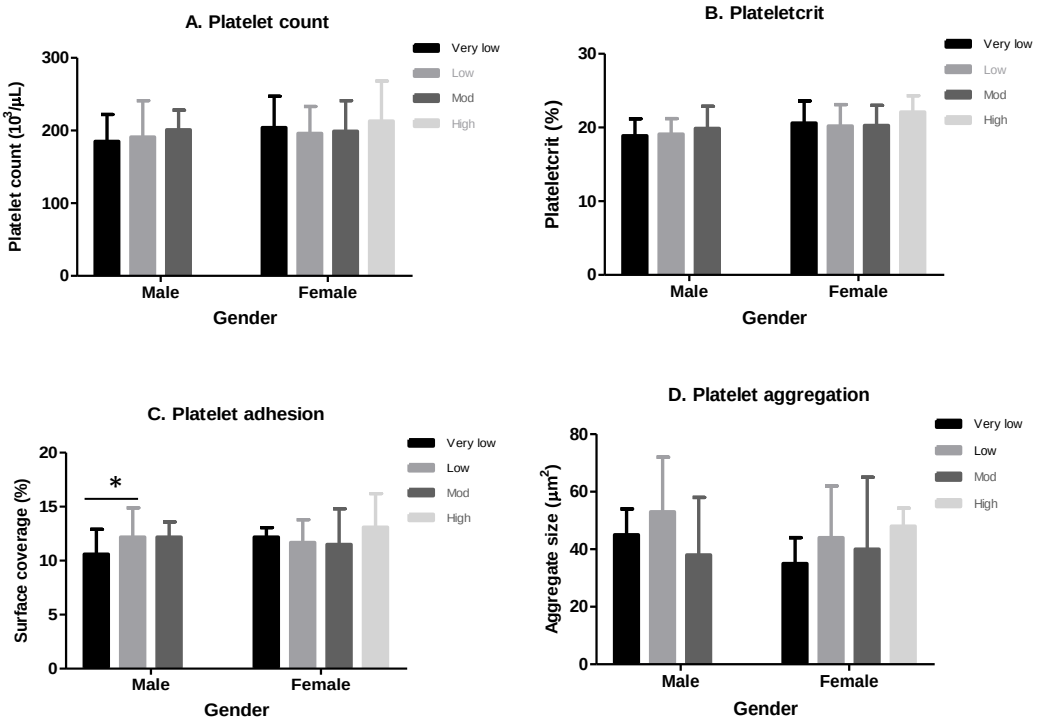


Figure 3.8: Difference in platelet function between fat mass groups. Graphs represent mean ± SD variance in A – PLT, B – PCT and C – SC. *P< 0.05. One-way ANCOVA adjusting for age.

3.2.4 Self-reported physical activity levels

Questionnaire data enabled the stratification of subjects depending on self-assessed fitness levels. Figure 3.9 shows variances in platelet indices and function according to fitness levels. Although no significance was reached between groups, as fitness levels increased, platelet indices decreased, and this was particularly evident in males.

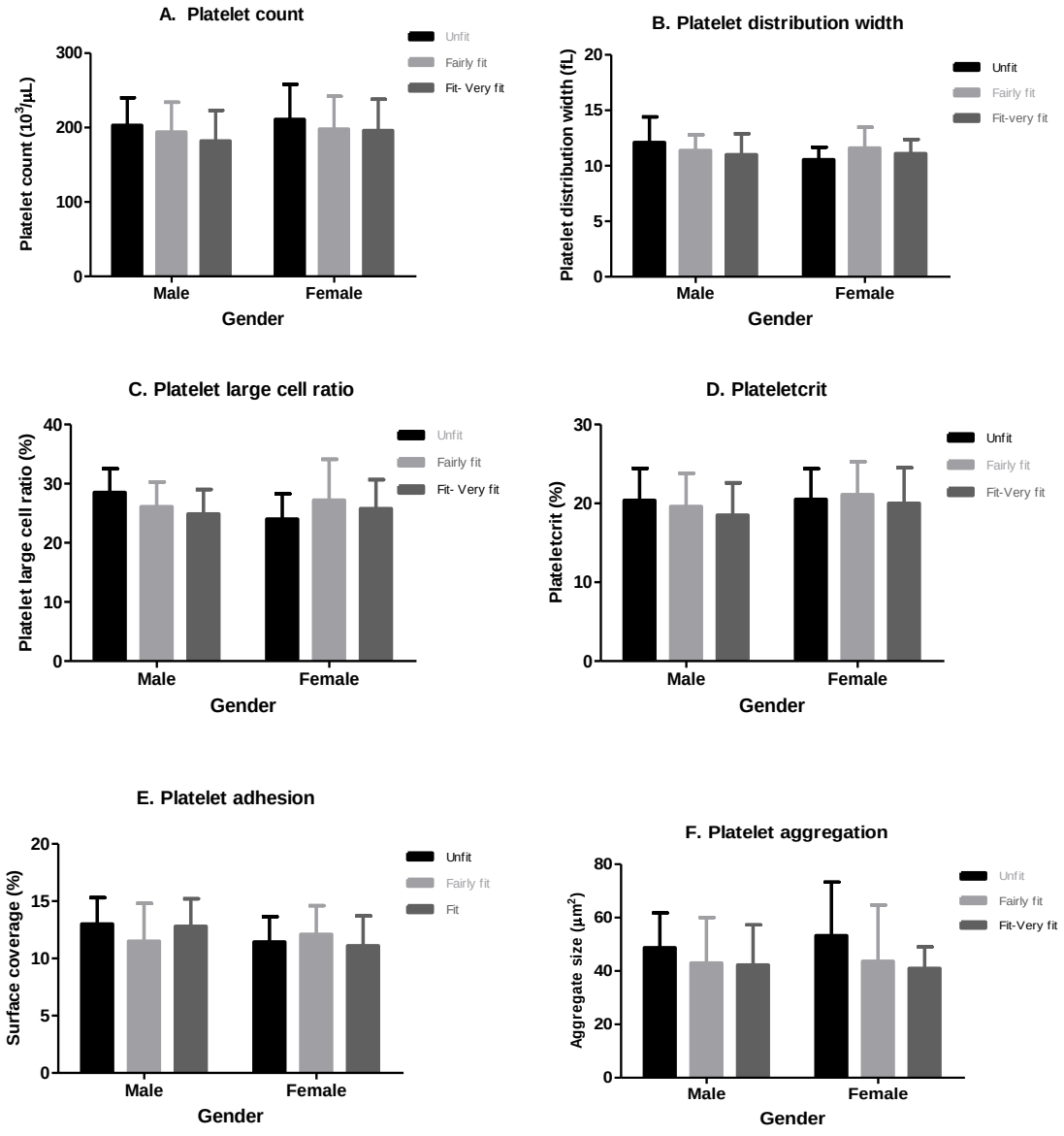


Figure 3.9: Comparison of platelet parameters between physical fitness categories. Graphs represent mean ± SD variances in A – platelet count, B – Platelet distribution width, C – Platelet large cell ratio, D – Plateletcrit, E – Platelet adhesion and F – Platelet aggregation. One-way ANCOVA adjusting for age.

3.3 Discussion

Modifiable lifestyle components including physical inactivity, obesity, smoking, and hypertension, and non-modifiable risk factors comprising ethnicity, gender and age, contribute to CVD and atherosclerotic risk. There is increasing evidence that these traditional CVD risk factors such as type 2 diabetes mellitus (T2DM) and hypertension in diseased patients are associated with increased platelet reactivity (Kakouros et el. 2011) and that improvements in these risk factors are linked with a reduced prothrombotic tendency (Mittendorfer, 2008).

Studies have shown that platelet function is altered in CVD or in obese individuals with multiple CVD risk factors (Santilli *et al.*, 2011). Whilst it is known that platelet indices and markers of platelet health are expressed differently in diseased populations (Goshal, 2014), only a minority of studies have solely examined the relationship between overall physiological health, CVD risk factors and platelet function in healthy subjects. Some studies have presented data on the relationship between PLT, MPV and PDW according to gender and age (Zhang *et al.,* 2015).

However, the relationships between physical inactivity, overweight, amongst other lifestyle habits with platelet function are less well recognised, in particular in healthy subjects. This study aimed to address the deficit in this area by analysing the relationships between these parameters and also to evaluate the differences in platelet parameters according to non-modifiable risk factors including gender and age. We therefore explored the feasibility of platelet indices and whole blood platelet function measurements, as useful, non-invasive initial biomarkers of early/subclinical CVD risk and unhealthy lifestyle.

To accomplish this, correlations analysis and comparisons were performed for each lifestyle factor and platelet parameter. In this single site cross-sectional study of an apparently healthy, random population, platelet count, plateletcrit and platelet adhesion measurements were slightly elevated as physiological health measurements reflective of lifestyle, reached unhealthy levels. This was particularly evident in female subjects.

3.3.1 Characteristics of the study population

Table 3.1 shows the clinical characteristics of the study population. The average age was 37.4 years for males and 39.4 for females. The distribution of age in the population shows a centring around age 20-35 years (Figure 3.1) The average BMI was similar between male and females and as anticipated body fat % was significantly higher in women compared to men as is generally the case due to sex-characteristic fat related to child bearing (Blaak, 2001). Fat mass was significantly higher in females compared to males and this has been reported frequently in the literature (Schutz *et al.,* 2002). VAT levels were higher in males compared to females, the only other adiposity measure besides WC which was greater in males. Although women generally have a higher body fat percent than men, much of their adipose tissue is stored in the gluteal-femoral region, whereas men have been shown to store more fat in the abdominal area (Blaak, 2001) explaining this observation. Women had a significantly higher resting heart rate (RHR) than males as shown in Table 3.3. Gender related differences in leukocyte and erythrocyte parameters are illustrated in Table 3.5. As expected, erythrocyte parameters including RBC, HGB and HCT were significantly increased in males compared to females, a common finding in other studies (Murphy *et al.,* 2010) whereas RDW and MCV were higher in females. WBC was similar across gender (Charles *et al.,* 2007). The majority of the population was of Irish descent.

3.3.2 Gender differences in platelet function and platelet indices

CVD develops approximately 7-10 years later in women than men (Maas and Appelman, 2010). It has been suggested that exposure to endogenous estrogens delays this development as men are at greater risk of heart disease than pre-menopausal women, but once past the menopause, a woman's risk is parallel to a man's (WHF). Tables 3.6 and 3.7 compare platelet parameters between male and female subjects. All platelet indices were slightly elevated in females compared to males. Supporting this data, platelet indices were increased in females in the large cross-sectional Moli-Sani study (Santimone *et al.,* 2011) but, analogous to other studies, there were no significant differences observed for platelet indices (Giovanetti *et al.,* 2011).

Baseline PLT in females was 5% higher than males compared to other studies where the gender-related variances in adults was slightly higher at 10% (Balduni and Noris, 2014; Sloan *et al.,* 2015). Further studies have also shown similar results whereby female PLT have exceeded those of their male counterparts (Segal *et al.,* 2006; Zhang *et al.,* 2015). The minimal changes in MPV between gender (.15 fL) are consistent with findings by Sloan *et al.,* (2015) and Butkiewicz *et al.,* (2006) where MPV was not found to be dissimilar between the sexes. There were negligible changes in PDW between males and females and (0.28 fl) whereas slightly higher changes in PLCR and PCT were observed. Giovanetti *et al.,* (2011) and Zhang *et al.,* (2015) have reported significant differences in PCT between males and females.

It has been postulated that the higher PLT and PCT levels in females may partially exist due to hormonal influences and in particular the presence of estrogen which has a role in generating platelet production in mice, although no human studies have been performed to date (Nagata *et al.,* 2003). Estradiol has been revealed to stimulate proplatelet production in megakaryocytes and furthermore, estrogen–receptor antagonists prevent platelet production *in vivo,* ancillary to a role of estrogens in platelet production (Segal and Moliturno, 2006). Changes in hormonal profiles between gender therefore may be proposed to end in different platelet phenotypes.

Regarding platelet function parameters, mean platelet adhesion values for male and female were 12.2 and 11.8% respectively, similar to values reported for healthy volunteers in the literature (Shenkman *et al.,* 2008). Mean platelet aggregation was almost identical. Evidence that platelet function varied according to gender was first recognised by Johnson *et al.,* (1975) and has been established in more recent years where females have displayed increased platelet aggregation compared to males after stimulation with a number of agonists in whole blood and platelet rich plasma (PRP) (Becker *et al.,* 2006). Other studies did not find any sex related platelet function disparities (Rubak *et al.,* 2012; Beyan *et al.,* (2006) Furthermore, Cho *et al.,* (2008) found no difference in platelet function between gender in a Korean population using the PFA-100. Overall, we found minimal differences in platelet function between gender but a larger sample size may display differences between gender.

3.3.3 Body composition/adiposity measures

Body composition is used to describe the percentage of fat, bone, water and muscle in the body and can be measured using a variety of techniques including body mass index (BMI), waist circumference (WC), bioelectrical impedance analysis (BIA) which are important health outcomes (Wells and Fewtrell 2006). Overweight and obesity are defined as excessive fat accumulation and are rapidly growing global problems (WHO, 2015). Estimations of global trends in obesity has put Ireland on course to become the most obese country in Europe within a decade, with Irish men already having the highest BMI in Europe (Singh *et al.,* 2016). This study aimed to examine the relationship between platelet parameters and body composition before a disease threshold is apparent, to determine if overweight subjects display a similarly enhanced platelet profile to obese subjects. A number of adiposity measures were assessed in each subject.

3.3.3.1 Body mass index (BMI)

BMI measurements were performed to determine its relationship with platelet parameters due to its useful population measure of overweight and obesity. It is widely accepted that a high BMI is a predictor of CVD morbidity (WHO, 2015). In adults, being overweight is defined as a BMI of 25 - 29.9 kg/m^2 and obesity is defined as having a BMI $\geq$ 30 kg/m^2 (Lavie *et al.,* 2009). For the majority of people, obesity related health problems increase after a BMI of 25 kg/m^2. In this population, 33% of males and 22% of females had a BMI of greater than 25 kg/m^2, classed as overweight.

Table 3.8 displays the relationship between BMI and platelet parameters. There was a positive relationship between BMI and PLT in males and females, with a weak significant correlation only amongst females (Figure 3.2). This finding is in concordance with other studies who have demonstrated a correlation between BMI and PLT in women only (Sloan *et al.,* 2014; Chen *et al.,* 2016; Kotani *et al.,* 2007). As shown in Figure 3.2, PCT was positively correlated to BMI in females but not males, which also remained significant when adjusted for age. PCT has been correlated with C-reactive protein (CRP), a marker of inflammation (Sahin *et al.,* 2012) supporting the hypothesis that platelet activation is elevated in overweight individuals. While all platelet volume indices were inversely correlated to BMI, none of these associations were significant. Another large cross-sectional study found no association between BMI and MPV in healthy subjects (Sansanayudh *et al.,* 2016).

Samuel-Bonet *et al.,* (2008) suggest that the relationship between BMI and PLT in females can be explained by simultaneously high CRP levels, indicating inflammation (Samuel-Bonet *et al.,* 2008) and alterations in platelet production from megakaryocytes can be modified by cytokines involved in inflammation. The latent chronic inflammation could have a certain bearing for females who generally have a higher body fat percent than men with the same BMI. Additionally, higher thrombopoeitin (TPO) (the major governor of platelet production) levels have been observed in inflammation and this could partly explain the elevated platelet production and count in overweight subjects (Cerisa *et al.,* 2007).

Comparisons of platelet indices and function was facilitated by stratifying subjects into two BMI groups, outlined in Table 3.9 (healthy weight and overweight). Figure 3.3 compares platelet parameters according to BMI category. PLT was elevated in overweight males and females compared to healthy weight and this trend was more pronounced and statistically significant in females. Similar results have been reported amongst women elsewhere (Farhangi *et al.,* 2013). PCT was significantly higher in overweight females and remained unchanged in overweight males.

Platelet adhesion is intricately linked to activation in obesity however, it is unclear whether platelet activation is enhanced in overweight subjects. In a cross-sectional study by Leit *et al.,* (2016), obese subjects demonstrated reduced platelet nitric oxide synthase (NOS) activity and increased platelet adhesion compared to non-obese subjects. Other large population based studies demonstrate that obese subjects present enhanced markers of platelet activation including P-selectin plasma concentrations (Pergola *et al.,* 2008), and urinary 11-dehyrdo-TxB2 (Davi *et al.,* 2002). Likewise, we found a slight significant positive relationship between platelet adhesion (SC%) and BMI (Table 3.9). Figure 3.3 shows that there was also a significant difference between SC% between the healthy and overweight BMI group in males (10.7-12.8%) and females (11.2 – 13.4%), showing increased platelet adhesion in the overweight groups suggesting that platelet activation is heightened in overweight subjects.

In obesity, platelets develop a resistance to their main anti-aggregating mediators, PGI_2 and NO. This is likely caused by the impairment of cyclic nucleotide production and subsequent action (Anfossi *et al.,* 2004). Cyclic nucleotides are the primary anti-aggregatory mediators. Therefore, a resistance to them could be a factor in platelet hyperactivity in obesity, making platelets more prone to adhesion. The increase in platelet adhesion in the overweight group could be as a result of a chronic resistance to these mediators, as a result of lifestyle as Leite

et al., (2016) suggested that long-term conservation of excessive body weight is detrimental to platelet function.

The lack of correlation between BMI and platelet aggregation (AS) indicates that although subjects with an increasing BMI appear to possess a more adhesive platelet profile, these changes are not being translated at the aggregatory level. Although these subjects were overweight and beginning to show signs of subclinical platelet activation, they have not yet reached a disease state and therefore the risk of CVD and thrombosis is substantially lower. Accordingly, there were minimal differences in aggregation between both groups (Figure 3.3).

The limited number of studies investigating the association between BMI and platelet aggregation have focused on the link between platelet aggregation and BMI in diseased populations such as metS, T2DM, or in obese subjects. Angiollio *et al.,* (2004) found that platelet aggregation was elevated in patients with an overweight BMI compared to normal weight patients. ADP-induced platelet aggregation was significantly elevated in overweight patients than in normal-weight patients undergoing percutaneous coronary intervention (Bonello-Palot *et al.,* 2009). Corsonello *et al.,* (2003) examined platelet function in normal weight, overweight and obese males in a small (n=49) cross-sectional study. They assessed ADP-induced platelet aggregation and platelet calcium release in PRP with varying concentrations of leptin which can encourage platelet aggregation through activation of its receptor on the platelet surface. ADP-induced platelet aggregation was increased in overweight and obese subjects compared to controls. However, our study had low numbers of obese subjects, and perhaps a larger number would have shown similar results.

3.3.3.2 Waist circumference and visceral obesity

Although obesity predisposes to CVD, the pattern of body fat distribution is also recognised as an extremely important predictor of the health risks of obesity. Visceral obesity is the most dangerous type of fat and is specifically linked to cardiovascular morbidity (Davi *et al.,* 2002). Visceral obesity relates to an excess of intra-abdominal adipose tissue and is distinguished from gynoid obesity by more fat on the trunk, carrying increased risk of hypertension and CVD compared with gynoid obesity. The pathophysiology of visceral obesity comprises distinct intertwined elements including an accrual of ectopic fat, insulin resistance and an increased ability to release inflammatory cytokines and free fatty acids than other types of fat deposition, all of which contribute to CVD. Therefore, while BMI is

an extremely useful measure of obesity, not every overweight or obese subject (BMI >30) carries visceral fat, and, non-obese individuals can accumulate disregarded visceral fat. We measured visceral obesity by waist circumference (WC), which can be used alone as an indicator of health risk as it directly measures abdominal obesity (Janssen *et al.,* 2004).

Table 3.11 illustrates that visceral obesity appeared to manifest itself more in females as 24% of the population compared to 16% in males had a WC which was in a high risk category. Partial correlation analysis shown in Table 3.10 indicate a positive relationship between WC and PLT in females and no evident relationship in males. This has been found in other cross-sectional studies of platelet indices where platelet number increased with a rising WC (Vuong *et al.,* 2014). PCT was also positively correlated to WC in females. As PCT has been employed as a biomarker of platelet activation, one could hypothesise that WC is accompanied by elevated platelet activation in overweight females. In fact, this is supported by the observation that platelet adhesion was positively correlated to WC in females, indicating slight platelet hyperactivity when visceral obesity is present (Figure 3.4). This increased platelet adhesiveness is probably due to the same multitude of mechanisms involved in central obesity such as LDL oxidation (Colas, 2011), insulin resistance (Ferreira *et al.*, 2006), increased TxA_2 production, (Davi *et al.,* 2002) and the resistance to anti-aggregating effects of NO, PGI_2 and their mediators (Anfosso,2004; Russo, 2010).

Increased MPV as a consequence of inflammation has also been noted in visceral obesity (Ozhan *et al.,* 2010; Sansanayudh *et al.*, 2016), however, there was no evident association between WC and platelet volume indices MPV, PDW or PLCR in this population, probably due to the fact that subjects were healthy/overweight and not obese. Additionally, there were positive correlations between RBC and WC and between WBC and WC in females only, suggesting an overall increase in haematological parameters in females.

To examine the variation between platelet indices according to WC, subjects were divided into four groups shown in Table 3.11, according to their overall disease risk for T2DM, hypertension and CVD, relative to normal WC and gender (Thompson *et al.,* 2009). This was subsequently reduced to two groups (low and high risk) to facilitate comparisons. Figure 3.5 compares platelet parameters between the risk groups. There were minimal differences in platelet volume indices between low and high risk subjects, reflecting the lack of correlations between the parameters. In females, PLT and PCT were significantly higher in the high risk group compared to the low risk group.

Furthermore, SC% in females was significantly elevated in the high risk group (11.3% compared to 13%) suggesting a more reactive platelet profile. Similarly, to variations in platelet aggregation when stratified by BMI, there were no large changes in AS (μm^2) in either gender.

Mechanisms for increased platelet activation in visceral obesity are under investigation. A number of metabolic alterations such as enhanced free fatty acids, oxidised LDL, TNF-α, are evident in patients with visceral obesity which contribute to reactive oxygen species (ROS) production. High ROS subsequently affects platelet function by reduction of NO bioavailability, overexpression of integrin αIIbβ3 and production of isoprostanes, the latter activating platelets through interaction with the TxA_2 receptors (Anfossi *et al.,* 2009). Visceral fat plays a key role in the development of metS, a condition epitomising a group of cardiometabolic risk factors including central obesity, insulin resistance and hypertension. metS is characterised by increased adipose tissue, which secretes a number of pro-inflammatory cytokines (e.g. Leptin and interleukin-6 (IL-6)) which lead to chronic low-grade inflammation and can influence platelet count and function. Leptin is a saiety hormone mainly produced from adipose tissue which increases platelet Ca^{2+} levels and simultaneously reduces the effect of platelet inhibitors such as cAMP (Elbatarney and Maurice, 2005; Konstantinides *et al.,* 2001; Santilli *et al.,* 2012).

Platelet count in females with metS are significantly higher than those without metS, whereas males with metS only show a slight increase (Park *et al.,* 2012) agreeing with results from this study. Studies using the Impact-R system have demonstrated that those with metS have enhanced baseline platelet adhesion compared to their healthy counterparts (Vaduganathan *et al.,* 2008). Although subjects in this study did not have metS, it appears that the females with increased WC have a tendency to show similar platelet profiles to those with visceral obesity and metS.

It is also possible that the systemic effects of visceral obesity are influencing the properties of platelets before/during their synthesis from megakaryocytes. Alterations in megakaryocyte ploidy numbers has been described in TD2M as a result of high IL-7 levels present in visceral obesity (Brown *et al.,* 1997).

3.3.3.3 Body fat percentage and fat mass

Although correlated with body fat percent, BMI or measures of visceral adiposity cannot distinguish between body fat and lean mass or muscle. More specific measurements of body composition, including body fat % (weight of fat/total body weight) and fat mass (FM) (actual weight of fat in the body) were measured by BIA. Some body fat is essential for physiological health, with a range of 10-22% and 20-30% for men and women considered acceptable for health. Contrarily, too much fat is detrimental for cardiovascular long-term health and reducing excess levels of body fat has been shown to directly reduce the risk of various conditions such as hypertension, T2DM, and CVD.

Table 3.12 shows the relationship between platelet indices and BIA measurements. PLT and PCT were positively associated with FM in both genders. However, only these relationships in females were statistically significant, consistent with the other adiposity measures in females. Interestingly, there were small significant positive correlations between BF% and PLT and between BF% and PCT in both genders. Scatterplots depicting these relationships are shown in Figure 3.6.

Similarly, to BMI, the platelet volume measurements MPV, PDW and PLCR were inversely related to BF% and FM in males, however these associations were not significant. A solitary study has solely examined the relationship between PLT and body fat % using 15 healthy males and finger prick for a blood draw and saw no relationship with PLT and body fat %, possibly due to very low sample size and dated techniques. (Marley and Linnerd, 1978). Other studies have illustrated changes in MPV with increasing body fat %. Muscari *et al.*, (2008) examined the relevance of body fat on MPV in an elderly population and found that body fat percent was independently associated with high MPV. However, this was measured by skinfolds.

Table 3.12 illustrates the significant positive relation (weak) between FM and platelet adhesion in both genders. This was further highlighted by the disparity in platelet adhesion between FM groups in Figure 3.8. There was no correlation between FM and platelet aggregation in either gender and no study has reported on a specific correlation between fat mass and platelet function, even though other adiposity measures (BMI, WC) already discussed, are linked to enhanced platelet activation.

Table 3.12 shows positive significant associations between platelet adhesion and BF in males and females. In adults, there have been no studies to date investigating platelet adhesion and body fat %, however a recent study in an adolescent population demonstrated that at an early age, platelet activation is associated with increasing body fat %. Garcia *et al.,* (2014) demonstrated that the platelet activation marker P-Selectin was positively correlated with BIA measured body fat % in obese children. Again there were no tangible correlation between aggregation and BF in either gender.

To compare platelet parameters according to body fat %, subjects were categorised into groups based on body fat % norms for their age and gender according to the American College of Sports Medicine normative values (Thompson *et al.,* 2009). The number of subjects in each category is displayed in Table 3.13. There was a significant difference in platelet adhesion (SC %) between groups in females, as shown in Figure 3.7. PLT and PCT appeared to be the most reliable platelet indices measured when groups were stratified by body fat %, as MPV, PDW and PLCR showed no major differences between groups. The data presented in Figure 3.7 show a stepwise increase in PLT between the three BF % groups across genders, which was more pronounced in females. In males, PCT was increased in the group displaying highest BF levels compared to the other two groups. There was a significant difference in PCT between the good and fair groups in females.

We expected that platelet aggregation would be enhanced in those with higher body fat percentages, however we saw no major differences between groups. This inconsistency could be explained by the limited ratio of subjects in the high fat groups. Platelet adhesion was significantly increased in the fair body fat group compared to the good body fat group in females, similar to results obtained for BMI. The higher adhesion and PLT values in those with higher body fat percentage point toward a more reactive platelet profile in overweight.

Although an association between obesity and platelet activation is evident, the cellular and molecular mechanisms responsible have only begun to surface (Blokhin and Lentz, 2013). RNA from platelets are reflective of pathological disease states and inflammatory transcript profiles from platelets, including INFG, IL1R1, IL6, and TLR2, all of which were reported to be significantly correlated with increasing BMI (Freedman *et al.,* 2010), supporting the hypothesis that surplus fat could unfavourably alter the inflammatory potential of platelets. However, obesity can also cause dysregulation of other factors which control haemostasis such as microRNA (miRNA). miRNA are involved in the pathogenesis of obesity (Zampetaki *et al.,* 2010) where plasma levels of miR-223 are reduced in obese compared to

lean subjects, proposing that the miR-223/$P2Y_{12}$ alliance could signify a contributing mechanism of platelet activation in obesity (Bray *et al.,* 2013).

Each of the adiposity measurements (BMI, WC and body fat %) showed increased adhesion levels in subjects displaying unhealthy physiological measurements, whereas in general, aggregation levels remained unchanged. Platelet adhesion and aggregation are frequently considered as distinctive processes by which platelets create contact with extracellular exteriors or stick to one another. However, they are intrinsically linked as both mechanisms involve the alteration of platelets from free flow to attach to a surface, sometimes facilitated by the same adhesive ligand and receptors (Ruggieri *et al.,* 2007). In this study, it appears that adiposity may regulate the adhesive receptors such as α2β, GPVI and GPIb-IX-V, but is not at this point affecting the aggregatory potential of platelets and activation of their main adhesion receptor, αIIbβ3, which is present at extremely high density on the platelet surface. A comparison of gene and protein expression of adhesive receptors in overweight and healthy subjects would provide further detail on the platelet function changes occurring in these cohorts.

3.3.4 Physical activity and fitness

The primary lifestyle factor investigated in this chapter was various measures of overweight, which is associated with physical inactivity and sedentary behaviour. Epidemiologic studies have illustrated an undisputable relationship of increased fitness/exercise/physical activity with reduced cardiovascular risk (Knoops *et al.,* 2004). Self-assessed physical fitness questionnaires enabled the comparison of platelet indices across subjects of varying fitness levels (Unfit, fairly fit and fit). Approximately 50% of the study population reported themselves as fairly fit or fit (Table 3.5), which was interesting considering sedentary behaviour patterns in Ireland have increased dramatically in the last 10 years (Department of Health, 2013). Since platelets are involved in the pathogenesis of atherosclerosis, the protective effect of exercise against cardiovascular diseases may be partially due to alterations of platelet function.

Figure 3.9 compares platelet indices and platelet function between fitness groups adjusting for age and gender. Although not significant, there appeared to be a linear decrease in PLT from unfit to fit in females. The same trend appeared in the male cohort and this may be indicative of varying platelet phenotypes relating to habitual exercise. MPV remained relatively unchanged between groups but PDW was elevated in the unfit group compared to

both the fairly fit and fit groups across both genders. As PLCR and PCT have only emerged as important markers in platelet health, there is limited data concerning their variance in an exercise capacity. There was a linear decrease in PLCR in males from unfit to fit. PCT showed the largest changes out of the platelet indices parameters. These patterns are interesting as although qualitative data does not compare to quantitative measures of aerobic fitness (such as a VO_2 test), the trend suggests an inverse relation between aerobic fitness and platelet indices.

Karakilcik *et al.,* (2014) showed that PLT, MPV and PCT were decreased after exercise training in soccer players compared to those who did not partake in exercise training, suggesting a less thrombotic profile in trained individuals. Furthermore, a comparison of resting platelet indice levels on athletes (aged 22 years) who participated in regular sports for 5+ years with those of sedentary subjects, showed that PLT and PCT were significantly decreased in the athletes (Rba *et al.,* 2015).

We could identify no clear trend with platelet adhesion. Wang *et al.,* (1995) demonstrated that long-term exercise training in sedentary males had the potential to suppress *in vitro* platelet adhesion. Furthermore, Singh *et al.,* (2006) showed that baseline platelet activation is enhanced in trained males compared to sedentary males. Figure 3.9 shows there were no changes in platelet aggregation between the three groups in males. Others report that exercise training does not affect resting platelet aggregability (Davis *et al.,* 1990). In females, there was a linear decline in aggregate size from the unfit to the very fit group. Consequently, 12 weeks of exercise training decreased resting platelet aggregation in sedentary females (Wang and Chen 1985). Possible mechanisms for beneficial effects of exercise training on platelets include a reduction in resting plasma catecholamine levels and platelet α_2- adrenergic levels, diminishing platelet P-Selectin expression. eNOS and NO are increased after exercise training, thereby increasing platelet cGMP levels and potentially reducing platelet activation under shear stress (Wang *et al.,* 2005).

Overall, regular exercise participation appears to have a favourable effect on platelet function, with reports that exercise training is associated with encouraging effects on platelet aggregation and activation in both men and women. Long-term intervention studies in this area are required to elucidate the effects of habitual exercise on platelet function.

This chapter primarily focused on the relationship between platelet function and modifiable CVD risk factors (overweight and obesity, and physical inactivity) and physical activity. We collected additional data on other modifiable CVD risk factors including smoking and hypertension with regard to both platelet and haematological parameters. We also examined the role of non-modifiable CVD risk factors (ethnicity and age). This data can be found in appendix A.

3.4 Limitations

This study represents a snapshot of the population and a larger sample size may improve the strength of associations and subsequently highlight better the effects of lifestyle/CVD risk factors on platelet function in healthy people. The sample size was small in certain subgroups and the ratio of female to male subjects was slightly uneven and could have been a limiting factor for some results. The cross-sectional design of the study suggests that caution should be used in causal interpretations. Subjects were informed to arrive for testing in the morning, having abstained from vigorous exercise/alcohol/a high fat meal in the previous 24 hours to reduce the effect of these confounders as each can affect platelet function (Guerrero *et al.,* 2004, Pearson *et al.,* 2005; Bonacciao *et al.,* 2014; Sinzinger and Berent, 2012). However, it cannot be said for certain that this was the case and this could have impacted the results.

While no solitary measure of body composition is globally accepted as the gold standard (Ackland *et al.,* 2012), dual-energy X-ray absorptiometry (DEXA) is considered the most precise technique but is costly and time consuming. BIA is often used in population-based studies due to its promptness of results and non-invasive approach. However, BIA may compromise accuracy for efficiency of use and although studies have shown it is comparative to other measurements, it is dependent on factors including food and fluid intake, temperature, menstruation and exercise, all of which could have influenced results (Heyward, 1991; Deghan, 2008; Mialich *et al.*, 2014). Physical activity/fitness levels were self-assessed by individuals using a questionnaire. Ideally, aerobic fitness levels should be quantitatively assessed and therefore results must be carefully interpreted given the lack of distinction between self-reported and objective physical activity. However, this study aimed to acquire a broad view of the subjects' lifestyle.

3.5 Summary and conclusion

This chapter examined a range of CVD risk factors in a nationally representative population to examine the relationships between platelet function and physiological health. Results showed that higher platelet counts and plateletcrit were associated with less favourable cardiovascular risk profiles, in particular with measures of overweight Other platelet indices (PDW, MPV and PLCR) were not associated with overweight. Sahbaz *et al.*, (2016) also found that plateletcrit had higher sensitivity and specificity than other platelet indices when predicting disease states.

Importantly, platelet adhesion was significantly increased in male and female adults who had a BMI classed as overweight compared to those with a healthy weight BMI. BMI and body fat percent were the most sensitive adiposity measures in reflecting the changes in platelet parameters, whereas waist circumference was specific for alterations in females only. Although BIA measurements such as fat mass and body fat % showed similar results to WC and BMI, the VAT scale was not a reliable indicator of body composition. The elevated PLT, PCT and SC levels in overweight subjects indicate a more reactive platelet profile and could imply the occurrence of subclinical CVD symptoms.

It is important to note that while some changes were significant, often there was not a huge difference between subgroups. A more effective approach to detect changes in platelet function in overweight subjects could be to stress the cardiovascular system by exercise, rather than taking single one-point measurements. Furthermore, as the association of the variables was mainly directed toward a risk profile for CVD, such a relationship may be stronger in pathological conditions such as obesity, TD2M and hypertension. Nonetheless, it is possible that changes in platelet parameters in overweight subjects are associated with disease risk and these patterns may offer new insights into platelet function.

The search for simple biomarkers that allow for early identification of subclinical/CVD risk is ongoing. Work from this chapter demonstrates that platelets can reflect changes in unhealthy lifestyle patterns. The Impact-R test is a relatively inexpensive test which can reliably detect changes in platelet adhesion and could be employed for CVD risk evaluation amongst subjects who are asymptomatic. Platelet indice and function markers should be further tested in larger cross-sectional studies and during lifestyle interventions, to determine their reliability as surrogate markers for evaluating cardiovascular health.

Chapter Four

Outputs:

- Varying levels of cardiorespiratory fitness (CRF) resulted in no major basal differences in platelet function in male adults or adolescents
- Male adults with a moderate CRF had higher basal platelet counts and plateletcrit than those with a high CRF
- Acute aerobic exercise resulted in increased platelet adhesion and aggregation
- This increase was more pronounced in those with a low CRF, particularly in adolescents

Contributions from others:

- The adolescent cohort from this study was part of a large study investigating cardiorespiratory fitness, physical activity and vascular health in Irish adolescent males, conducted in DCU by Prof Niall Moyna

Chapter Four: The Effect of Acute, Strenuous Exercise on Platelet Function

4.1 Introduction

Physical inactivity and obesity are prominent behavioural risk factors, which lead to cardiovascular disease (CVD). It has been recognised that regular exercise may reduce the risk of major vascular thrombotic events and protect against cardiovascular disease (Blair and Norris, 2009). Differences in known factors explain a large percentage of the inverse relationship between physical activity and CVD risk (Mora *et al.*, 2007; Kwasniewska *et al.*, 2016). However, over 40% of the inverse association remains unexplained. Although the beneficial effects of regular exercise on blood lipids and blood pressure have been well accepted, research focusing on platelet function has only recently gained greater attention.

Cardiorespiratory fitness (CRF), also known as aerobic fitness/capacity, is defined as the ability to deliver oxygen to the muscles and to use it to produce energy during exercise. It consequently relies upon pulmonary, cardiovascular and haematological constituents of oxygen delivery and the oxidative mechanisms of the exercising muscles (Armstrong *et al.*, 2006). Low CRF represents a major risk factor for atherosclerosis. Since platelets play a key role in the pathogenesis of atherosclerosis, the protective effect of exercise against CVD may be partially due to alterations in platelet properties.

Chapter 3 examined a cross-sectional cohort of the population to determine the relationship between platelet function and physiological health. Rather than taking basal single point measurements, stressing the cardiovascular system using *in vivo* instead of *in vitro* or *ex vivo* stress models, by prescribed exhaustive exercise may provide much more valuable and conclusive evidence in relation to the body's response to stress. This chapter extended our research using a 'human laboratory' approach, evaluating the effect of controlled aerobic exercise, as a cardiovascular stressor, on platelet function in male adolescents (n=30) and adults (n=16) of varying CRF. Inconclusive information is available on the effect of acute exercise on platelet function in adults and even less have investigated platelet function in healthy adolescents and with respect to acute exercise. (Heber and Volf, 2015). ***Therefore, the purpose of this study was to assess the relationship between CRF and platelet function and to examine the effect of varying CRF levels on the platelet response to acute aerobic exercise.***

4.1.1 Chapter aims and experimental approach

Hypothesis:

Basal platelet function measurements will differ between low and high fit adolescent and adult males. Acute exercise will induce a change in platelet function in both groups, with a more pronounced change in subjects with a lower cardiorespiratory fitness level.

Main aims:

- Investigate differences in basal platelet function in males of varying age and CRF levels.
- Examine the effect of acute strenuous exercise on platelet indice markers (Platelet count, plateletcrit and platelet volume markers of platelet activation) and platelet function (adhesion and aggregation) in high fit, and moderately – low fit adolescent and adult male subjects.
- Elucidate the relationship between CRF and platelet function in both healthy adolescent and adult males.

4.1.2 Study design

Subject recruitment

The subject selection for adults and adolescents was explained in detail in Chapter two (Section 2.2.5.5). In brief, the thirty adolescent subjects in this study were selected upon completion of a 20 m MSST (multi-stage shuttle test) to determine fitness levels and then provisionally categorised as high fit (HF) or moderate – low fit (MLF). Permanent fitness categorisation into separate groups was based on VO_2 max (ml/kg/min) values using the percentile data from a study undertaken on youths aged 12-19 years, illustrated in Chapter 2. This was part of a larger study investigating CRF, physical activity, sedentary behaviour and vascular health in male adolescents.

The adult cohort (n=16) was recruited from the School of Health and Human Performance and provisionally defined as moderately fit (MF) or high fit (HF) based on responses from a physical activity questionnaire. This determined their VO_2 max testing protocol and based on their VO_2 results they were categorised into two definite groups according to normative VO_2 data for healthy males for their age (Adapted from Astrand *et al.*, 1960). The MF group had a VO_2 between 44-55 ml/kg/min, and the HF group between 58-66 (ml/kg/min). All subjects (and parents for the adolescents) gave informed consent. Ethical approval for both cohorts was provided by DCU ethics.

Blood sample

Two bloods sample were taken from each subject in a resting position, one pre exercise and one immediately post exercise. Blood was obtained by standard venepuncture from the antecubital forearm vein and collected into vacutainer tubes containing 3.2% sodium citrate. Platelet, Erythrocyte and Leukocyte indices were measured by the Beckman Coulter Ac·T diff™ Analyser.

Platelet function

Two parameters of platelet function were evaluated using the Impact R: surface coverage (SC, %) representing platelet adhesion, and the average size (AS, μm^2) of the polystyrene-bound platelet clusters/aggregates representing platelet aggregation.

Exercise test

Cardiorespiratory fitness (VO_2 max) was assessed using an incremental exercise test on a treadmill with continuous breathing gas analysis and HR monitoring using a modified Bruce protocol (Bruce *et al.*, 1973). Two minutes of warm up were performed before subjects performed the selected test described in Chapter 2. Blood pressure and rate of perceived exertion were continuously monitored during the test. Subjects were verbally encouraged throughout the test until the finish. End criteria used for VO_2 max attainment were VO_2 levelling off, $RER_{max} \geq 1.0$, >18 on the Borg $Scale_{6–20}$ rating, and $HR_{max} \geq 95\%$ of the age-predicted HR_{max} (220 – age) and subject requesting to stop.

Body composition

Height and body weight were measured to the nearest 0.5 cm and 0.1 kg, respectively, with subject wearing no shoes and light clothes. BMI was calculated as previously described in Chapter 3. Waist circumference was measured in both adults and adolescents as an indicator of visceral obesity. Body fat % was measured by BIA analysis in the adult cohort and by skinfold analysis in the adolescent cohort.

Assessment of covariates

Participants abstained from any exercise for >24 h prior to testing and (Adults) were tested after an overnight dietary fast. The adolescent cohort was allowed to have a simple cereal based breakfast on the morning of the test. On the morning of testing, participants were permitted and encouraged to drink water. All participants were required to abstain from non-steroidal anti-inflammatory (NSAID) use for at least 7 days prior to testing. The adolescent cohort signed consent and assent forms and completed the PAR Q & U questionnaire, while the adult cohort completed an adapted physical activity questionnaire (Appendix B).

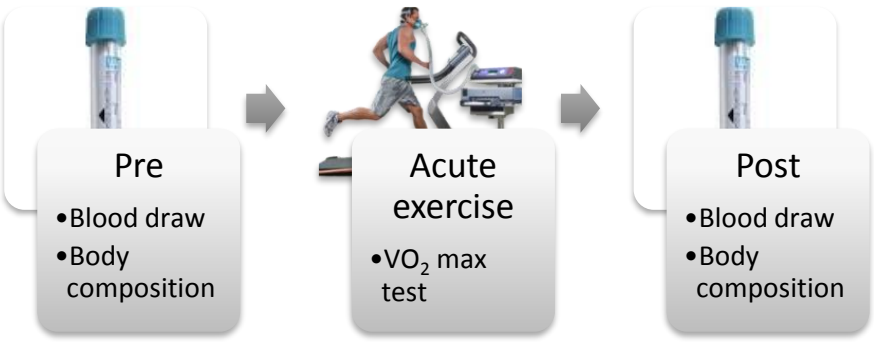


Figure 4.1: Workflow for testing both adults and adolescents. Subjects had basal measurements and blood samples taken and then performed acute exercise. Post exercise, the same measurements were performed.

4.2 Results

4.2.1 Characteristics of the study population

Characteristics of the adolescent subjects are displayed in Table 4.1, categorised into two groups according to their VO_2 max moderate-low fit (MLF) (16) and high fit (HF) (14). There were significant differences between the MLF and HF group for all characteristics except for height. Physical fitness characteristics of the adolescents are summarised in Table 4.2. HF subjects had significantly higher VO_2 max and MSST shuttle parameters compared to MLF subjects. Characteristics of the adult cohort are displayed in Table 4.3. VO_2 max was significantly higher in the HF group compared to the MF group. Body composition characteristics were similar between groups, with the largest difference in body fat % between the two. Figure 4.1 shows the difference in CRF between the two groups in both cohorts. Table 4.4 shows the categorisation of subjects according to BMI and Figure 4.2 highlights the association between BMI and VO_2 max in both cohorts. There was a significant inverse relationship between BMI and VO_2 max in the adolescent cohort, but not in the adult cohort.

Table 4.1: Anthropometric characteristics and RHR characteristics of the adolescent cohort. Values are mean ± SD. BMI - Body mass index, Waist – Waist circumference, SBP – Systolic blood pressure, DBP – Diastolic blood pressure. *P<0.05. Independent samples *t*- test.

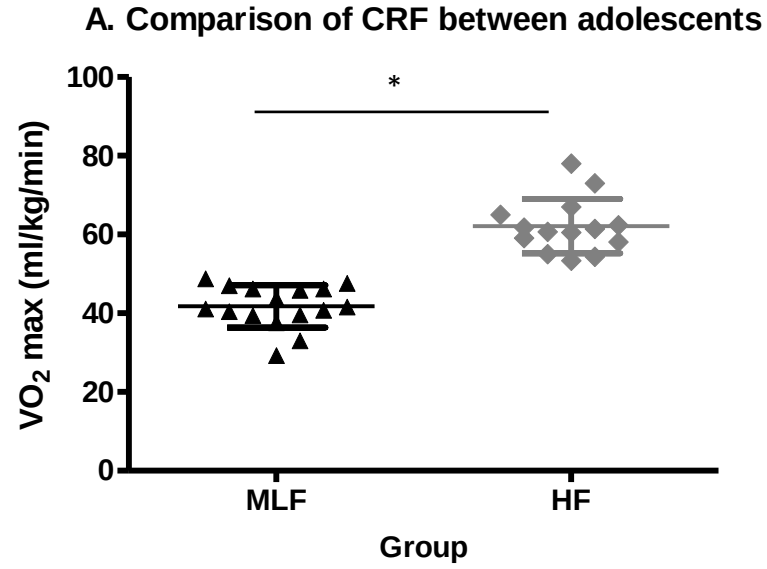
	Adolescent cohort		
	MLF (n=16)	**HF (n=14)**	**P value**
Age (yrs)	15.5 ± 0.63	16.07 ± 0.73	*
Height (cm)	177.36 ± 5.42	177.46 ± 6.41	.965
Weight (kg)	85.4 ± 14.51	68.4 ± 8.08	*
BMI	27.4 ± 5.58	21.7 ± 2.32	*
Waist (cm)	84.9 ± 9.28	73.01 ± 5.35	*
Body fat (%)	21.9 ± 8.19	10.6 ± 6.29	*
SBP (bpm)	130 ± 8.98	115 ± 6.52	*
DBP (bpm)	81 ± 4.51	73 ± 4.21	*

Table 4.2: Multi-Stage shuttle test and VO_2 characteristics of the adolescent cohort. Max HR - Maximal heart rate attained during exercise test, Max RER - Maximal respiratory exchange ratio attained during exercise test. VO_2 max – Maximal aerobic capacity. Values are mean ± SD. *P<0.05. Independent samples *t*-test.

Adolescent cohort			
Performance parameter	**MLF**	**HF**	**P value**
Shuttle level	5.8 ± 1.15	10.2 ± 2.34	*
Shuttle number	40.1 ± 10.42	87.4 ± 20.85	*
Shuttle distance	802 ± 208.12	1748 ± 337.13	*
Max HR	206.1 ± 14.01	200.1 ± 4.70	.250
Max RER	1.08 ± 0.05	1.12 ± 0.04	.071
VO_2 max	41.7 ± 5.01	62.2 ± 5.9	*

Table 4.3: Anthropometric characteristics and RHR and VO_2 characteristics of the adult cohort. BMI - Body mass index, WC - Waist circumference, SBP - systolic blood pressure, DBP - Diastolic blood pressure, VO_2 max - Maximal aerobic capacity. RHR - Resting heart rate. Values are mean ± SD. *P<0.05. Independent samples *t*-test.

Adult cohort			
	MF (n=8)	**HF (n=8)**	**P value**
Age (yrs)	21.75 ± 2.06	21.38 ± 1.61	.653
Height (cm)	181.08 ± 7.25	181.5 ± 7.53	.986
Weight (kg)	80.12 ± 2.3	77.12 ± 3.6	.598
BMI (kg/m^2)	24.3 ± 2.61	23.4 ± 2.08	.421
WC (cm)	78.1 ± 1.9	79.2 ± 3.1	.809
Body fat (%)	15.7 ± 1.5	12.1 ± 1.1	.073
SBP (mmHg)	121 ± 3.5	122 ± 2.2	.811
DBP (mmHg)	77 ± 2.6	81 ± 3.5	.258
VO_2max(ml/kg/min)	50.1 ± 3.91	60.2 ± 3.19	*
RHR (bpm)	77 ± 6.5	66 ± 4.9	.230



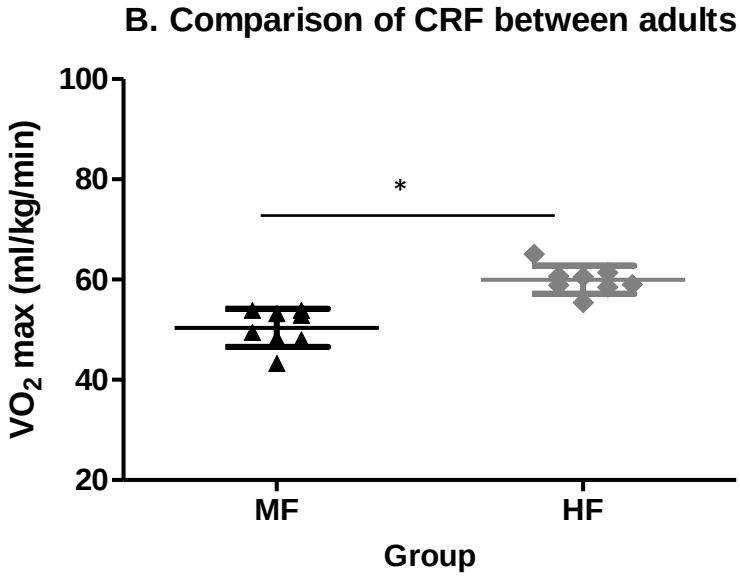


Figure 4.2: Comparison of the differences in CRF between the adolescent and adult cohorts. A: Comparison of CRF levels between the HF and MLF adolescents, B: Comparison of CRF levels between the HF and MF adults. Each subject is represented on the graph with mean ± SD shown by the lines. *P<0.05. Independent samples *t*-test.

Table 4.4: BMI classification of adult and adolescent cohorts. A BMI between 18-25 kg/m^2 is considered a healthy weight, a BMI > 25 kg/m^2 and < 30 kg/m^2 is considered overweight, and a BMI > 30 kg/m^2 is categorised as obese**.** Values are numbers and percentages of subjects in each category.

	BMI category	
	Healthy	**Overweight/Obese**
Adolescents	19 (64%)	11 (36%)
Adults	12 (75%)	4 (25%)

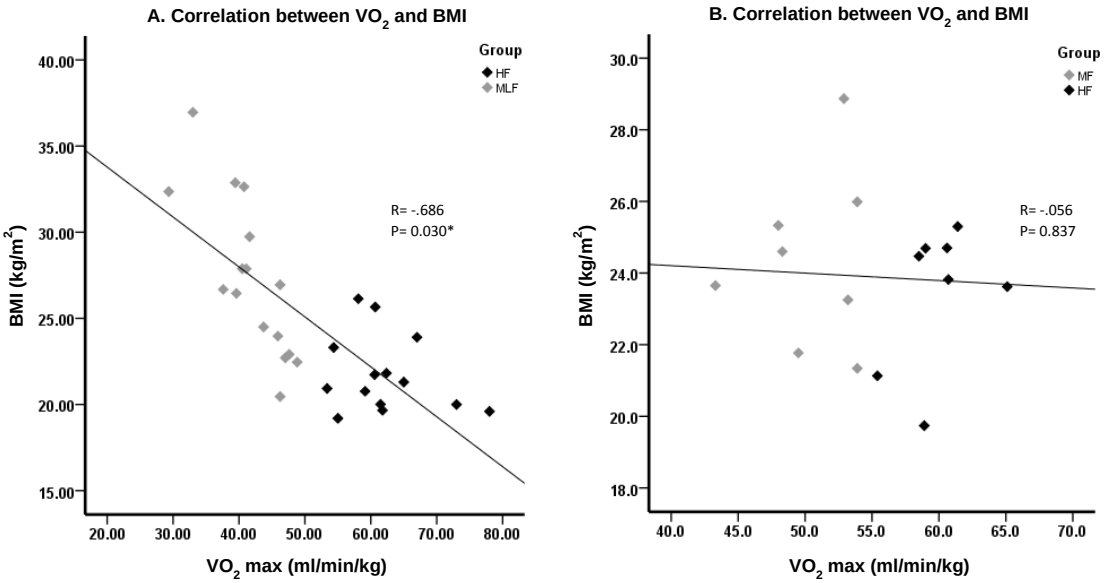


Figure 4.3: Relationship between BMI and VO_2 max. A - shows the correlation between BMI and VO_2 in the adolescent cohort and MLF and HF subjects are displayed. B shows the lack of relationship between BMI and VO_2 in the adult cohort and HF and MF subjects are shown by the black and grey colours respectively. *P<0.05. Partial correlations adjusting for age.

4.2.2 Basal platelet and haematological characteristics of the study population

To compare the basal platelet and RBC/WBC characteristics between adults and adolescents of varying CRF levels, the same groups were used. Tables 4.5 and 4.6 below compare white blood cell and red blood cell counts in addition to the difference in morphological features. As seen in Table 4.6, WBC was higher in the MF group compared to the HF group in adults, almost reaching statistical significance. There were no major differences between any of the other haematological parameters at basal time point. Figures 4.4 – 4.6 compare basal platelet indices and functions in the adult and adolescent populations.

Table 4.5: Basal RBC and WBC characteristics of the adolescent cohort. Values are mean ± SD. RBC – Red blood cell count, WBC – White blood cell count, HCT – Haematocrit, HGB – Haemoglobin, MCV – Mean corpuscular volume, RDW – Red cell distribution width. Independent samples *t*-test.

	Adolescent cohort		
	MLF	**HF**	**P value**
WBC (10^3/µl)	5.64 ± 1.54	6.15 ± 1.69	.404
RBC (10^6/µl)	4.98 ± 0.31	4.86 ± 0.65	.705
HCT (%)	44.37 ± 2.33	42.64 ± 3.64	.991
HGB (g/dl)	14.84 ± 0.81	14.19 ± 1.27	.160
MCV (fl)	88.86 ± 2.32	87.84 ± 1.91	.465
RDW (%)	13.02 ± .90	13.35 ± 0.98	.230

Table 4.6: Basal RBC and WBC characteristics of the adult cohort. Values are mean ± SD. RBC – Red blood cell count, WBC – White blood cell count, HCT – Haematocrit, HGB – Haemoglobin, MCV – Mean corpuscular volume, RDW – Red cell distribution width. *P<0.05. Independent samples *t*-test.

	Adult cohort		
	MF	**HF**	**P value**
WBC (10^3/µl)	7.95 ± 2.03	5.98 ± 0.98	.051
RBC (10^6/µl)	4.72 ± 0.25	4.61 ± 0.28	.439
HCT (%)	43.35 ± 1.87	42.7 ± 0.1.97	.702
HGB (g/dl)	14.1 ± 0.82	14.2 ± 0.79	.782
MCV (fl)	89.40 ± 1.12	92.70 ± 0.97	*
RDW (%)	12.81 ± 0.99	12.73 ± 0.87	.826

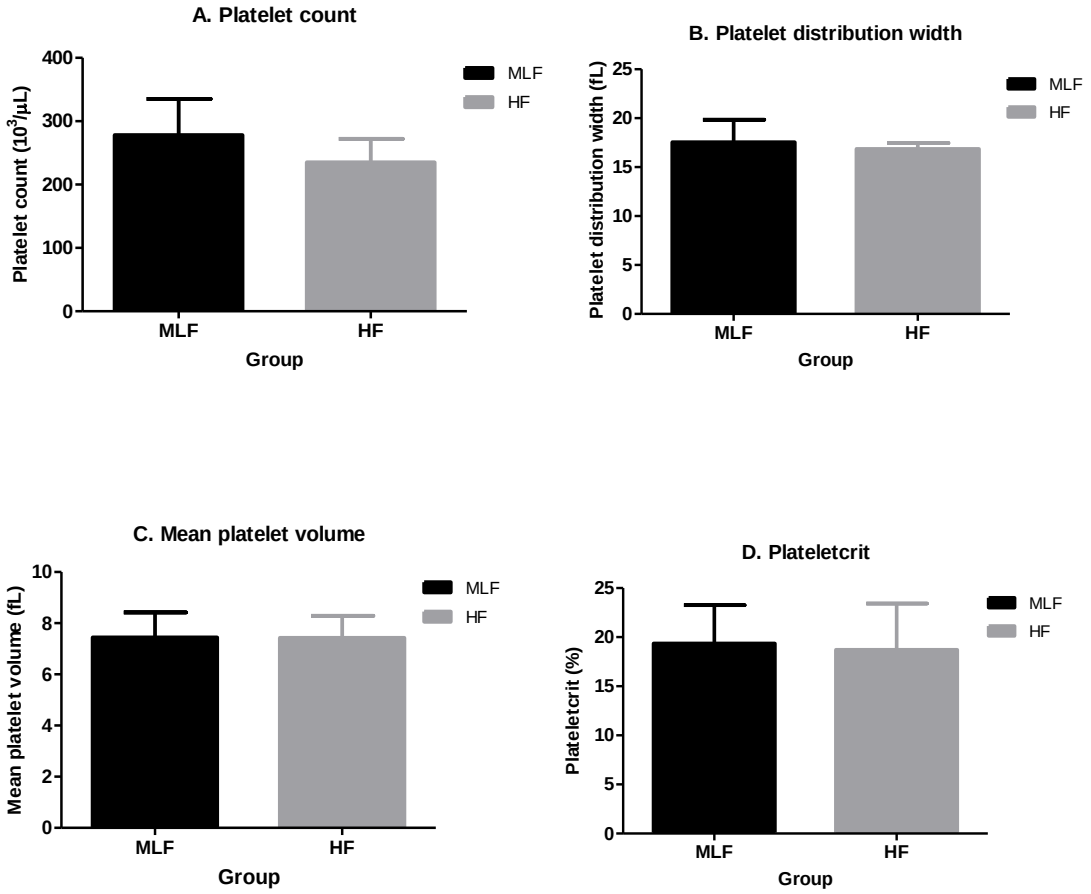


Figure 4.4: Comparison of adolescent platelet indices at baseline. Graphs represent the mean ± SD value of each group. A – Basal platelet count comparisons, B – basal platelet distribution width comparisons, C – Basal mean platelet volume comparisons, D – Basal plateletcrit comparisons. Independent *t*-test and One-way ANCOVA adjusting for age.

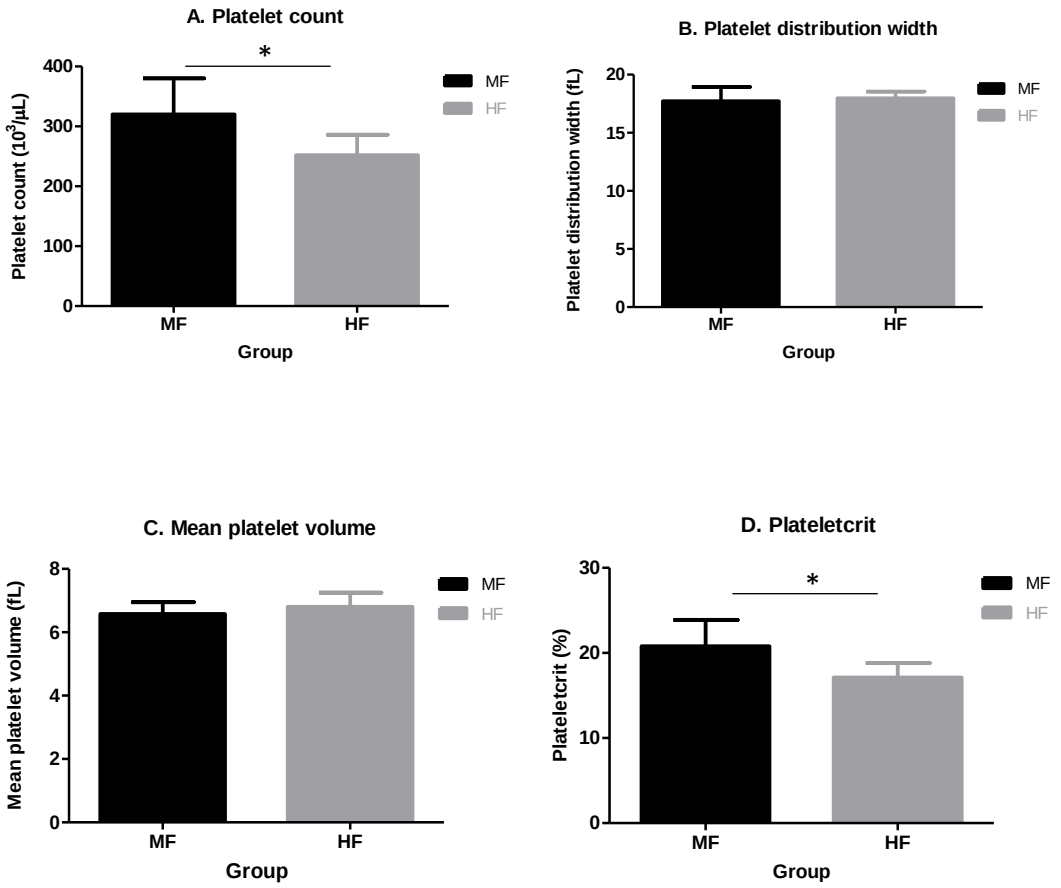


Figure 4.5: Comparison of adult platelet indices at baseline. Graphs represent the mean ± SD value of each group. A – Basal platelet count comparisons, B – basal platelet distribution width comparisons, C – Basal mean platelet volume comparisons, D – Basal plateletcrit comparisons. *P<0.05. Independent *t*-test and One-way ANCOVA adjusting for age.

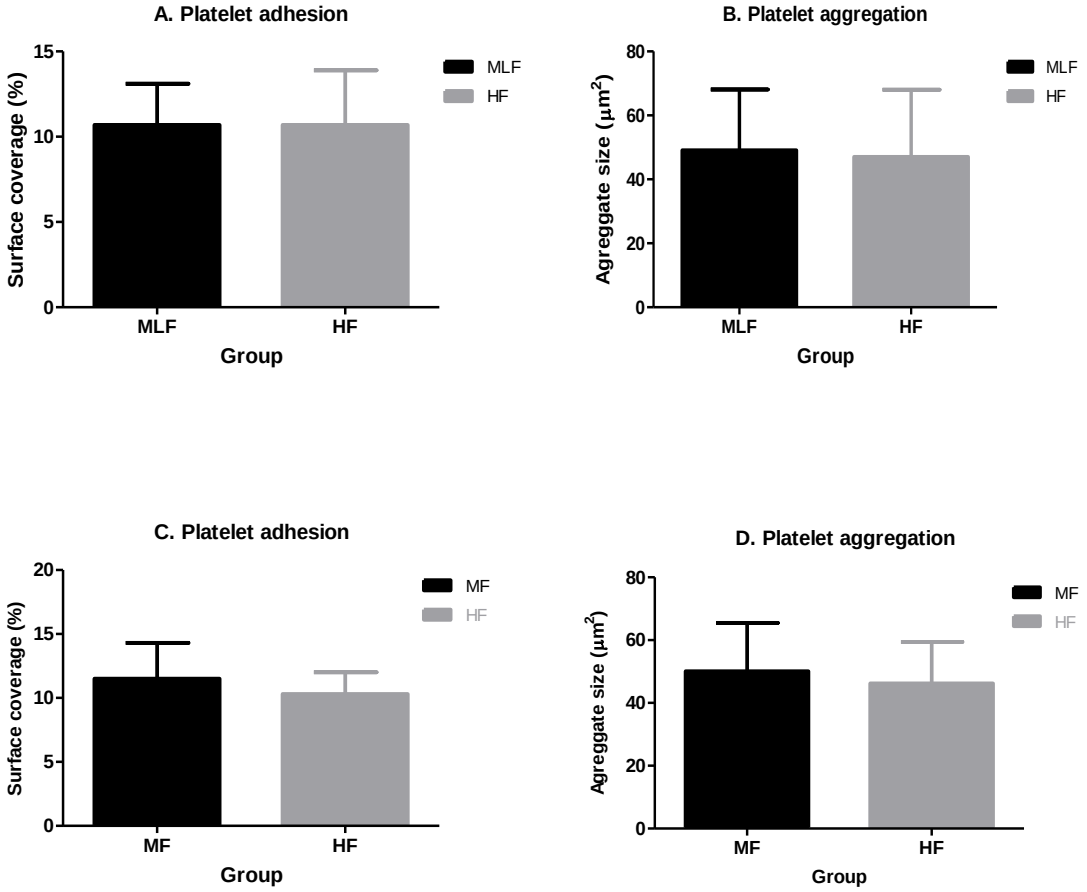


Figure 4.6: Comparison of adolescent and adult platelet function at baseline. Graphs represent the mean ± SD value of each group. A – basal comparison of platelet adhesion in adolescents, B – basal comparison of platelet aggregation in adolescents. C – Basal comparison of platelet adhesion in adults and D – basal comparison of platelet aggregation in adults. Independent *t*-test and One-way ANCOVA adjusting for age.

4.2.3 Effect of acute, strenuous exercise on platelet indices

The second and main focus of this chapter was to assess the effect of a single bout of strenuous exercise on platelet properties in adolescent and adult males of varying CRF levels. Post-exercise, there were significant changes in the platelet indices, platelet count and plateletcrit, in adolescents (Figure 4.7) and adults (Figure 4.8). There were no significant changes in platelet volume indices.

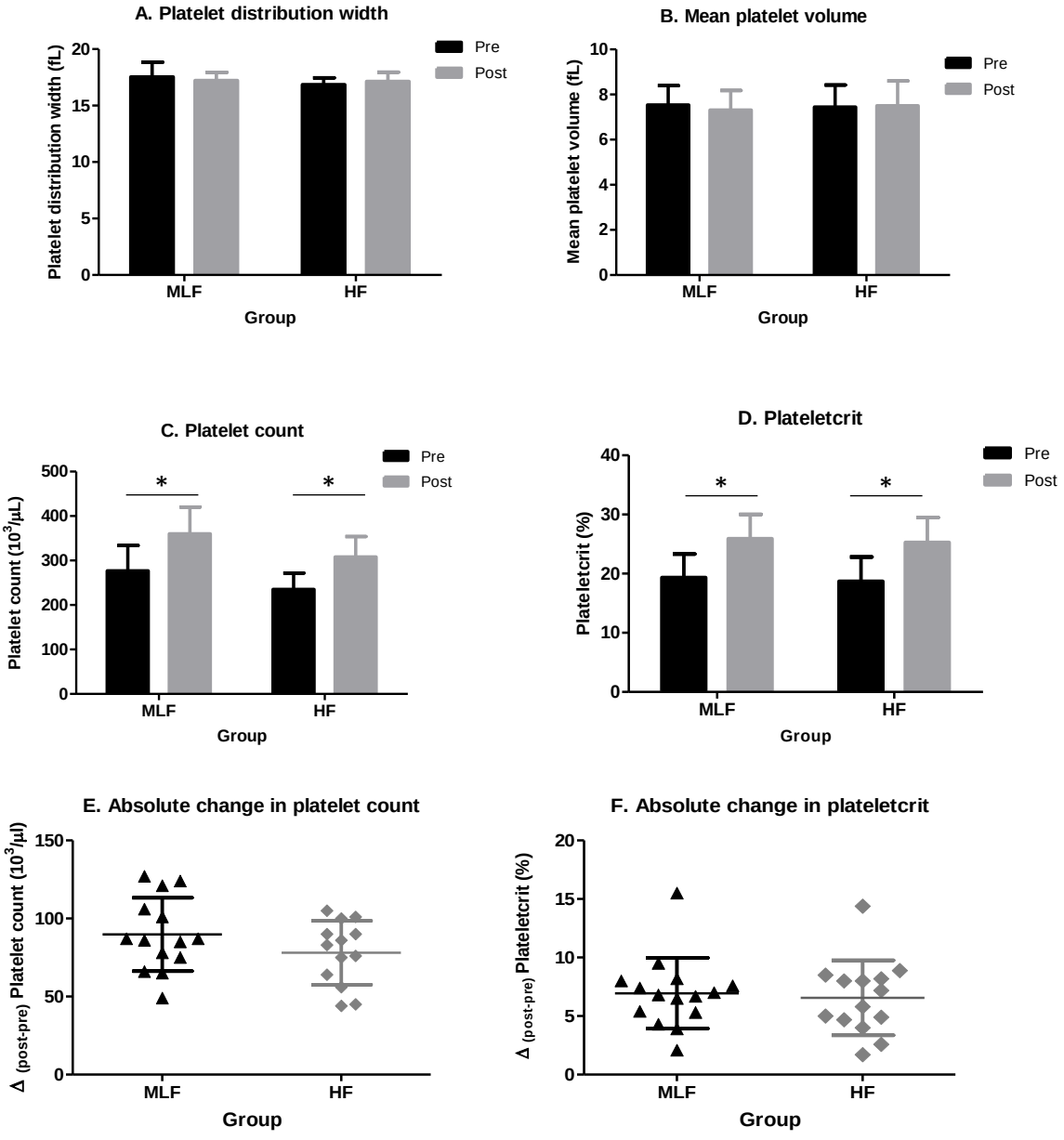


Figure 4.7: Effect of acute, strenuous exercise on platelet indices in adolescents. Graphs represent the mean ± SD value of each group. Graph A shows changes in platelet distribution width after exercise, Graph B shows changes in the mean platelet volume, Graph C shows changes in platelet count and Graph D shows changes in plateletcrit. Image E and F depict the absolute significant changes in platelet count and plateletcrit. *P<0.05. Paired samples *t*-test.

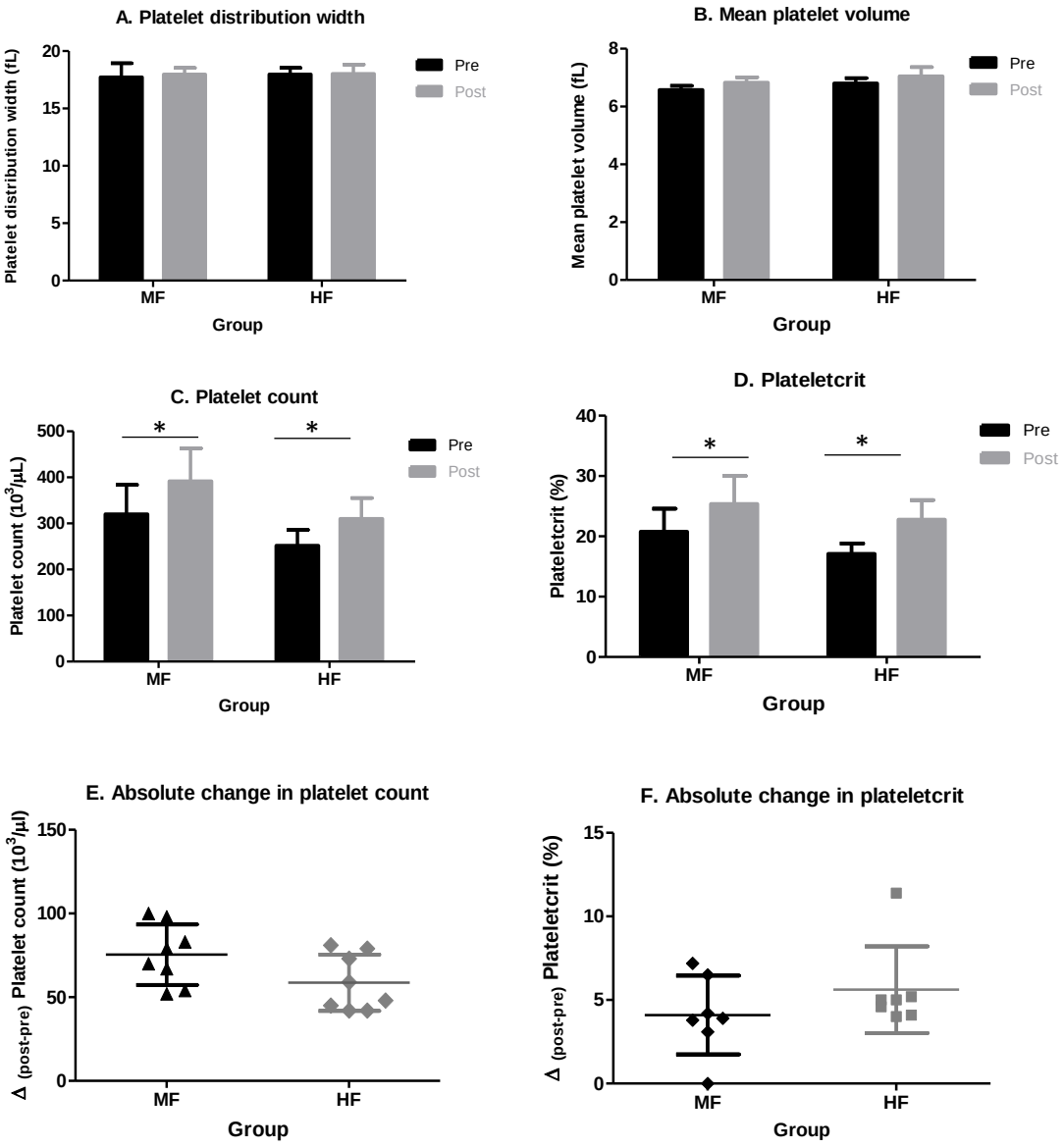


Figure 4.8: Effect of acute strenuous exercise on platelet indices in adults. Values are mean ± SD. Graph A shows the changes in platelet distribution width after exercise, Graph B shows the changes in mean platelet volume, Graph C shows the changes in platelet count and Graph D shows the changes in plateletcrit. Graphs E and F depict the absolute changes in platelet count and plateletcrit respectively. *P<0.05. Paired samples t-test.

4.2.4 Effect of acute, strenuous exercise on platelet function

The effect of a single bout of strenuous exercise on platelet function in male adults and adolescents of varying CRF levels was assessed by Impact R analysis. Figure 4.9 shows the adolescent responses to exercise. There was a significant increase in platelet adhesion and aggregation in the MLF adolescents after exercise, whereas a minor increase was observed in the HF group. This is highlighted in Figure 4.10 – Outputs from the Impact R cone and plate analyser show a visual representation of the changes seen in a randomly selected HF and MLF subjects. Figure 4.11 shows the effect of exercise on platelet function in the adult cohort. While platelet adhesion was elevated in the MF group after exercise, there were no statistically significant changes observed.

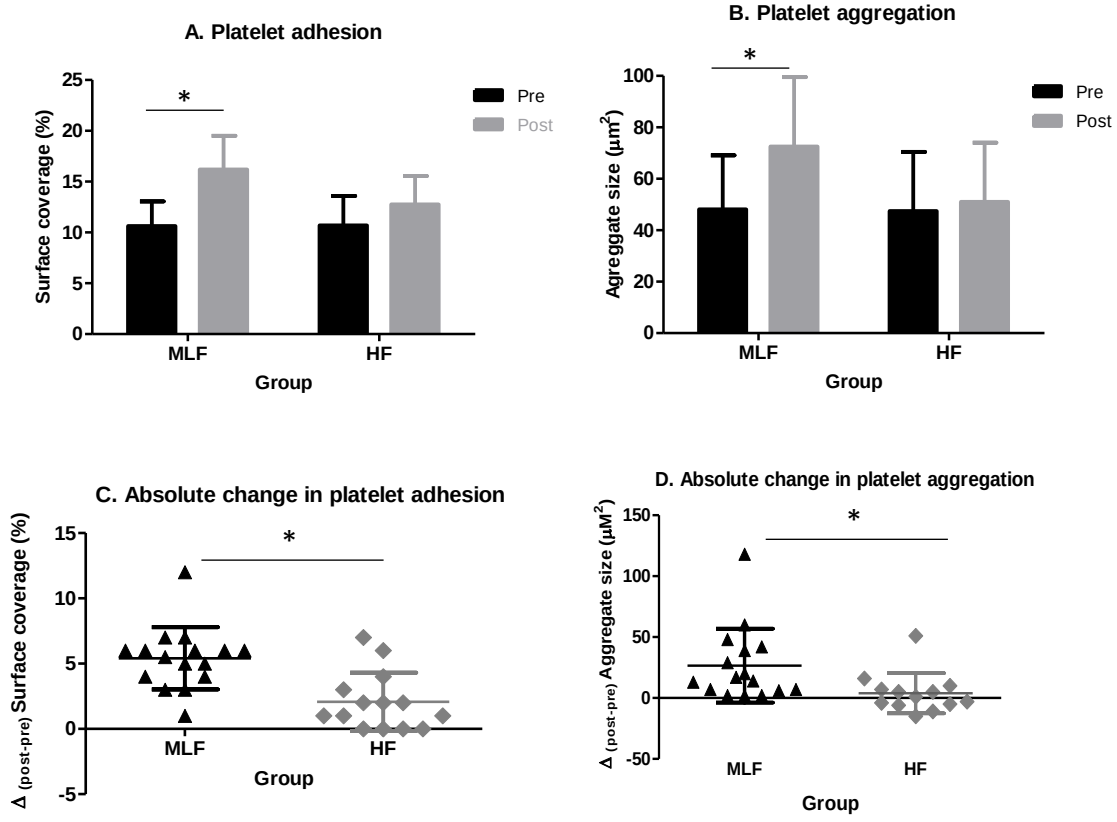


Figure 4.9: Effect of acute strenuous exercise on platelet function in adolescents. Bar charts A and B represent the mean ± SD and show the effect of exercise on platelet adhesion and platelet aggregation respectively. Graphs C and D show the absolute change in adhesion and aggregation for each subject. HF and MLF subjects are identified. *P<0.05. Paired samples t-test.

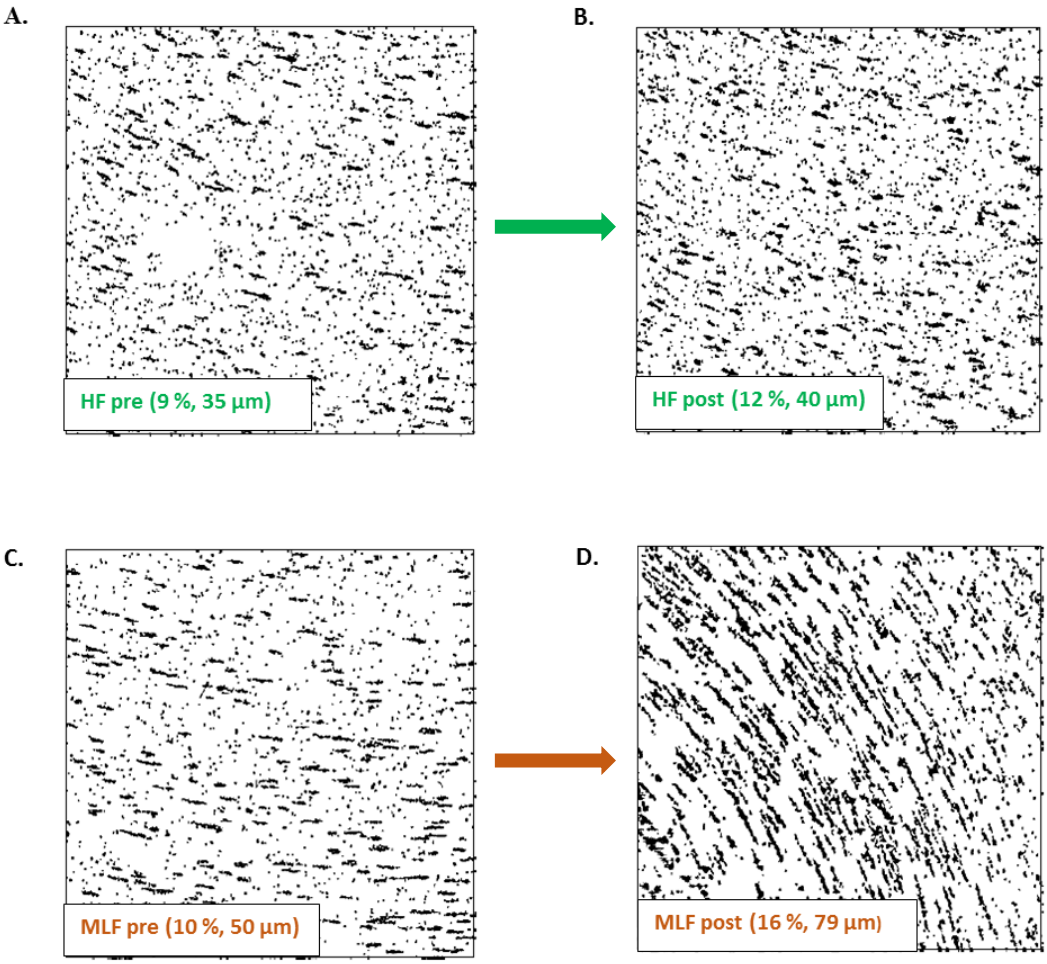


Figure 4.10: Visual representation of the platelet function changes in adolescents after acute strenuous exercise. Images are taken from the Impact R device after capture analysis and represent the Graphs in Figure 4.8. Image A represents basal platelet function in a HF male adolescent with Image B the corresponding post exercise test. Image C represents basal platelet function in a MLF male adolescent with Image D showing the change in platelet function in the same subject after acute exercise. Two values are shown in each bracket (platelet adhesion -SC % and platelet aggregation – AS μm^2).

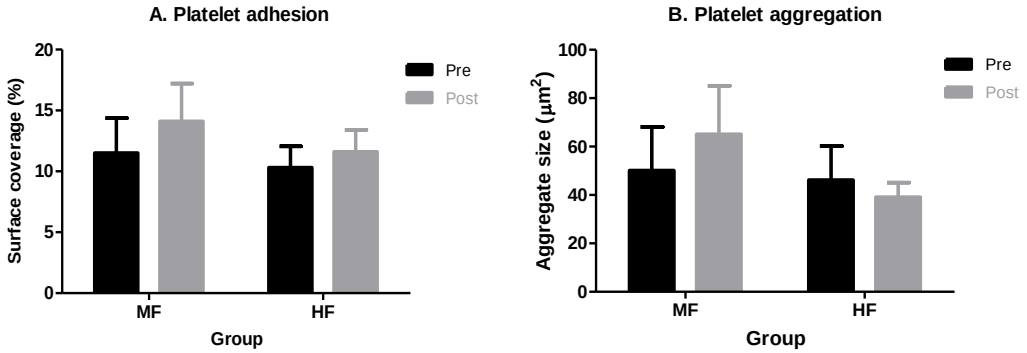


Figure 4.11. Effect of acute strenuous exercise on platelet function in adults. Values are mean ± SD. Graph A shows the effect of exercise on platelet adhesion and graph B shows the effect of acute exercise on platelet aggregation. MF and HF subjects are identified in each graph. Paired samples t-test.

4.2.5 Relationship between CRF and platelet function

To assess the relationship between CRF and platelet function, VO_2 max was correlated to parameters of platelet function in the adolescent and adult male cohorts. In adolescents, the platelet parameters PLT, MPV, PDW, as well as platelet adhesion and aggregation were inversely correlated with VO_2 max at baseline, however, these associations were not significant. Partial correlation coefficients adjusting for age and BMI showed significant inverse relationships between VO_2 max and post exercise platelet adhesion and aggregation and significant inverse relationships between PLT pre (adults) and PLT pre and post (adolescents) shown in Table 4.7 and Figure 4.12/4.13. There were no significant relationships between other haematological parameters and VO_2 max.

Table 4.7: Correlation between CRF and platelet parameters in adults and adolescents. *P<0.05. VO_2 max – Maximal aerobic capacity, PLT – Platelet count, SC – Surface coverage and AS – Aggregate size. *P<0.05. Pearson product and partial correlations adjusting for age.

		Platelet parameter and time point			
		PLT (pre)	**PLT (post)**	**SC (post)**	**AS (post)**
Adolescents	**VO_2 max**	-.217	-.381*	-.403*	-.256
Adults	**VO_2 max**	-.574*	-.564*	-.674*	-.560*

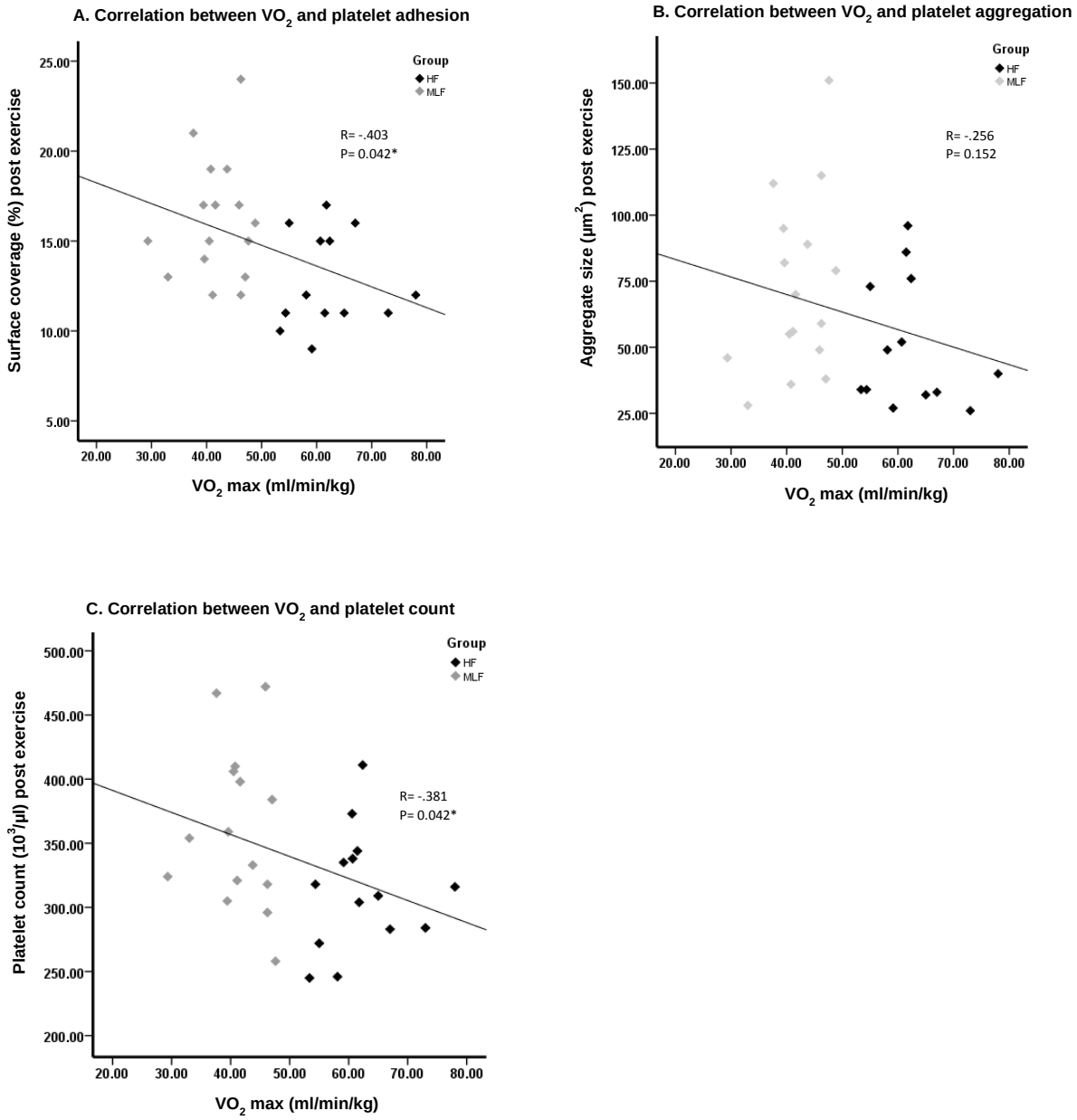


Figure 4.12: Correlation plots between VO_2 max and the pre/post exercise values for platelet parameters in adolescents. MLF and HF subjects are identified for the adolescent population. A – Correlation between VO_2 max and platelet adhesion post exercise in adolescents, B - Correlation between VO_2 max and platelet aggregation post exercise in adolescents and C – Correlation between VO_2 max and platelet count post exercise in adolescent. *P < 0.05. Pearson product and partial correlations adjusting for age.

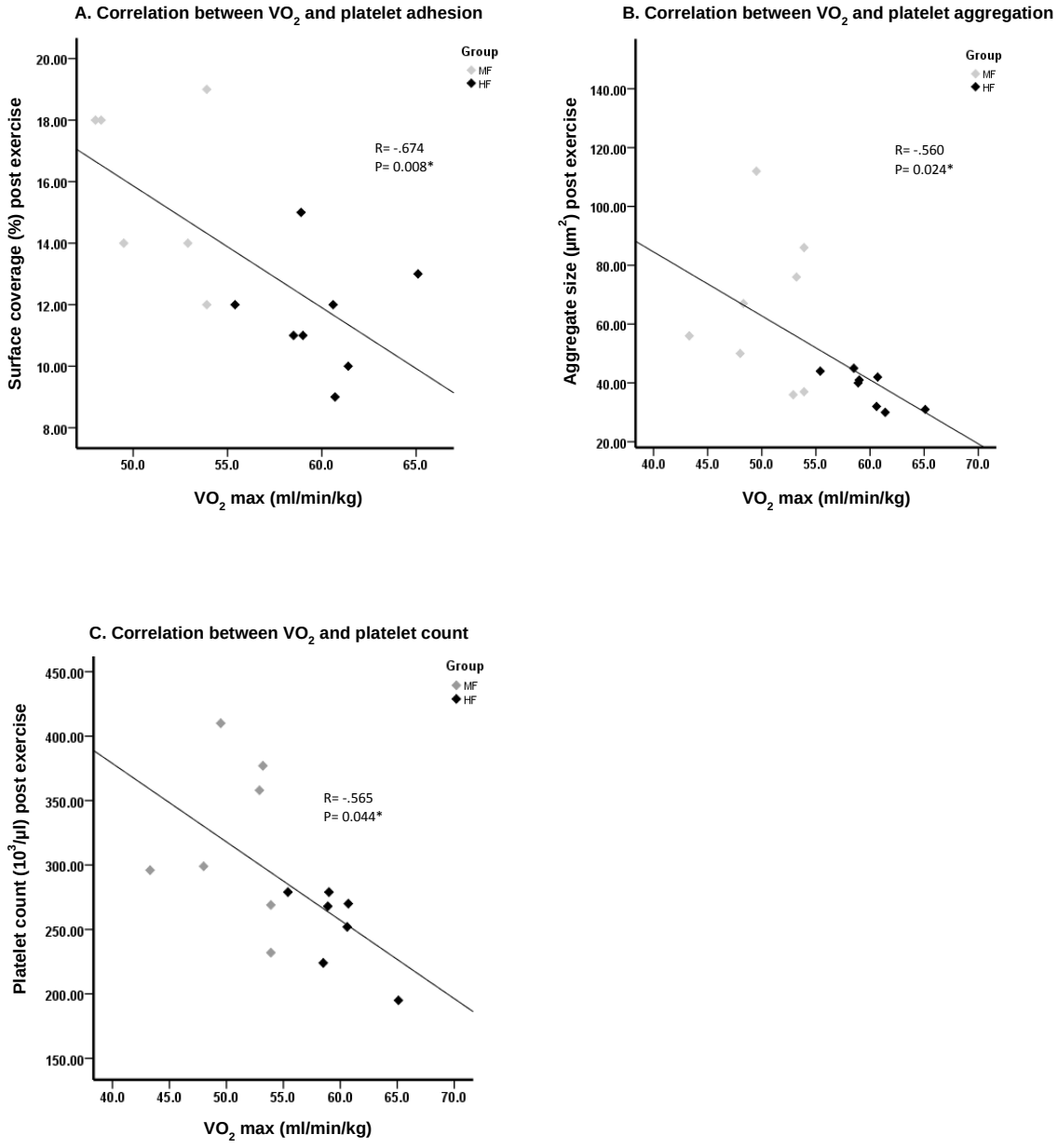


Figure 4.13: Correlation plots between VO_2 max and the pre/post exercise values for platelet parameters in adults. MF and HF subjects are identified for the adult population. A – Correlation between VO_2 max and platelet adhesion post exercise, B – Correlation between VO_2 max and platelet aggregation post exercise and C – Correlation between platelet count and VO_2 max pre exercise. *P < 0.05. Pearson product and partial correlations adjusting for age.

4.3 Discussion

The purpose of this study was to assess the relationship between CRF and platelet function and to examine the effect of varying CRF levels on the platelet response to acute aerobic exercise. Concurrent with the obesity and overweight epidemic, there has been a striking decline in physical activity levels in recent years. Physical activity is the most robust modifiable determinant of cardiorespiratory fitness (CRF), the ability to perform large-muscle whole-body exercise at a moderate to high intensity (Saltin *et al.,* 1973). Maximal oxygen uptake (VO_2 max), is considered the gold standard measurement of CRF. While CRF is a sensitive measure of regular physical activity (Wang *et al.,* 2010), it is also an important inexpensive and valuable health indicator for both healthy individuals and CVD patients (Gulati *et al.,* 2005; Myers *et al.,* 2009). Physical inactivity with a simultaneous low CRF epitomises the most important independent risk factor for atherosclerosis (Lee *et al.,* 2010; Barry *et al.,* 2014; Lavie *et al.,* 2015; Farrell *et al.,* 2015; Holterman *et al.,* 2015).

Cardiovascular disease (CVD) begins in childhood, primarily due to exposure to lifestyle-mediated risk factors such as inactivity and low CRF. Alarming data from Woods *et al.,* (2010) indicate that 88% of Irish adolescents do not meet the current PA recommendations of 60 minutes of daily moderate to vigorous physical activity. The Growing up in Ireland (2012) study of over 8000 children showed that 1 in 4 children were either overweight/obese. High CRF in late adolescence is linked with a reduced risk of MI later in life whereas inactivity pooled with obesity/overweight during adolescence increases the risk of future CVD (Hogstrom *et al.,* 2014).

The relationship between CRF and platelet function has only been referred to once in recent literature (Heber *et al.,* 2016) and therefore we investigated this relationship further. We hypothesised that acute exercise would induce increases in platelet function in adult and adolescent males, with a more pronounced change in those with a lower CRF. Using a clinically relevant human experimental model of thrombosis, we evaluated the effect of controlled aerobic exercise, as a cardiovascular stressor, on platelet function in male adolescents (n=30) and adults (n=16) of varying cardiorespiratory fitness (CRF). *The main finding was that a low CRF favoured a pro-thrombotic platelet phenotype after exercise in both groups with more pronounced changes in the adolescent group. Young male adolescents with a low CRF exhibit a hyperreactive platelet phenotype in response to acute exercise compared with their fitter counterparts.*

4.3.1 Characteristics of the study population

The first aim was to compare basal anthropometric, platelet and haematological parameters. The adolescent cohort was split into two groups according to their VO_2 max for their age – a high fit (HF) and moderate to low fit (MLF) group. The adult cohort was split into two groups – a high fit (HF) and moderately fit (MF) group based on their VO_2 max for their age. This was an all Irish male population. Table 4.1 outlines differences between anthropometric characteristics in the adolescent cohort. As anticipated, the HF group had significantly lower body fat %, BMI and waist circumferences than the MLF group. Similar results were observed in a large study examining physical fitness and body composition in adolescents (Monyeki *et al.,* 2012).

Resting blood pressure was measured twice when the subjects arrived in the morning. The mean systolic and diastolic blood pressures were also significantly higher in the MLF group, with eight of the MLF subjects classed as prehypertensive for SBP. This observation is consistent with research by Mansour *et al.,* (2016) who observed that the mean systolic and diastolic BP in obese adolescents was higher than non-obese, suggesting that the inflammatory condition of obesity could contribute to increased BP. As expected, the HF group had significantly higher physical fitness characteristics than the MLF – in terms of all aspects of their shuttle test and had distinctly higher VO_2 max results (Table 4.2). A graphical representation of the difference in CRF between the groups is shown in Figure 4.1. Max RER (respiratory exchange ratio), one of the criteria for completion of VO_2 max was slightly higher in the HF group compared to the MLF group (Edvardsen *et al.,* 2014) suggesting the HF group were pushing themselves slightly harder to exhaustion than the MLF group.

Table 4.3 shows differences in anthropometric, RHR and fitness levels in the adult cohort. The mean age of both the MF and HF groups was similar, 21.7 years and 21.3 years respectively with the eldest subject 25 years old. Unlike the adolescents, there were no major differences between the MF and HF groups in terms of body composition, with the mean BMI and body fat % only slightly higher in the MF group. Of the cardiovascular parameters measured, RHR was higher in the MF group compared to HF, whereas their blood pressure measurements were similar.

Although CRF was the primary method of comparison for this chapter, it was relevant to examine the extent of obesity and overweight amongst the two cohorts. As outlined in Table 4.4, a substantial percentage (23%) of the adolescent cohort were overweight, with an additional 4% categorised as obese. The smaller adult cohort had only four subjects categorised as overweight. Figure 4.2 examines the relationship between BMI and CRF in both subject groups. The scatterplots illustrating the relationship between CRF and BMI in both cohorts show as significant inverse relationship between BMI and CRF in adolescents but not in adults. As there was not a huge discrepancy between BMI in the MF-HF adults, results showed a non-significant relationship between BMI and VO_2 max. A larger, more disperse adult cohort with subjects with a very low CRF would probably have followed the same trend as the adolescents.

4.3.2 Haematological characteristics of the study population

Table 4.5 shows red and white blood cell characteristics between the two groups in adolescents. Though all values except WBC were higher in the MLF group, there were no significant differences between the two groups at a resting level. It was expected that WBC may be elevated in the MLF group as prior studies have described a chronic low-grade inflammatory state in obese (Tenorio *et al.,* 2014; Ferrari *et al.,* 2015) and overweight (Oliveria *et al.,* 2014) adolescents. Similarly, there were no significant differences between basal RBC and WBC parameters in adults, even though the MF group had higher WBC levels (Table 4.6). This baseline observation was expected as results from Chapter 3 showed that those who were categorised as "fit" had lower WBC levels than unfit adults, and furthermore, overweight adults displayed increased WBC compared to healthy weight adults. Similarly, Church *et al.,* (2002) also showed that healthy male adults with increased fitness have lower WBC concentrations. Athletes tend to have an increased total mass of RBC and HGB in circulation compared to sedentary individuals, but often in endurance sports, trained subjects can have a decreased HCT generated by increased plasma volume (Mairbaul, 2013). Some of the HF adults and adolescents were extremely fit and their slightly lower resting RBC values and subsequent morphological features could be explained by this occurrence.

4.3.3 Comparison of basal platelet indices according to CRF

Chapter 3 illustrated elevated platelet indices in adult subjects with overweight/obesity or indications of unhealthy lifestyle compared to healthy subjects. This chapter used CRF compare differences between groups. In our first approach, we examined basal platelet parameters. Figure 4.5 outlines basal differences in platelet indices between the HF and MF adults. The platelet indices PLT and PCT were significantly higher in MF compared to HF adults. Other studies have shown similar results (Mansour *et al.,* 2016). This is similar to results from the cross-sectional study in Chapter 3 whereby PLT and PCT were the platelet indices displaying most variance between the groups when stratified by adiposity measures. However, as the MF and HF groups were comparable in terms of BMI and other adiposity measurements, this suggests that CRF is a superior indicator of basal platelet activity. We observed no differences in basal MPV and PDW between the MF and HF adult cohort groups. A recent study comparing platelet indices according to fitness levels showed comparable results whereby PLT was slightly higher in sedentary subjects compared to trained, but no major differences in the rest of the platelet indices were observed (Alis *et al.,* 2016).

Research around platelet indices is in it's infancy with regard to younger populations. Some studies have shown basal platelet indice alterations in child and adolescent populations with respect to disease, such as elevated MPV levels in children with bicuspid aortic valve condition Ayan *et al.,* (2015). However, there is a lack of research surrounding platelet indices in healthy adolescents.

Figure 4.4 compares basal platelet indices in the adolescent cohort. The MLF group had slightly higher platelet indice values compared to the HF group. Similarly, to the adults, the trend showed increased PLT levels in MLF group. There was no difference in basal MPV and PDW levels. MPV has been identified as higher in obese adolescents compared to healthy controls (Arslan and Makay, 2010) and has also been linked with common carotid artery (CCA) thickness in obese adolescents (Arslan *et al.,* 2013). However, as outlined in Table 4.4, only four adolescent subjects had a BMI > 30 (obese), a possible explanation for the similarity in MPV between the two groups. Overall, further research around the indices of platelet count and plateletcrit are required, to determine their usefulness as biomarkers of CRF.

4.3.4 Comparison of basal platelet function according to CRF

Figure 4.6 compares basal platelet function characteristics of the adult and adolescent cohorts. In the adolescents, there were no notable differences between platelet adhesion or platelet aggregation between the two groups. Both the HF and MLF groups had a mean basal surface coverage (platelet adhesion) of 10% and platelet aggregate size was also similar revealing values of 49 μm^2 and 47 μm^2 for the MLF and HF groups respectively.

There have been no specific studies on platelet adhesion variabilities in adolescent cohorts in relation to CRF to date. However, basal platelet function in obese children and adolescents has previously been investigated. Platelet aggregation in PRP was significantly increased in hypertensive obese adolescents compared to healthy adolescents (15.5 years old) (Hazon *et al.,* 2003). Recent work by Desideri *et al.,* (2009) evaluated endothelial dysfunction and platelet activation in 40 obese and 40 non-obese children (mean age 10 yrs). Also utilising PRP, they established that obese children had higher plasma levels of soluble P-Selectin and CD40L compared to non-obese children, suggesting increased platelet activation. When they stratified subjects based on their BMI, biomarkers of endothelial cell and platelet activation were similar despite varying body fatness levels.

As referred to in Chapter Three, the physiological milieu of whole blood is sometimes preferred for platelet function studies. Using whole blood lumi-aggregometry, Lohse *et al.,* (2010) reported that paediatric overweight subjects displayed platelet hyperaggregability compared to healthy weight controls. They also noted a positive correlation between triglycerides, total cholesterol and collagen-induced aggregation in the overweight children and adolescents. Desideri *et al.,* (2009) did not find significant correlations between platelet activation and the adiposity measures BMI, visceral fat or WHR in obese children.

Unlike the adolescent cohort there were subtle changes in basal platelet function values between MF and HF adults with the MF group showing slightly increased platelet adhesion (11.5% compared to 10.3% in HF) and aggregation (50 um^2 compared to 46 um^2 in the HF). This indicated that rising measures of adiposity may not necessarily be responsible for all changes in platelet function and that physical fitness status may be a participating factor, as the MF subjects were not overly different in terms of body fat % or BMI compared to the HF subjects. Based on results from Chapter Three, we were expecting to see minimal basal differences between the two groups.

Lippi *et al.,* (2006) also compared platelet function between elite male cyclists and healthy sedentary males at rest. Platelet adhesion and aggregation were tested by the PFA-100. Platelet adhesion and closure time to collagen-ADP was significantly increased in the sedentary controls compared to elite athletes, highlighting that changes in platelet function are more pronounced in sedentary individuals. However, their population size was significantly larger (n=140).

A recent breakthrough study by Heber *et al.,* (2016) investigating platelet function and CRF in women (n=62), was the first research to combine CRF and platelet physiology in an adult population. Platelet function was tested by determination of P-Selectin and CD40L expression and quantification of platelet ROS generation in PRP. Basal platelet activation (reflected by CD62P expression) and agonist induced platelet activation (ROS, CD62P and CD40L) were higher in the LF compared to the MF and HF. The group found no difference between basal CD40L expression (non-agonist-induced). Interestingly, basal platelet function in the MF and HF were almost equal. Similar to this group, we found no major differences in basal platelet activation between the MF and HF adults, and addition of LF subjects could have resulted in interesting results and would be of benefit in future studies. However, the study cannot be applied to a male population as platelet function can be varied between gender.

4.3.5 Effect of acute, strenuous exercise on platelet indices

The main aim of this chapter was to compare platelet indice and function responses to acute exercise in male adolescents and adults of varying CRF. At the onset of exercise, the physiological systems respond, proportional to the intensity and duration of exercise, to maintain homeostasis (Riviera-Brown and Frontera, 2012). Indeed, in the cardiovascular system there are various responses to the increased demands of exercise. Cardiac output increases linearly with increasing oxygen demand. Blood flow patterns are dramatically altered in response to exercise where more blood is distributed to skeletal muscle.

Figure 4.7 displays the effect of acute exhaustive exercise on platelet indices amongst adolescents. PLT was significantly increased in both HF and MLF after exhaustive exercise. This difference was slightly more enhanced in the MLF group but there were no significant changes in absolute PLT numbers between the MLF and HF groups as shown in Figure 4.7 (E). In the adult cohort, PLT was significantly increased after exhaustive exercise (Figure 4.8 (A)), and similarly to adolescents, the increase was more pronounced in the MLF group. An increase in PLT in adults after acute exercise has been described by numerous groups in the literature (Ghaediyan, 2012; Cadroy *et al.*, 2002; Dabis *et al.*, 1990) with only one study showing the same trend in adolescents (Ribeiro *et al.*, 2007).

The spleen harbours one third of the body's platelets in an interchangeable pool which can be discharged into circulation by alpha adrenergic stimulation (Chamberlain, 1990; Shepard, 2016). The increase in PLT after acute exercise is attributed to platelet release from the liver, lungs and spleen, and hemoconcentration – the decrease in plasma volume which causes an increase in other blood cell components. Increased epinephrine after intense exercise causes splenic contractions (Bakovic *et al.*, 2013; Gao *et al.*, 2016). Additionally, disintegration of confined megakaryocytes in the lungs and bone marrow may result in exercise induced thrombocytosis. As the change in platelet count was similar between all groups (shown in Figure 4.7 and 4.8) after exercise, the activated and hyper-aggregable profile of the platelets observed in the MLF group in adolescents could be a result of platelet integrity loss initiated by the low CRF – where subjects may have higher oxidative and inflammatory conditions in addition to alterations in lipid and body composition profiles.

As shown in Figure 4.7, there was no major change in platelet volume markers MPV or PDW in either adolescent group after exercise. This is consistent with recent work by Alis *et al.*, (2015) who described the increase in MPV after acute strenuous exercise as less than their within-subject variation stated for that parameter, concluding that increased MPV does not suggest clinical importance. In the adult cohort, there were no major changes in MPV in both groups post exercise (Figure 4.8). To support our findings, other studies have shown that MPV does not change hugely with concurrent thrombocytosis (excessive platelet count) after exercise (Watts, 2009; Singh *et al.*, 2006). and therefore, MPV was not a significant marker of performance in exhaustive exercise. The effects of marathon running on platelet activation markers demonstrated no significant changes in MPV or PDW post marathon (Kratz *et al.*, 2006). In the adult cohort there was a very small increase in PDW in both

groups post exercise. Some studies have reported a small increase in PDW and MPV after acute exercise in healthy subjects (Ghaediyan, 2012).

There is less information on the effect of acute exercise on PCT, which increased drastically in both groups by nearly 25% after the VO_2 max test, in both adults and adolescents (Figure 4.7 and 4.8) likely due to the increase in PLT after exercise. Only two studies have measured PCT with regard to training status. Karakilcik *et al.,* (2014) showed that PCT (along with PLT) was significantly decreased with exercise in young (mean 23 years) male soccer players. Rba *et al.,* (2015) showed that regular exercise participation decreased PLT and PCT levels compared to sedentary subjects. Similarly, to our results, novel data from Alis *et al.,* (2016) found that although exhaustive exercise also caused an increase in all platelet parameters PLT, MPV, PDW and PCT in sedentary and trained males, training status did not produce any alterations in baseline or post exercise increases in platelet indices.

4.3.6 Effect of acute, strenuous exercise on platelet adhesion

Potential effects of acute exercise on platelet function (mainly aggregation) have been investigated by a plethora of studies in adult subjects with varying intra and inter individual results, making interpretation problematic. Differences in population type (e.g. CVD vs healthy), methods employed to assess platelet function and techniques to examine reactivity are the main reasons for discrepancies between research groups (Aurigemma *et al.,* 2007).

There have been no studies to date examining the effect of acute exercise on platelet adhesion in adolescents. Figure 4.9 (A) show that acute exercise resulted in elevated platelet adhesion in both MLF and HF adolescents. However, the MLF group displayed much larger changes than the HF group. This is further illustrated in Figure 4.10 with a visual representation of the changes in platelet adhesion in a MLF and HF adolescent subject. Platelet function and exercise in children or adolescents is in it's infancy. A sole study by Ribieria *et al.,* (2007) showed that platelet count increased after acute exercise in 10 sedentary males with a mean age of 13 years. Other available studies have mainly examined the effect of aerobic exercise in disease cohorts. Kumar *et al.,* (2016) investigated the effect of aerobic exercise on haemostatic indices in paediatric subjects with haemophilia. Platelet function analysis was performed using the PFA-100 before and after 3 minutes of cycling at 85% of max heart rate.

Similarly, in the adult cohort, acute exercise caused an increase in platelet adhesion in both the HF and MF group, with a significant increase in the MF group only (over 25% increase from 11.5% – 14.5%) (Figure 4.11). While a substantial amount of research has been devoted to the exercise and platelet function in adults, different platelet adhesion experimental protocols have provided no definite response to exercise in healthy adult subjects. Wang *et al.,* (1994) were the first to investigate the effects of aerobic exercise on platelet adhesion, quantified by a tapered parallel plate chamber. Results from the adult cohort are consistent with results from Wang *et al.,* (1994) who showed that platelets from sedentary male subjects displayed increased adhesion after an incremental exercise test performed on a bicycle ergometer, compared to physically active male subjects. More recent research by Wang and colleagues found increased platelet adhesion after strenuous exercise, suggesting a resilient platelet-surface collaboration post exercise stimulus. These studies used an assay developed to measure the resistance of adhered platelets against detachment by shear stress (Wang 1995, Wang 1997, Wang and Chen 1999, Wang 2002).

The change in platelet adhesion in adults after exercise was not as profound as the variations observed in adolescents. However, the difference in CRF between the two adult groups was not as large as the gap between the groups in the adolescent cohort and could be partially responsible for this (VO_2 max differences). Other studies investigating platelet function in sedentary and trained males have had VO_2 averages of 59 ml/kg/min for high fit and 37 ml/kg/min for the low fit (Singh *et al.,* 2006) compared to our averages of 50.1 ± 1.3 ml/kg/min for the MF and 60.2 ± .98 ml/kg/min for the HF.

4.3.7 Effect of acute, strenuous exercise on platelet aggregation

Effects of platelet acute exercise on platelet aggregation were also analysed. A number of studies have shown that acute exercise in healthy individuals or CVD patients results in an increase in platelet function and aggregation (El Sayed, 2002; Chung *et al.,* 2008, Perneby *et al.,* 2007; Scalone *et al.,* 2009). These studies typically used *in vitro* platelet aggregation tests to assess platelet function.

Figure 4.9, graphs B and C show the change in platelet aggregation after exercise in adolescents. There was an increase in aggregate size in both the HF and MLF group, with significant increases in aggregation post exercise in the MLF group (mean size increased from 49 um^2 to 73 um^2). Figure 4.11 (B) shows the effect of acute exercise on platelet aggregation in the adult cohort. Similarly, exhaustive exercise incurred an increase in platelet

aggregation in the MF group. However, these changes were not significantly different to pre exercise measurements. Interestingly, average platelet aggregation dropped slightly in the HF group, post exercise.

Acute exercise was shown to increase platelet reactivity in healthy sedentary subjects but not in healthy subjects were regularly engaged in physical activity (Wang *et al.,* 1994; Kestin *et al.,* 1993). Coppola *et al.,* (2005) also compared sedentary with active subjects and showed that exercise increased circulating platelet aggregates, whose levels were much higher in the sedentary compared to active group. Furthermore, ADP induced P-Selectin expression in addition to platelet aggregation were only increased in the sedentary group after exercise.

The platelet integrin αIIbβ3 is found at high densities on the platelet surface and platelet α-granules, existing in an inactive form on resting platelets (Rumbaut and Thiagarajan 2011). Upon platelet activation, it experiences conformational alterations resulting in a drastically heightened affinity for fibrinogen, facilitating platelet aggregation. Some studies have found increases in αIIbβ3 activation post exercise. Whittaker *et al.,* (2013) used flow cytometry to test platelet activation after high intensity aerobic exercise in 12 healthy males, and reported increases in the platelet activation marker PAC-1, which binds to the activated form of αIIbβ3. Peat *et al.,* (2010) assessed the platelet response to acute exercise in trained and sedentary middle aged subjects using an incremental treadmill test. Platelet function was assessed by flow cytometry and results displayed an increase in platelet αIIbβ3 expression post exercise. Wang *et al.,* (2004) have also shown that intense exercise increases shear induced platelet aggregation in males. They also advocate that this is a result of increased Von Willebrand factor (vWF) binding, αIIbβ3 and heightened P-Selectin expression. These results could have been a factor in the increase in platelet aggregation in the MLF adolescents and MF adults post exercise in our study.

As previously mentioned, the PFA-100 measures platelet activation in whole blood by the combined action of shear stress and various platelet agonists. It is representative of platelet aggregate formation. Acute strenuous exercise has resulted in shorter closure times induced by collagen-epinephrine and collagen-ADP in healthy sedentary males (Cadroy *et al.,* 2003). Madsen *et al.,* (2009) also showed that brief strenuous exercise enhanced platelet aggregation in healthy men, however LTA using PRP showed minor increases when compared to whole blood PFA-100 measurements, reinforcing the need for whole blood analysis. They also observed increases in plasma vWF which could be partially responsible for increased aggregation.

Interestingly there was a slight decrease in aggregation post exercise in the HF group, as outlined in Figure 4.11 (B). Some studies have revealed an inhibition of platelet aggregation by exercise, however the majority of these showed that submaximal, not maximal exercise induced these changes (Aldemir and Kilic, 2005) reported that ADP-induced platelet aggregation was decreased after exercise in 10 moderately active males. It is possible that in healthy subjects with an intact endothelium possessing normal anti-thrombotic properties, vigorous exercise does not present a risk of thrombosis.

Of note, the change in platelet aggregation post exercise was much greater in the adolescent MLF cohort compared to the change observed in the adult cohort, suggesting that in low fit adolescents, there exists a baseline hyper-aggregable platelet state. The absolute change in platelet aggregation is shown in Figure 4.9, (D) whereby all subjects are represented on the graph. There was a significant difference in the change in platelet aggregation between the adolescent groups.

4.3.8 Overall effect of acute, strenuous exercise on platelet function

Some hypotheses have been suggested to explain the mechanism whereby acute exercise affects platelet function. The responses depend on various factors including exercise intensity, duration, fitness of subject and assays employed to test platelet function (Aurigemma *et al.,* 2007). Evidently, exercise causes circulatory activation, which could result in the mobilisation of newly produced platelets and more metabolically active ones from the pulmonary vessels (Bakovic, 2013).

After high intensity exercise, there is a large production of ROS resulting in oxidative stress and cellular damage. Singh *et al.,* (2006) suggest that excess ROS production can counteract the natural cellular antioxidant protection and that this process is more prominent in low fit subjects. Exercise-induced oxidative stress may be responsible for platelet hyperactivity in sedentary males. Strenuous exercise resulted in diminished total antioxidant capacity and enhanced susceptibility of LDL to *in vitro* oxidation in sedentary males (Tozzi-Ciancarelli *et al.,* 2002). The oxidative stress elicited by acute strenuous exercise might affect platelet responsiveness by encouraging oxidised LDL-mediated platelet activation.

The elevation of intracellular free calcium levels represents an initial factor in platelet activation and signalling. (Wu, 2012). A rise in cytosolic Ca^{2+} in platelets has frequently been reported after exercise (Tozzi-Ciancarelli *et al.*, 2002; Wang *et al.,* 2002) which could

be a factor contributing to the platelet hyperreactivity noticed in the MLF subjects. Lee *et al.,* (1999) also reported that cytosolic free Ca^{2+} concentrations were increased after aerobic exercise in sedentary, but not fit males.

The release of ADP and thromboxane $A_{2,}$ (TxA_2) initiate positive feedback loops to maintain aggregation after platelet activation. Increased urinary and plasma levels of the TxA_2 degradation product thromboxane B_2 have been reported post exercise (Weber *et al.,* 2007; Whittaker *et al.,* 2013; Santilli *et al.,* 2013). Degranulation of platelets after acute exercise results in the expression of soluble mediators P-Selectin, platelet factor 4 (PF4) and thromboglobulin, whose levels have been increased after acute exercise. This, in combination with increased αIIbβ3 activation could have contributed to excessive adhesion and aggregation in MLF subjects (Mockel, 2001; Weber 2007; Peat and Dawson, 2010). Elevated catecholamine levels after maximal exercise have also been suggested to increase the platelet surface α2 -adrenoreceptor numbers, augmenting their binding affinity to fibrinogens. However, unlike maximal exercise, acute moderate exercise does not seem to elicit platelet activation to the same extent and would be an interesting area of research (Chen and Lip, 2014).

Juskens *et al.,* (2016) indicated that vigorous exercise induces a hyperreactive overall rebalanced haemostatic state, concurrent with increased platelet reactivity in healthy men. In addition, other markers of risk factors for CVD were also increased after exercise including cardiac troponin T level, inflammatory markers IL-7, Il-8, MCP-1, RANTES and PDGF, and endothelial markers vWF were increased in this study.

4.3.9 Influence of exercise training and CRF on platelet responses to exercise

A high CRF level is a result of exercise training and habitual physical activity. Therefore**,** research on the effects of longitudinal exercise training on platelet function has mainly shown that habitual exercise has favourable effects on platelet function. Therefore, CRF might represent a serious determinant for changes in platelet function in response to acute exercise.

Platelet derived microparticles (PMPs) can be released upon platelet activation. After strenuous exercise, some studies have reported an increase in PMPs in sedentary men which could contribute to the platelet hyperaggregability seen in the MLF subjects in our study (Chen *et al.,* 2013; Chen *et al.,* 2014). Sossdorf *et al.,* (2011) examined the effect of acute,

moderate exercise on the manifestation of PMPs and their procoagulant action. In sedentary and trained subjects, 90 minutes of cycling at 80% individual anaerobic threshold (IAT) triggered a significant elevation of PMPs and an increase in their procoagulant activity. Conversely, two hours later the concentration of PMPs remained elevated in the sedentary group only. The reduced PMP clearance in the sedentary subjects could result in increased circulating levels of proatherogenic elements (Augustine *et al.*, 2014).

Prostacyclin (PGI_2) and nitric oxide (NO) inhibit platelet function. Training can cause a rise in NO released from platelets and endothelial cells and perhaps a long-term adaptation to exercise resulting in inhibition of platelet aggregation. (Chen *et al.,* 1993; Green *et al.,* 2004). The aggregating agent TxA_2 was reduced by exercise training. The PGI2/ TxA_2 ratio has been suggested to have an influential role in defining the extent of platelet aggregation (Moncada and Vane, 1979). This could be one of the reasons why HF (and trained) subjects could be less sensitive to the same physiological stimulus compared to the MF/MLF.

Heber *et al.,* (2015) investigated the relationship between CRF and various aspects of platelet function in low fit, medium fit and high fit women. This study stratified subjects in a similar way to this chapter. Platelet function was assessed by basal and agonist induced surface expression of CD62P and CD40L and analysis of intraplatelet ROS. CD40L encourages atherosclerosis by altering platelet function (Lievens *et al.,* 2010; Wang *et al.,* 2013). In the LF group, basal platelet activation and agonist induced platelet activation were higher compared to MF and HF. After 8 weeks of aerobic exercise training (40 minutes of walking/running three times per week) in the LF group, their CRF improved and platelet function aligned with that observed in the MF and HF groups.

Culminating the above mechanisms, despite inconsistency in the literature, it appears that exercise exerts a substantial influence on nearly all facets of platelet activation and results in this chapter are consistent with that theory. Regular exercise training alleviates the activating consequence of acute strenuous exercise on platelet function, and recently, long-term habitual exercise training has been shown to affect platelet function at rest. High levels of physical activity represent the normal physiological state (Cordain *et al.,* 1998; Booth and Laye, 2010) and subsequently elevated platelet adhesion and aggregation in the MLF and MF groups can be viewed as non-physiological and therefore pathological platelet hyperreactivity.

4.3.10 Correlation analysis between VO_2 and platelet parameters

The relationship between CRF and platelet function has not been exactly defined. We assessed degree of bivariate relationship between CRF and platelet function using Pearson product coefficient and partial correlation analysis (Table 4.7). We found a significant inverse correlation between VO_2 and PLT (pre) in the adult cohort which remained significant when adjusting for age and BMI. The fact that partial correlation coefficients were almost equivalent to Pearson correlations coefficients without accounting for the control variables suggests that our results were not majorly influenced by the subjects' age and BMI. Additionally, PLT post was significantly correlated to VO_2, but this was attenuated when age and BMI adjusted. There was no significant association between basal values for platelet indices MPV, PDW, PCT and VO_2 max in either the adolescent or adult cohorts, consistent with recent research by Alis *et al.,* (2016). Another study investigating the relationship between platelet indices and fitness showed that basal MPV was directly correlated to endurance performance (21 k half marathon) after adjusting for age, sex, BMI and PLT, indicating a relationship between platelet metabolism and aerobic performance. (Lippi *et al.,* 2014)

We found no significant correlations between CRF and basal platelet function parameters SC and AS in adolescents or adults. Heber *et al.,* (2016) noted significant correlations between VO_2 max and platelet function in 62 young women. Basal CD62P expression was inversely correlated to VO_2 max and so was agonist induced expression of CD62-, CD40L and ROS formation. There was a significant inverse correlation between VO_2 and platelet adhesion (post) in the adolescent cohort and adult cohort as shown in Figure 4.12 and 4.13. There was also a significant inverse correlation between VO_2 max and aggregation (post) in the adult cohort (Figure 4.13) but not in the adolescent cohort. Perhaps epigenetic drift could affect older individuals (adults) more due to increased temporal changes as we were beginning to see changes in the adolescents, which were not yet significant but followed the same trend as the adults.

4.3.10 CRF related microRNA

Work in this chapter shows that platelet function changes are also associated with CRF. However, the molecular mechanisms by which exercise affects platelet function remain poorly defined. Recently identified miRNAs have gained attention as modulators of platelet function (Landry *et al.,* 2009). Evidence for miRNA involvement in exercise-associated gene expression changes in a number of cell types in non-trained and trained subjects has been illustrated (Radom-Azik *et al.,* 2012). Work by Baggish *et al.,* (2011) showed altered expression of circulating miRNA (c-miRNA) in response to acute and chronic exercise in athletes. Eight c-miRNA involved in cellular processes fundamental to exercise adaptation (muscle contractility and inflammation and angiogenesis) were examined. Four distinctive signatures of c-miRNA were observed, including; c-miRNA upregulated by acute exhaustive exercise pre and post exercise intervention, c-miRNA responsive to acute exercise pre but not post intervention, c-miRNA only responsive to exercise intervention and non-responsive miRNA. Furthermore, low CRF was linked with high expression levels of three c-miRNAs in the large Nord-Trøndelag Health Study (HUNT) study (Bye *et al.,* 2013). As platelets contribute substantially to the circulating miRNA pool (Willeit *et al.,* 2013), their unique miRNA profile could be representative of adaptations to exercise.

4.4 Limitations

This study was carried out in males only and therefore, the results cannot be generalised for the total population. Additionally, the sample size was small for the adult cohort (n=18) compared to the adolescent cohort (n=30) and the results of the study are also age limited for the adolescent cohort (15-17-year-old) and the adult cohort (mean age 21 years). It would be desirable to confirm the observations from this study in larger populations with varying ages and to include female subjects. This would add weight and further significance to platelet function and CRF following maximal exercise. The inclusion of a control group would be beneficial in reinforcing the results of the study highlighting the benefits of regular exercise and consequently high CRF levels. Although VO_2 max is considered the gold standard measurement of aerobic fitness, it is often not attained by those of an overweight/obese nature, due to its dependency of effort (Marinov *et al.,* 2013) and while every attempt was made to encourage low fit subjects to complete the treadmill test, some may not have reached their peak VO_2. The results are also limited to an acute bout of maximal exercise as we did not investigate submaximal exercise.

4.5 Summary and conclusion

This study aimed to develop the array of literature on the effects of acute vigorous exercise on platelet function and is the first to compare both basal and exercise induced platelet function in healthy Irish adolescents and adults. Results showed minimal basal differences between MLF and HF adolescents whereas MF adults had a significantly higher platelet count than HF adults. Acute exhaustive exercise increased platelet function in adults and adolescents, and this increase was more pronounced in moderate to low fit adolescents. The same effect of exercise in the HF adults and adolescents was not observed, possibly due to adaptive mechanisms from habitual physical activity and exercise.

It appears there is a collective proinflammatory milieu of all blood cells after strenuous exercise but nonetheless platelets are important players in this response. The results suggest this hyperreactive haemostatic state is much more pronounced in subjects with a lower CRF. Our results support past studies highlighting the different effect of CRF on platelet function in adults with novel findings showing more pronounced changes occurring in adolescents. Those with a low CRF level are at increased risk of thrombotic events following maximal exercise. These findings highlight the need to develop a strategy to increase activity amongst adolescents and adults to combat the early development of subclinical CVD.

In conclusion, this study demonstrates that acute, strenuous aerobic exercise enhances the thrombotic tendency depending on the individuals CRF level. CRF is an independent and reproducible quantitative measure reflecting physical activity patterns and may better mirror the adverse consequences of a sedentary lifestyle than adiposity measures.

Chapter Five

Chapter outputs

- Physical inactivity resulted in significant increases in platelet count, plateletcrit and platelet adhesion in healthy males
- Physical inactivity resulted in a slight elevation in aggregation, platelet reactivity index (PRI) and microvesicle concentration in healthy males
- Physical inactivity resulted in altered expression of 15 protein biomarkers
- We identified a set of 22 'physical inactivity' related miRNA, with potential targets involved in pathways associated with platelet function

Contributions from others

- Olink Bioscience (Sweden) performed the Proseek Biomarker Assay on isolated plasma samples.
- Applied Biosystems (France) ran the RT-PCR microRNA assay using The miRNA profile was determined by RT-qPCR using Applied Biosystems OpenArray® plate technology on the QuantStudio™ 12K Flex Real-Time System.

Chapter Five: The effect of Physical Inactivity on Platelet Function

5.1 Introduction

Chapters 3 and 4 demonstrated that both regular physical inactivity (PI), otherwise known as sedentary behaviour, and low cardiorespiratory fitness (CRF), both negatively impact platelet function. In particular, physical inactivity has major health effects globally, contributing to the 4th leading cause of death. Evidence has shown that physical inactivity and sedentary behaviour have direct effects on CVD risk factors including obesity and hypertension (Prentice and Jebb, 2004). Despite attempts to encourage a physically active lifestyle, rates of inactivity have remained high with a staggering 68% of Ireland's population not meeting minimum guidelines for regular physical activity (WHO 2009; Healthy Ireland, 2015).

However, in contrast to the accumulating evidence supporting the benefits of regular exercise, relatively little is understood about the deleterious mechanisms underlying the physiological, cellular and molecular responses to PI, specifically with regard to platelet function. Experimental models to mimic physical inactivity can be achieved through various techniques. Reducing ambulatory activity from >10,000 steps to <2000 steps per day has previously been employed (Krogh-Madsen *et al.*, 2010). The European Space Agency (ESA), use ground-based models of microgravity (the condition in which people or objects appear to be weightless). One model is that of dry immersion (DI), frequently used to study the effects of spaceflight on human physiology in a precisely controlled environment. DI involves immersing a subject in a bath of thermoneutral water covered by a waterproof fabric for a specific time period (Coupe *et al.,* 2013).

Several factors act simultaneously on the human body during immersion, including hydrostatic compression, supportlessness and extensive physical inactivity. Hypokinesia and hypodynamia are the major characteristics of physical inactivity induced by dry immersion. Hypodynamia involves a reduction in postural muscle load, whereas hypokinesia is a decline in motor activity. For these reasons, DI has been well accepted as a valuable tool to study physical inactivity (Widlansky, 2010). Subjecting twelve healthy men to three days of DI presented a unique opportunity to analyse the specific effects of physical inactivity on platelet and related biomarkers. ***Therefore, the aim of this study was to examine the effect of three days of acute physical inactivity on platelet function.***

5.1.2 Chapter aims and experimental approach

Hypothesis:

It was expected that the 3 days of Dry Immersion would be sufficient to induce the physiological effects of microgravity. In particular, the support withdrawal by dry immersion should induce alterations at a higher rate than bed rest.

We hypothesised that three days of physical inactivity, induced by DI would result in increases in platelet activation, adhesion and aggregation, as well as inflammatory processes. Furthermore, as microRNA (miRNA) expression patterns likely influence platelet function, we further hypothesised that physical inactivity would alter the platelet miRNA profile.

Main aims:

- The main aim of the study was to investigate the effects of 3 days of Dry immersion, acting as model of acute physical inactivity on platelet function – Platelet indices, Impact-R analysis and VASP phosphorylation in 12 healthy males.
- Investigate changes in cardiovascular and inflammatory related protein biomarkers, and microvesicle levels, in platelet-poor plasma as biomarkers of platelet activation.
- A secondary aim was to assess the clinical aspects (adverse events, comfort of the subjects etc.) of the study
- Establish a shortlist of the most highly expressed microRNA in platelets
- Determine if the miRNA profile of platelets is altered with physical inactivity by way of DI. Elucidate changes in miRNA profile pre- and post-immersion
- Discover biomarkers of platelet function induced by sedentariness/physical inactivity in healthy males.

5.1.3 Study design

A detailed overview of the experimental design was provided in Chapter 2 (Section 2.2.5.4). Briefly, 12 healthy French males participated in the study. All subjects provided informed consent in compliance with the Helsinki Declaration. The experimental protocol was approved by the local ethics committee (CPP Sud-Ouest Outre-Mer I, France) and the French Health Authorities. The study was organised by the Institute for Space Medicine and Physiology (MEDES) Toulouse, France. The subjects were selected based on a clinical investigation consisting of a detailed medical history, physical examination, an electrocardiogram, general blood screening, and urine analyses. Participants were free from muscular or neurological pathologies.

General dry Immersion method (outlined in detail in Chapter 2)

In brief, DI involves immersing the subject, who is covered with a unique elastic waterproof fabric, in a large tank of thermoneutral water (Figure 5.1). The subject, suspended in the water mass, remains dry, protected by the waterproof film. The fabric is thin and large enough to guarantee that the water's hydrostatic pressure is equally distributed over the body surface, generating conditions similar to the complete lack of structural support experienced in microgravity.

Figure 5.1: The dry immersion method. The left figure shows a subject in a dry immersion bath used in the study and the right figure depicts the supportlessness and lack of structure experienced in the bath.

Blood samples

Blood samples were drawn by a nurse in MEDES, at 7am in the morning after an overnight fast. Blood sampling in pre and recovery periods was also performed after the subject had rested in the supine position for 30 min. The division of blood samples is outlined Table 5.1

Table 5.1: Division of blood during the three day DI study. 10 millilitres of blood for platelet work was collected on BDC-3 (pre), R+0 (post) and R+1 (recovery).

Stage	**Baseline data collection**				**Dry Immersion**			**Recovery**	
Day	**-x**	**-3**	**-2**	**-1**	**1**	**2**	**3**	**0**	**1**
Blood volume (mls)	9	32	32	111	18	111	34	18	55
Platelet allocation (mls)		10						10	10

Complete blood count analysis

Complete blood counts were performed on every sample after blood draw, using the Sysmex XN-3000^{TM} Haematology system. Haematological and platelet indices parameters were obtained this way. Calculation of percent changes in plasma volume were calculated from changes in HGB and HCT using the Dill and Costil method (Dill and Costil, 1974). $DPV\,(\%) = 100 \times [HbB\,(1 - 0.01Hcti)]/\,[Hbi\,(1 - 0.01HctB)] - 100.$

Platelet function analysis

Platelet function (adhesion and aggregate size) was measured by Impact- R Cone device 1-hour post blood draw.). Platelet function was also measured by the assessment of platelet VASP phosphorylation. Platelet VASP phosphorylation was measured by flow cytometry and a standardised $P2Y_{12}$/VASP kit (Biocytex, Stago, France) according to the manufacturer's instructions.

Protein biomarker analysis

Platelet poor plasma (PPP) samples were sent to Olink Bioscience (Upsalla, Sweden) to measure the expression of 184 cardiovascular/inflammatory protein biomarkers using unique Proximity extension assay (PEA) technology. Protein biomarkers were measured each time point during the DI.

MicroRNA analysis

Platelet miRNA profiles were assessed at the pre and post DI time points. Total leukocyte-depleted RNA from platelets was extracted using the miRVANA RNA extraction kit. The miRNA profile was determined by RT-qPCR using Applied Biosystems OpenArray® plate technology on the QuantStudio™ 12K Flex Real-Time System. For a complete miRNA profile, 754 human miRNAs were quantified using two panels of 384 miRNA. The A panel contains miRNA targets which are highly expressed and tend to be functionally defined, whereas miRNA targets on the B panel are usually less abundant and not as well functionally defined.

Microvesicle analysis

PPP samples were analysed by the Nanosight NS300 to determine microvesicle size and concentration at each time point. This device employs Nanoparticle Tracking Analysis (NTA) technology using the properties of both light scattering and Brownian motion to analyse particles in liquid suspension. Mean ± SD output for the concentration, mean size and SD of the sample are provided from the NS300 software after sample analysis.

5.2 Results

5.2.1 Effect of dry immersion on physiological characteristics

Table 5.2 shows the characteristics of the study population. Heart rate (HR) and blood pressure (BP) were continuously monitored throughout the experiment. HR rose significantly at post and subsequently decreased significantly at recovery. Body weight during immersion decreased significantly by approximately 1.3 kg (Figure 5.2).

Table 5.2: Characteristics of the study population. BMI - Body mass index, VO_2 max – Aerobic capacity.

Characteristic	Mean ± SD
Age (yrs)	31.75 ± 4.81
Weight (kg)	74.54 ± 7.21
Height (m)	1.78 ± 0.07
BMI (kg/m^2)	23.48 ± 1.54
VO_2max (ml/kg/min)	38.75 ± 3.98

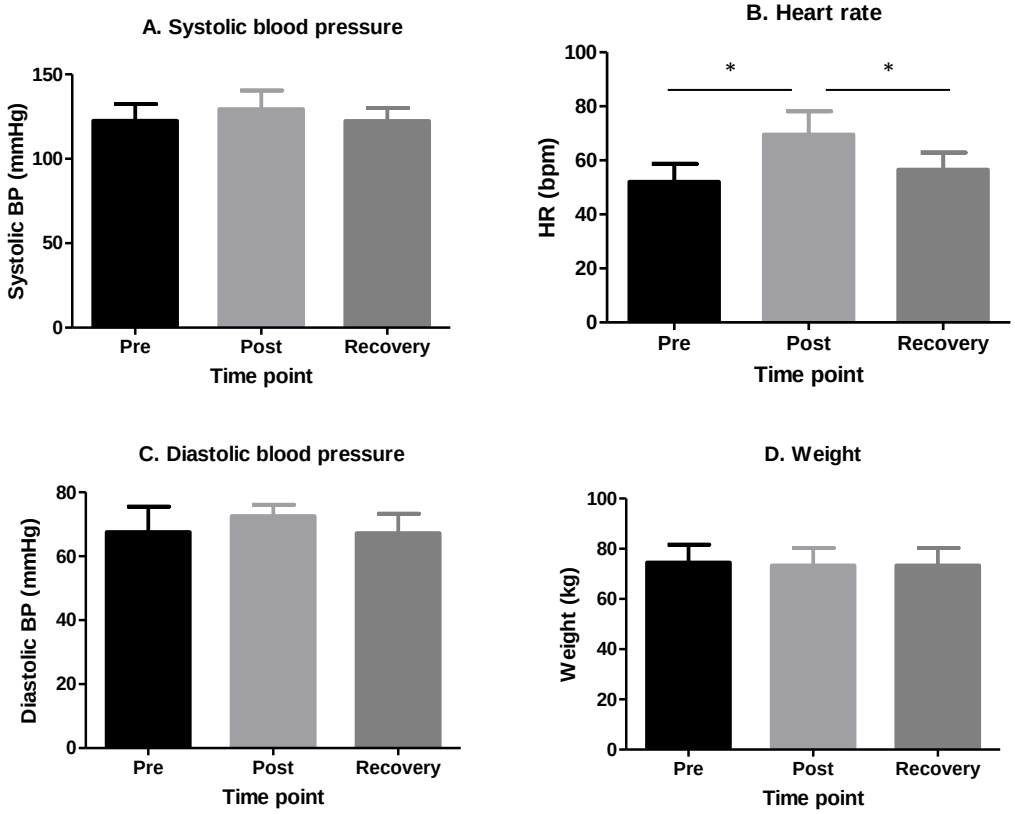


Figure 5.2: Changes in heart rate, blood pressure and weight during the dry immersion. Graphs represent the mean ± SD of each parameter at each time point. A – Systolic blood pressure, B – Diastolic blood pressure, C - Heart rate and D – Weight. * P<0.05. Paired samples t-test and repeated measures ANOVA adjusted for Age, BMI and VO_2).

5.2.2 Effect of physical inactivity on haematological characteristics

Changes in haematological parameters in response to DI were measured (Figure 5.3). There was a significant increase in WBC, RBC concentration, HGB and HCT at the post time point. There was a significant decrease in WBC, RBC concentration and HCT between post and recovery, almost to basal levels.

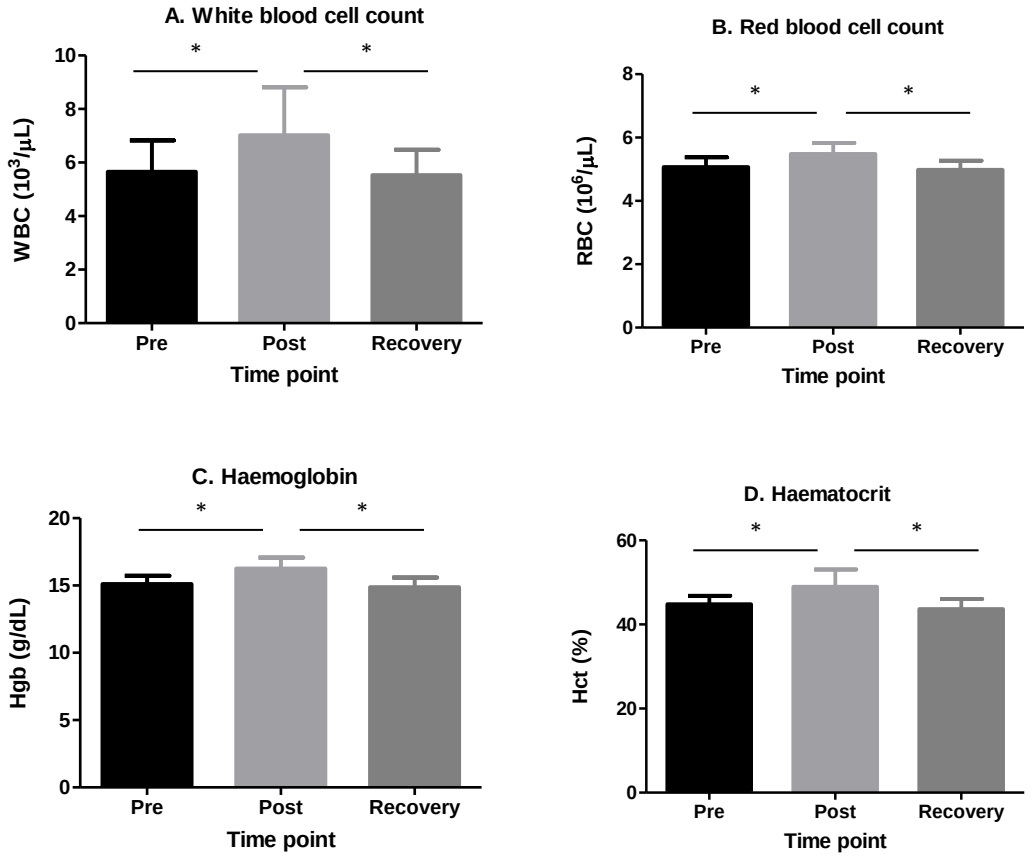


Figure 5.3: Effect of dry immersion and physical inactivity on RBC and WBC characteristics. Graphs represent the mean ± SD of each parameter at each time point. A – White blood cell count, B – Red blood cell count, C – Haemoglobin and D - Haematocrit. * P<0.05. Paired samples t-test and repeated measures ANOVA (adjusted for age, BMI and VO_2).

5.2.3 Effect of physical inactivity on platelet indices

The effect of DI on various platelet indices, as a marker of platelet activation, was also evaluated. There were no changes in platelet large cell ratio, mean platelet volume and platelet distribution width between any time points. There was a significant increase in platelet count and plateletcrit from pre to post immersion and subsequently, a significant decrease in these parameters from post time point to recovery (Figure 5.4).

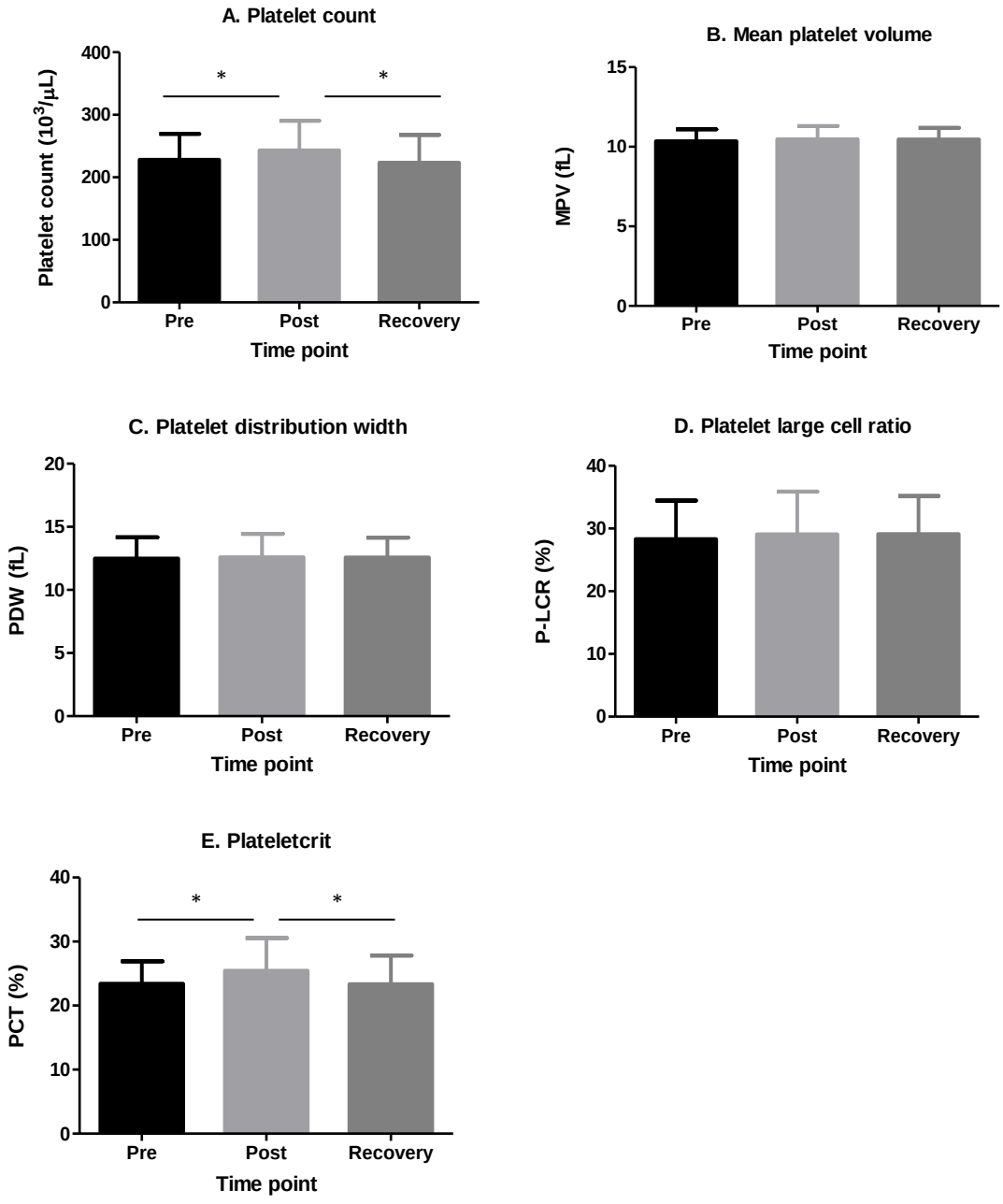


Figure 5.4: Effect of dry immersion and physical inactivity on platelet indices. Graphs represent the mean ± SD of each parameter at each time point. A – Platelet count, B – Mean platelet volume, C – Platelet distribution width, D – Platelet large cell ratio and E – Plateletcrit. * $P<0.05$. Paired samples t-test and repeated measures ANOVA (adjusted for age, BMI and VO_2).

5.2.4 Effect of physical inactivity on platelet function

Platelet adhesion and aggregation were measured by Impact R analysis pre, post and at recovery. Figure 5.5 and 5.6 show the effect of physical inactivity on platelet function. There was a significant increase in platelet adhesion post-immersion and a subsequent decrease between the post and recovery time points. There was also a significant increase in platelet aggregation from pre to post DI.

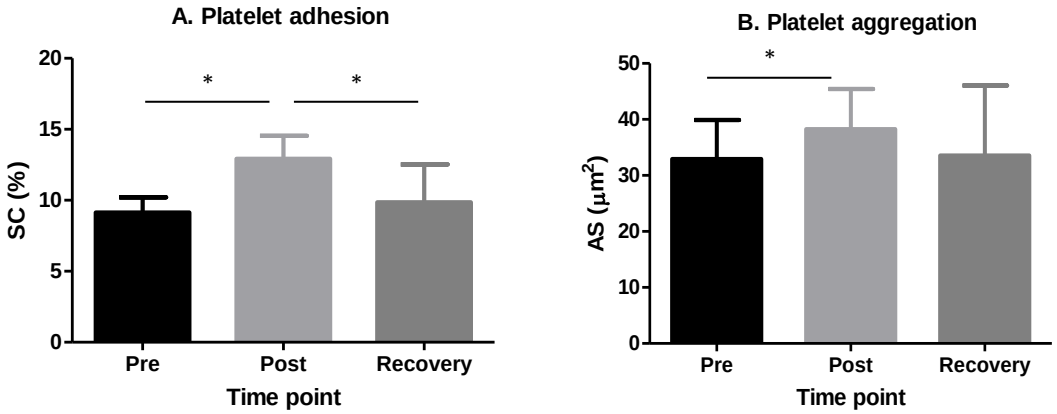


Figure 5.5: Effect of dry immersion on platelet function assessed by Impact R analysis. Graphs represent the mean ± SD of each parameter at each time point. A – Platelet adhesion and B- Platelet aggregation. * P<0.05. Paired samples t-test and repeated measures ANOVA (adjusted for age, BMI and VO_2).

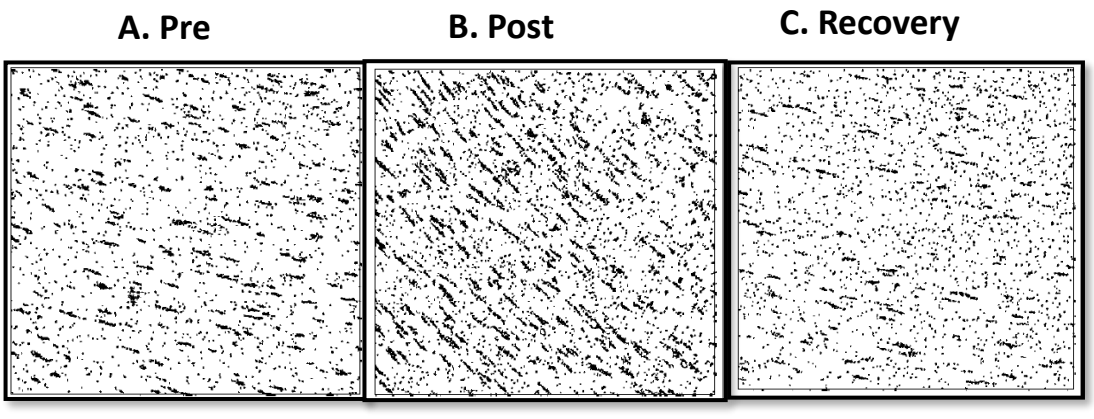


Figure 5.6: Impact R images from subject J at each stage of dry immersion. Image shows platelet adhesion and aggregation at A – pre immersion, B –post immersion, and C –at the recovery time point.

5.2.5 Effect of physical inactivity on platelet VASP phosphorylation

Platelet VASP phosphorylation is frequently employed as a marker of platelet activation. To assess if basal platelet VASP phosphorylation was affected by physical inactivity, the standardised $P2Y_{12}$/VASP kit was used in a flow cytometry assay. The platelet reactivity index (PRI) represents changes in VASP phosphorylation. There was a small insignificant increase in PRI post DI (Figure 5.8). There were individual fluctuations in VASP phosphorylation during the DI.

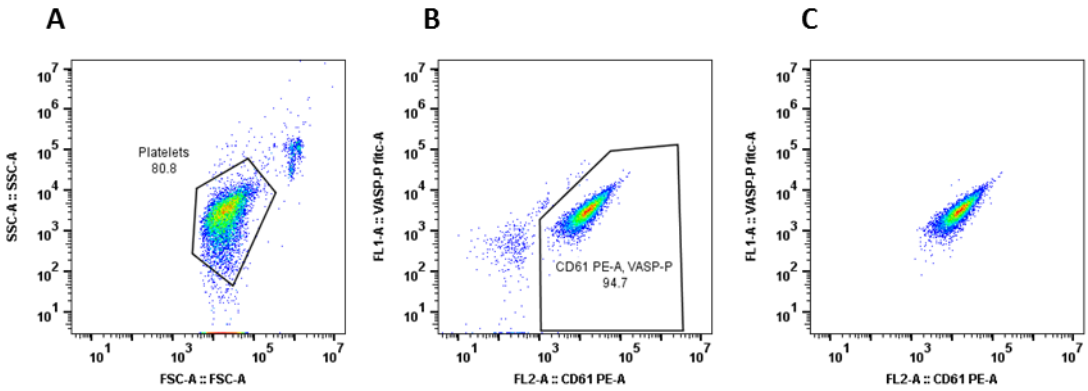


Figure 5.7: Gating strategy for the analysis of platelet VASP phosphorylation. Platelets were gated by side and forward scatter (A) and then by expression of CD61 (B). VASP-P phosphorylation was quantified by median and geometric mean fluorescence intensities (C). All data was analysed with Flowjo software.

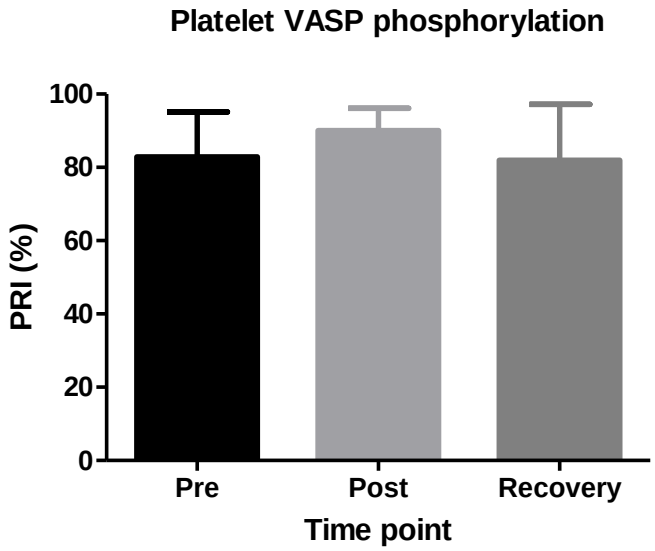


Figure 5.8: Effect of dry immersion on overall platelet VASP phosphorylation. Graph represents the mean ± SD of the PRI at each time point. PRI = Platelet reactivity index. Paired samples t-test and repeated measures ANOVA (adjusted for age, BMI and VO_2).

5.2.6 Effect of physical inactivity on protein biomarker expression

Protein biomarker expression of platelet poor plasma (PPP) was assessed using the Cardiovascular and Inflammatory protein biomarker panels (Olink Bioscience). Biomarker overlap between panels resulted in simultaneous evaluation of the expression levels of 157 protein biomarkers in each sample. The levels of 15 proteins differed significantly between time points (Table 5.3). Labelled in red are proteins discussed in this chapter. Results are expressed as normalised protein expression (NPX) on a log2 scale. Therefore, a normalised increase of 1 is equal to a two-fold increase in protein amount. Protein changes are shown in Figure 5.9 and 5.10.

Table 5.3: Protein biomarkers which were differentially expressed after the DI. The column on the left shows proteins from the CVD panel and the column on the right shows proteins from the inflammation panel**.** Highlighted in red are proteins discussed in this chapter.

Protein Biomarker Panel							
Cardiovascular panel				**Inflammatory Panel**			
Protein name	**Main function**	**Unique gene symbol**	**Change in expression post DI**	**Protein name**	**Main function**	**Unique gene symbol**	**Change in expression post DI**
Adreno-medulin	Vasodilation and regulation of hormone secretion	ADM	↓	Axin-1	Negative regulator of the WNT signalling pathway	AXIN1	↑
Dickkopf related protein-1	WNT signalling pathway inhibitor	DKK1	↑	Interleukin-6	Pro-inflammatory cytokine	IL6	↑
Heat shock protein-27	Stress resistance, actin organization	HSPB1	↑	STAM-binding protein	Cytokine-mediated signalling	STAMBP	↑
Lectin like oxidised LDL receptor-1	Binds, internalises, degrades oxidized LDL	OLR1	↑	Sulfotransferase 1A1	Catalyse the sulfate conjugation of hormones and neurotransmitters	SULT1A1	↑
NF-Kappa B essential modulator	Activation of Inflammatory, immune genes	IKBKG	↑	Sir-2 like protein	Unknown but possible role in epigenetic gene silencing	SIRT2	↑
Renin	Activation of angiotensinogen pathway	REN	↑	Matrix metalloproteinase-10	Degradation of extracellular matrix	MMP10	↓
Proto-oncogene non receptor tyrosine kinase	Regulation of cell growth	SRC	↑	Matrix metalloproteinase -3	Degradation of extracellular matrix normal	MMP3	↓
Tissue plasminogen activator	Disintegration of blood clots	PLAT	↑				

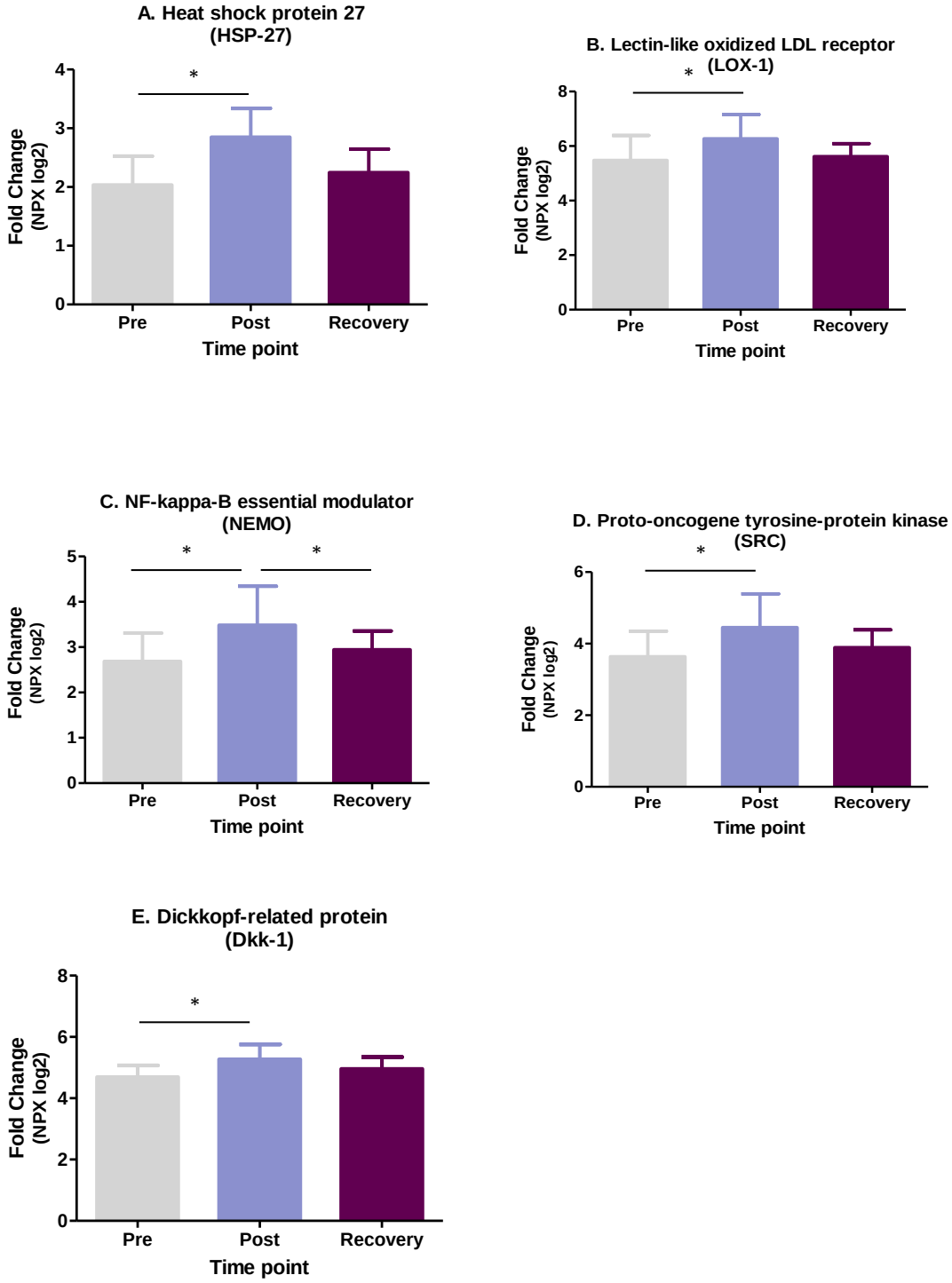


Figure 5.9: The effect of dry immersion on plasma cardiovascular protein biomarkers. The graphs represent mean ± SD of the subjects at time points pre, post and recovery. A – Heat shock protein 27, B – Lectin like oxidised LDL receptor, C – NF-Kappa-B essential modulator, D – Proto-oncogene tyrosine protein kinase and E – Dickkopf related protein. * P<0.05. Paired samples t-test and repeated measures ANOVA (adjusted for age, BMI and VO_2).

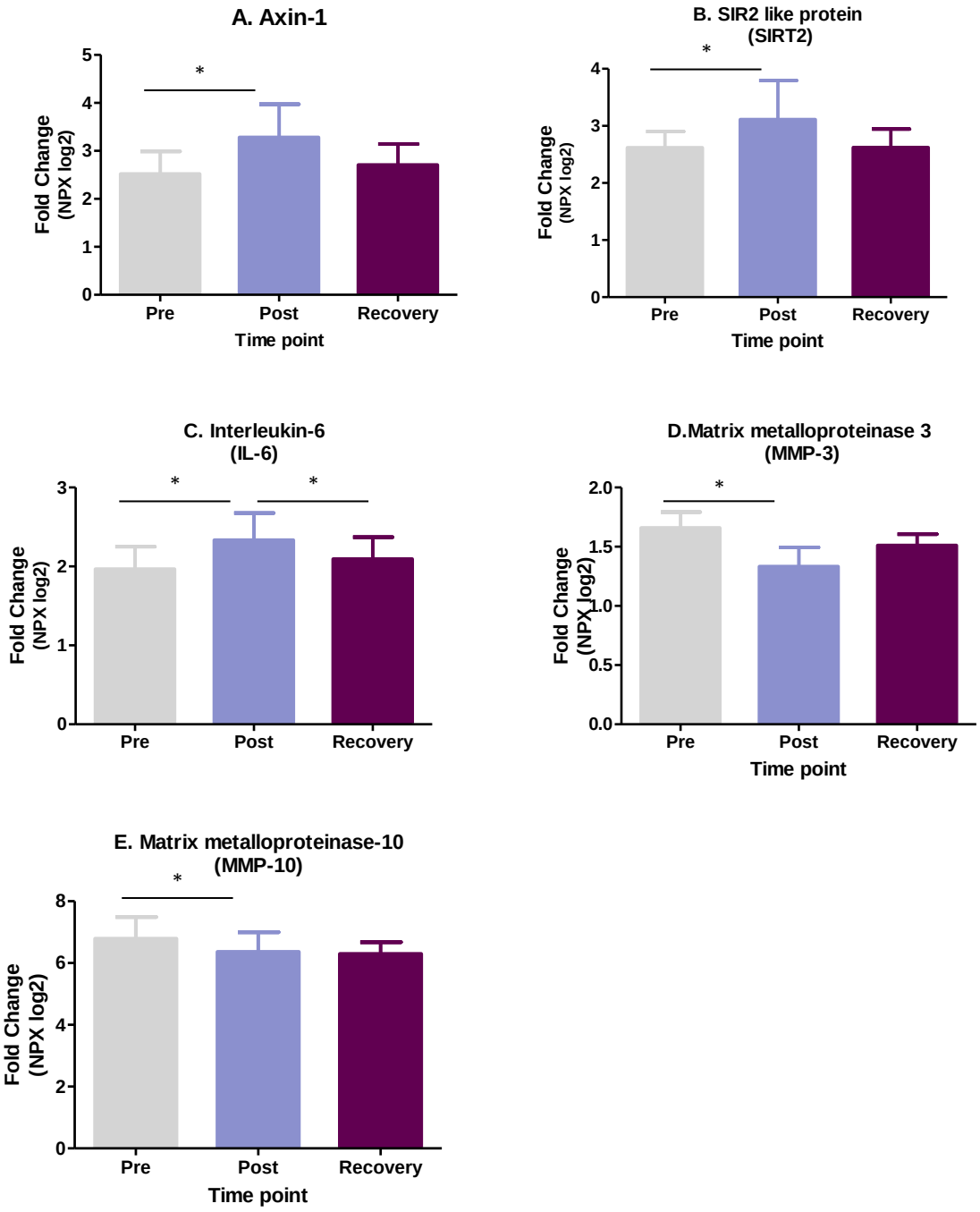


Figure 5.10: Effect of dry immersion on inflammatory protein biomarkers. Graphs represent the mean ± SD. A- Axin-1, B – Sir2 like protein, C - Interleukin 6, D – Matrix metalloproteinase 3 and E – Matrix metalloproteinase 10. Axin and MMP-3 were specific to INF panel while Il-6, SIRT-2 and MMP-10 were also found on the CVD panel. * $P<0.05$. Paired samples t-test and repeated measures ANOVA (adjusted for age, BMI and VO_2).

5.2.7 Effect of physical inactivity on platelet poor plasma microvesicles

Platelet poor plasma (PPP) samples were analysed by Nanosight technology to determine MV size and concentration at each time point. There was a decrease in the average MV size and an increase in average MV concentration at post DI. There was a significant decrease in MV size standard deviation after the DI (Figure 5.11). For further analysis, MVs were divided into three categories: Exosomes, microparticles and larger microparticles with modest increases in each category after DI, however the changes were not statistically different.

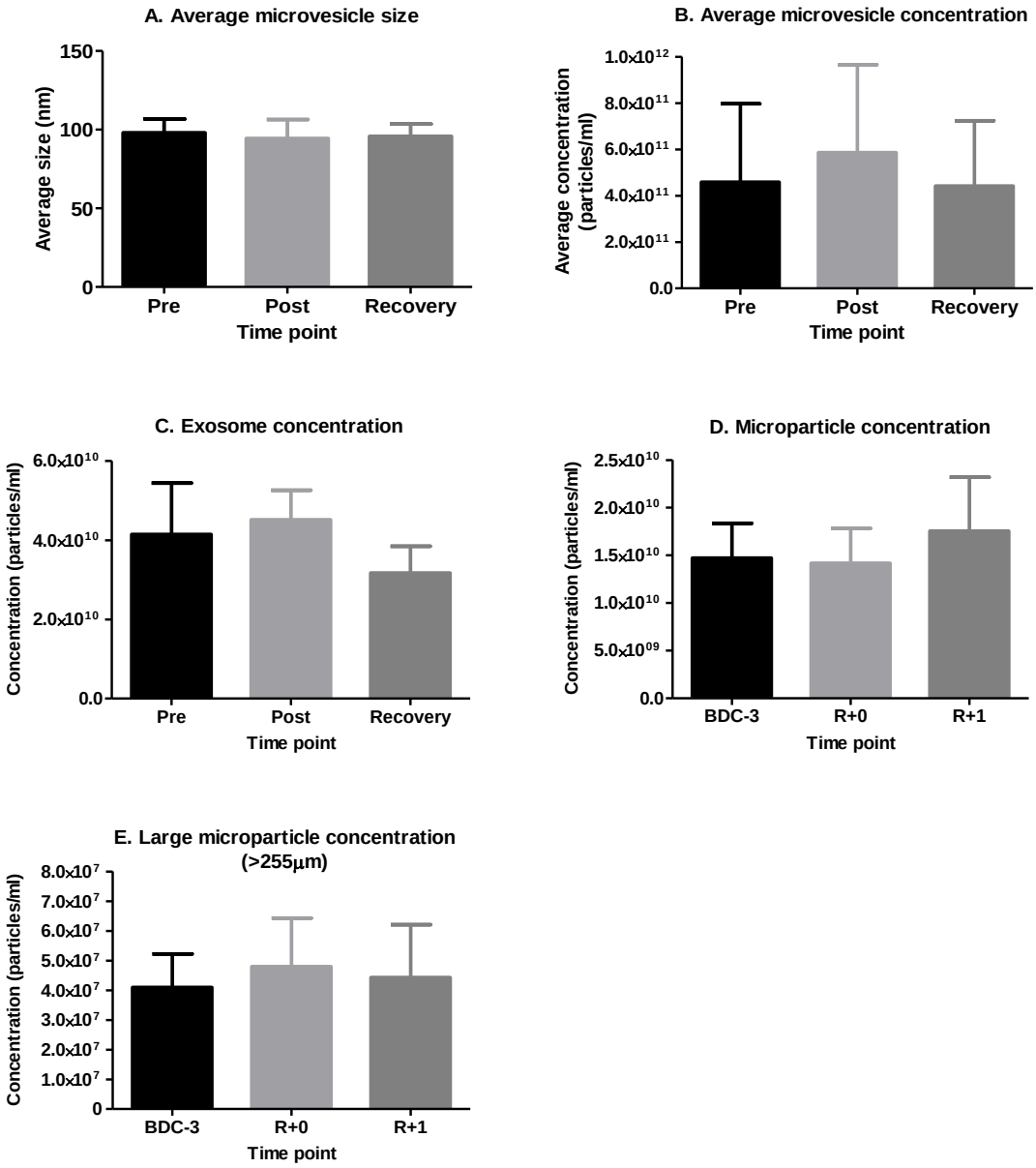


Figure 5.11: Effect of physical inactivity on microvesicle size and concentrations. Graphs represent the mean ± SD of each parameter at each time point. A – Average microvesicle size, B – Average microvesicle concentration, C - Exosome concentration, D – Microparticle concentration and E – Large microparticle concentration. * $P<0.05$. Paired samples t-test and repeated measures ANOVA.

5.2.8 Effect of physical inactivity on platelet microRNA expression

miRNA expression profiles were analysed from non-normalized leukocyte-depleted platelet RNA (ranging from 3.5 to 7.0 ng) extracted from equal volumes of platelet rich plasma from 8 of the 12 subjects at the time points pre and post immersion. Western blots of miRNA regulatory proteins are shown in Figure 5.12 (A) and the number of detectable miRNA after the DI is shown in part (B). Heat maps comprised of the most highly expressed platelet miRNA (miRNA across all subjects with the lowest CT value) were then constructed for both the A and B panel of miRNA. This allowed visualisation of basal miRNA expression profiles between subjects (pre DI) by comparing the expression of each subject's miRNA targets to that of a randomly selected subject, subject C. This is shown in Figures 5.13 and 5.14. A shift in colour from red to blue indicates decreasing expression of that miRNA compared to subject C.

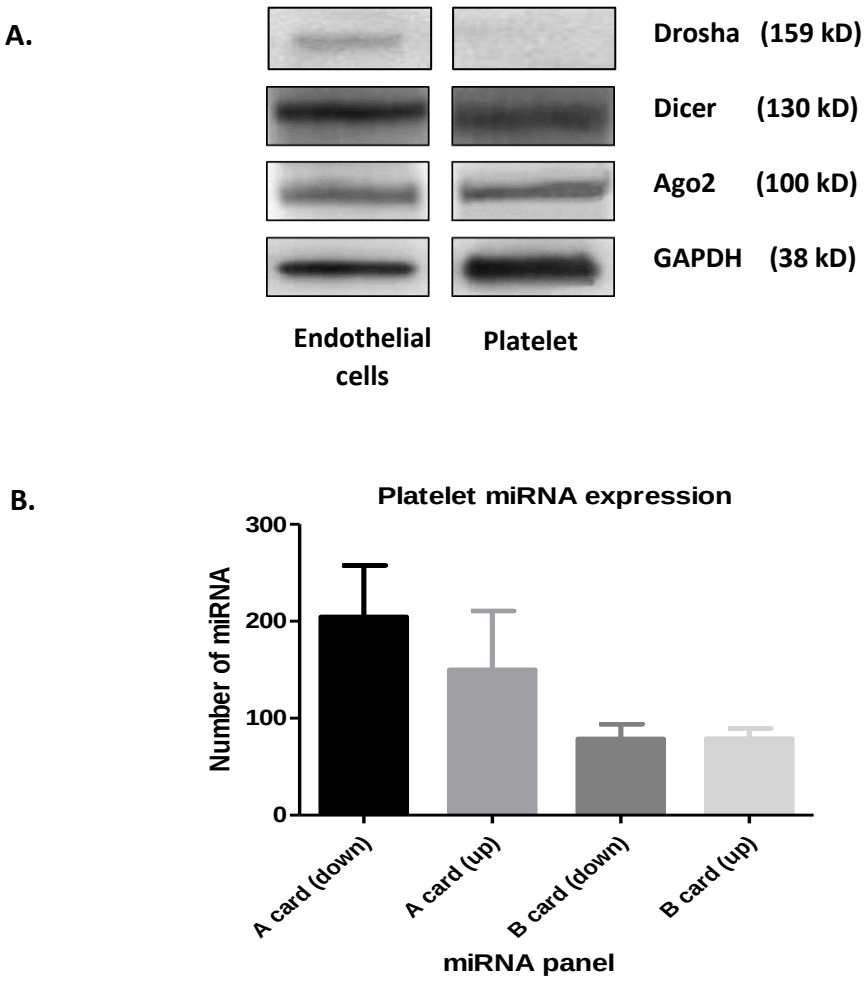


Figure 5.12: Confirmation of miRNA processing machinery in human platelets and miRNA numbers during the dry immersion study. Graphs represent the mean ± SD of each parameter. A – shows the presence of regulatory miRNA components Dicer and Argonaute 2 in platelets compared to endothelial cell controls. Western blot gels were loaded with 25µg of endothelial or platelet protein. B – shows the number of miRNA either up or downregulated after the dry immersion on the A and B panel.

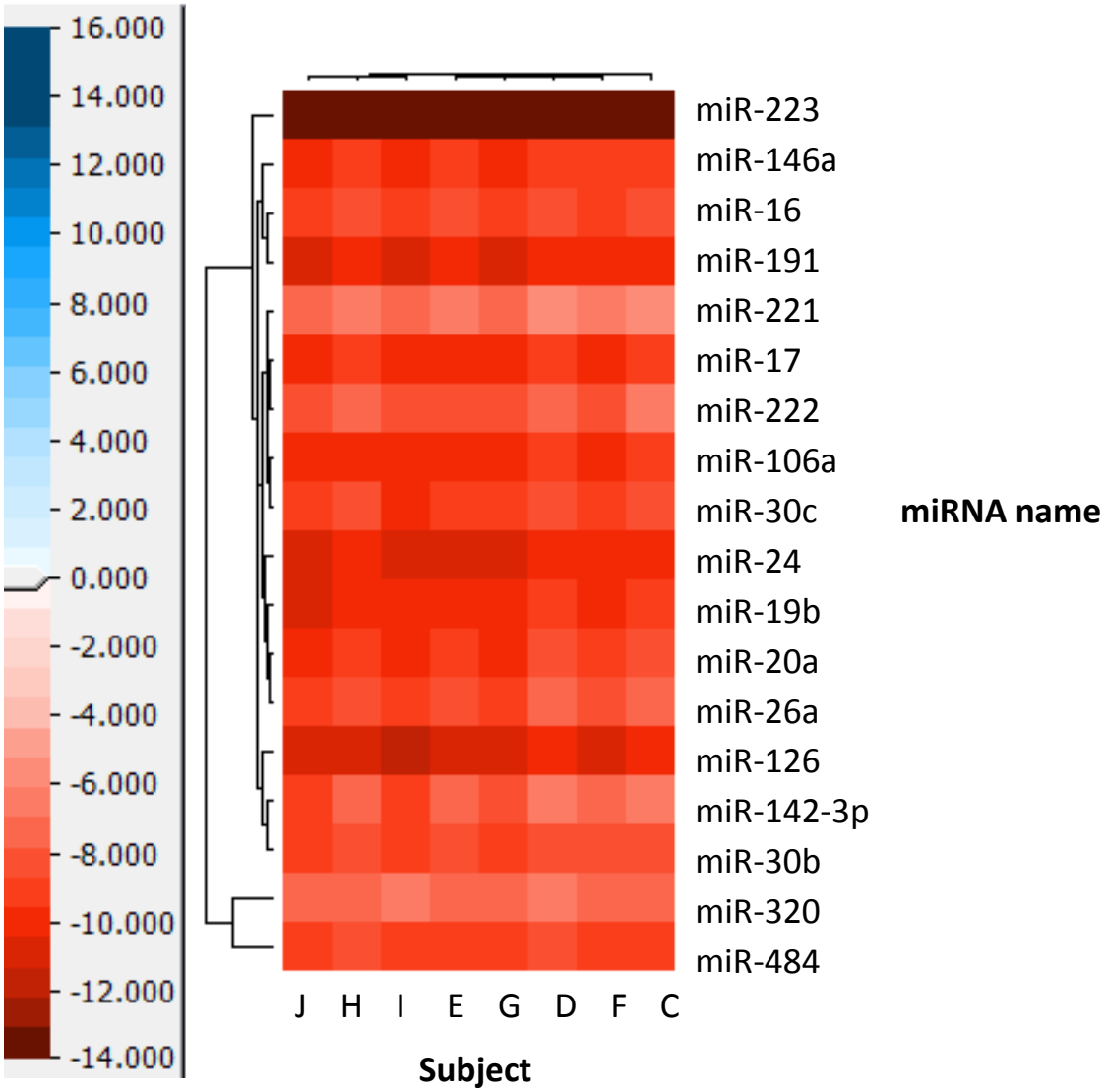


Figure 5.13: Heat map showing the most highly abundant A panel platelet miRNA before DI. Each miRNA is listed on the right hand side and the subjects are listed on the bottom of the panel. The colour key is shown on the left. A change in colour from dark red to blue indicates a decrease in miRNA expression levels compared to the miRNA target expression of subject C.

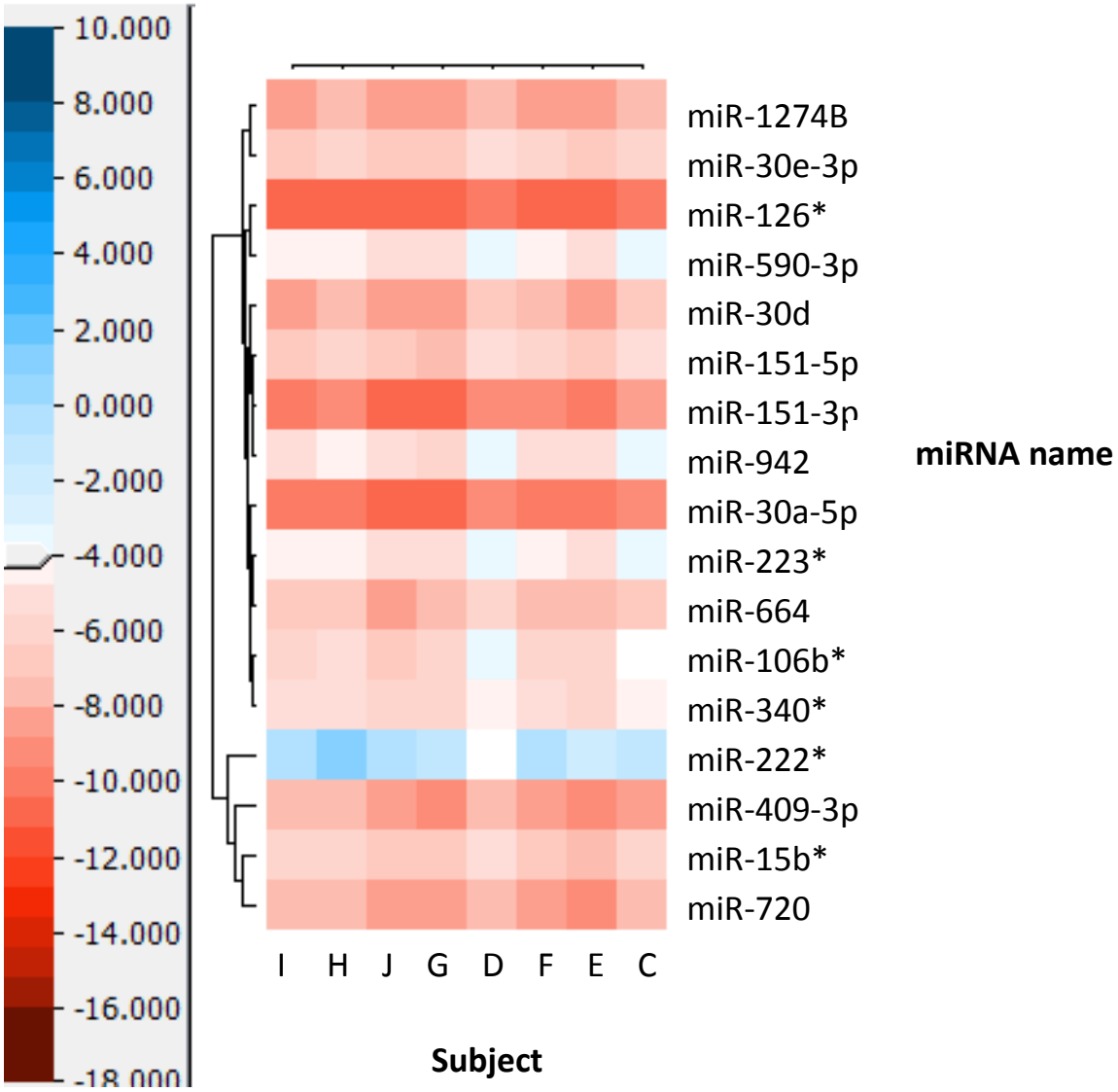


Figure 5.14: Heat map showing the most highly abundant B panel platelet miRNA before DI. Each miRNA is listed on the right hand side and the subjects are listed on the bottom of the panel. The colour key is shown on the left. A change in colour from dark red to blue indicates a decrease in miRNA expression levels compared to the miRNA target expression of subject C.

5.2.8.1 Identification of miRNA affected by physical inactivity

By comparing the miRNA expression profiles of the subjects between pre and post dry immersion, we identified 22 significantly differentially expressed miRNA with a fold change of more than 1.2 (12 of which were upregulated and 10 which were downregulated). Most reported miRNA fold changes are small (~ 1.5 fold) (Daniels *et al.*, 2014). The miRNA that were differentially expressed on the A card are shown in Figure 5.15, parts A and B, while the miRNA differentially expressed on the B card are shown in the same Figure, parts C and D.

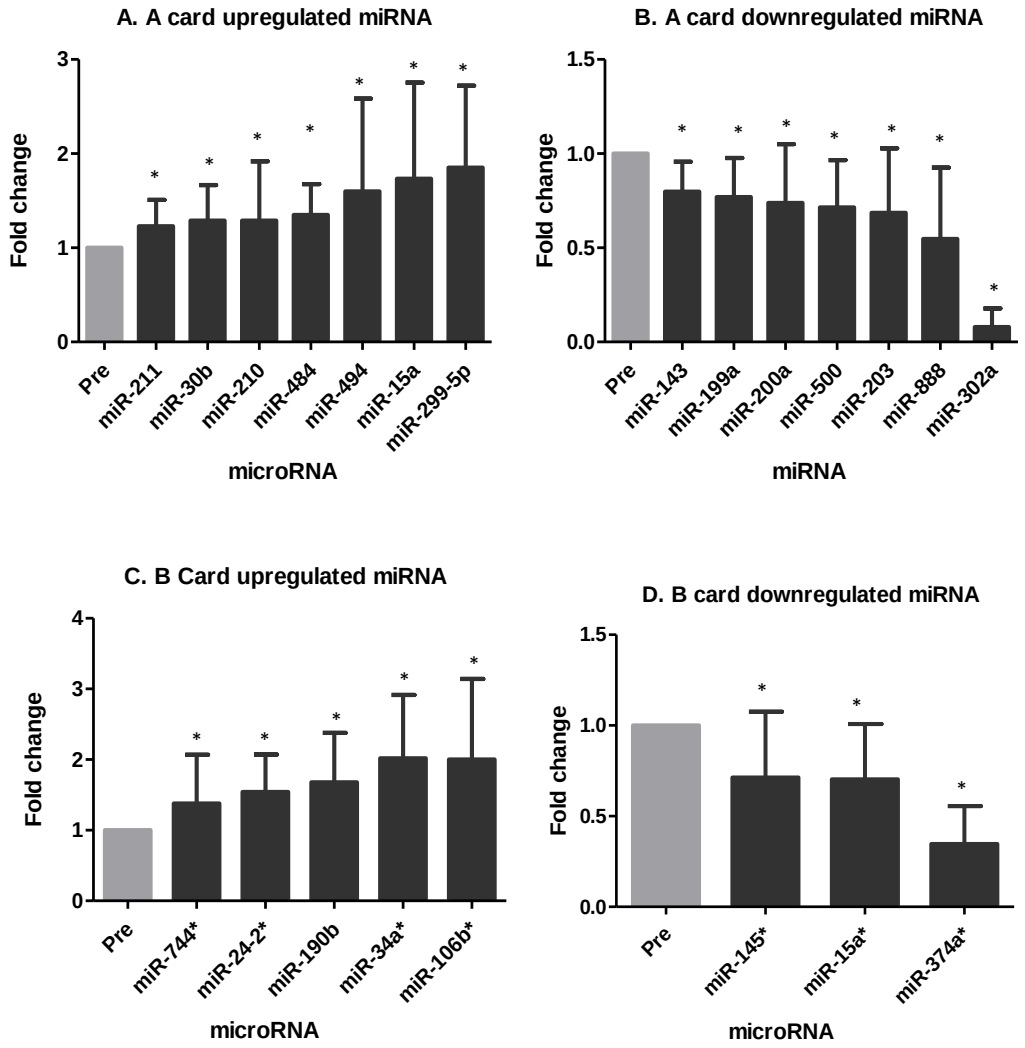


Figure 5.15 Differentially expressed miRNA at the post dry immersion time point. miRNA profiles were assessed only at the pre and post time points. This figure shows miRNA which were differentially expressed at the post compared to pre time point. All data are expressed mean + SD. Graph A shows miRNA that were up regulated after DI on the A card, and graph B shows miRNA that were downregulated after DI on the A card. Graph C shows B card up regulated miRNA, whilst graph D shows B card downregulated miRNA. * $P<0.05$. Paired samples t-test and repeated measures ANOVA.

5.2.8.2 Bioinformatic analysis of differentially expressed miRNA

To extrapolate biological meaning from miRNA that were significantly up or downregulated after the DI, bioinformatics was performed. This involved determination of putative targets using online software tools including Targetscan and microRNA.org. Following this, involvement of these targets in pathways of interest from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) were analysed using the DAVID bioinformatics database. To help visualise the regulatory potential of each miRNA we examined, a table was constructed illustrating the number of potential genes the miRNA could target and the number of KEGG pathways the predicted targets were part of (Table 5.4).

Table 5.4: Bioinformatics of differentially regulated miRNA. Table of miRNA regulated by DI from the A and B panels. Table shows the number of *in-silico* predicted conserved targets of each miRNA, in addition to the number of GO biological processes and KEGG pathways that these miRNA are putatively involved in.

	miRNA	**Fold change direction**	**Conserved targets**	**KEGG Pathways**
A card				
	miR-143	↓	497	24
	miR-199a	↓	631	16
	miR-200a	↓	896	18
	miR-500	↓	185	8
	miR-203	↓	964	32
	miR-888	↓	3350	36
	miR-302a	↓	1022	32
	miR-30b	↑	6957	14
	miR-211	↑	2809	46
	miR-210	↑	4046	38
	miR-484	↑	2696	26
	miR-15a	↑	3213	33
	miR-299-5p	↑	279	1
	miR-494	↑	620	28
B card				
	miR-145*	↓	891	22
	miR-15*	↓	1508	44
	miR-374a*	↓	4289	24
	miR-744*	↑	3696	22
	miR-24-2*	↑	741	18
	miR-190b	↑	224	10
	miR-34a*	↑	4289	22
	miR-106B*	↑	698	21

Table 5.5: Involvement of differentially regulated miRNA in KEGG cell pathways. Bioinformatics of differentially regulated miRNA**.** Table shows KEGG pathways common to a number of differentially expressed miRNA and which are involved in platelet function.

KEGG pathway			
Wnt signalling	**Regulation of Actin cytoskeleton**	**ECM receptor interaction**	**Toll like receptor**
miR-888	miR-143	miR-143	miR-143
miR-299a-5p	miR-199a	miR-302a	miR-15a
miR-484	miR-500	miR-199a	miR-34a*
miR-199a	miR-302a	miR-484	
miR-200a	miR-374a		
miR-500	miR-34a*		
miR-203	miR-744		
miR-302a	miR-494		
miR-190b	miR-15a*		
miR-15a*	miR-106b*		
miR-145			
miR-106b*			
miR-34a*			
miR-24-2			
miR-211			
miR-15a			
miR-494			

5.2.8.3 KEGG pathway analysis

Maps of key pathways involved in platelet function and activation (Figures 5.16-5.18) were downloaded from the KEGG database using DAVID. Pathways were chosen based on their involvement in platelet function and signalling, specifically in adhesion and aggregation, but also their inflammatory potential. Included in these were the Wnt signalling pathway, regulation of actin cytoskeleton, ECM interaction and Toll-like receptor pathway, of which the Wnt signalling pathway appeared repeatedly in both the up and downregulated miRNA targets. Genes within the pathways that were predicted targets for the miRNA were circled. Genes that were potential targets for multiple miRNA were circled red, genes that were targets for a single miRNA were circled yellow. Potential miRNA targets of genes involved in the Wnt signalling pathway are shown in Figure 5.16, potential miRNA targets of genes involved in the reorganisation of the actin cytoskeleton pathway are shown in Figure 5.17 and potential miRNA targets of genes involved in the ECM receptor-interaction pathway are shown in Figure 5.18.

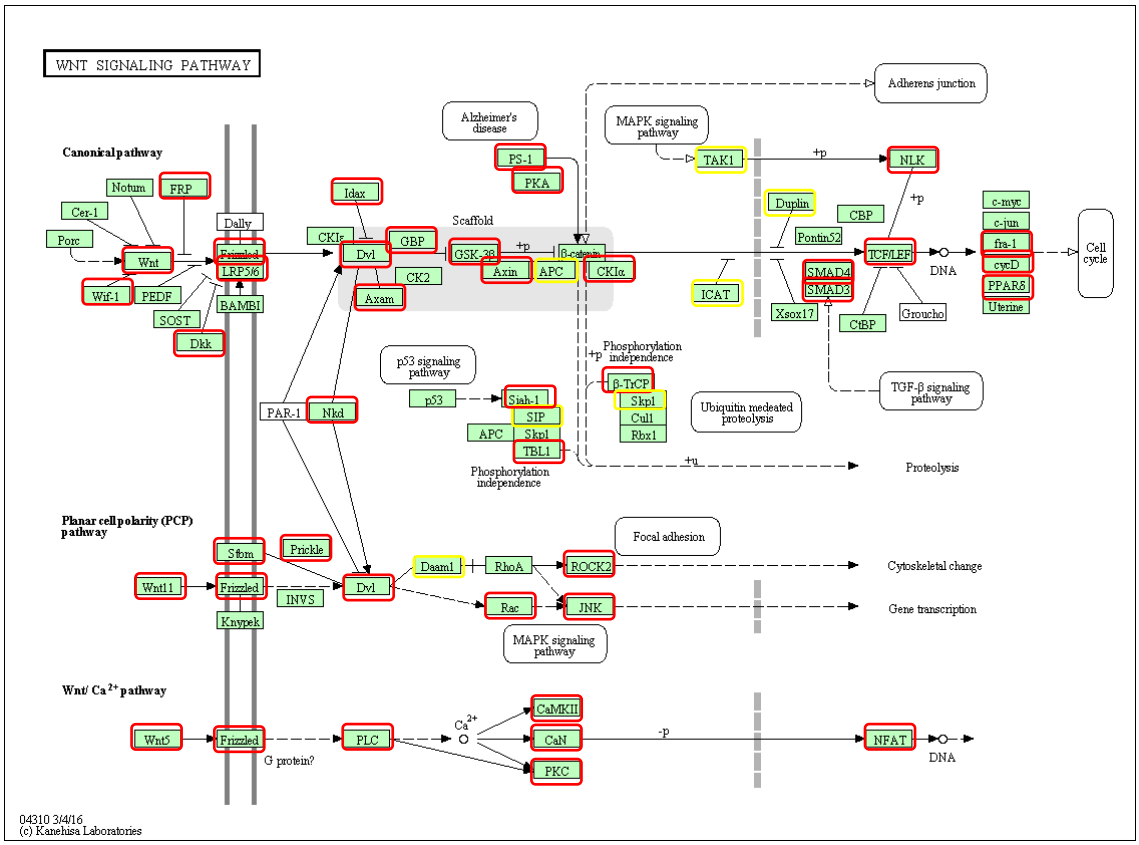


Figure 5.16: KEGG map of the Wnt Signalling pathway. Wnt signalling negatively regulates platelet function and miRNA targeting genes in this pathway were relevant to this study. Genes circled in red are predicted targets for multiple miRNA, and genes circled in yellow are predicted targets of single miRNA.

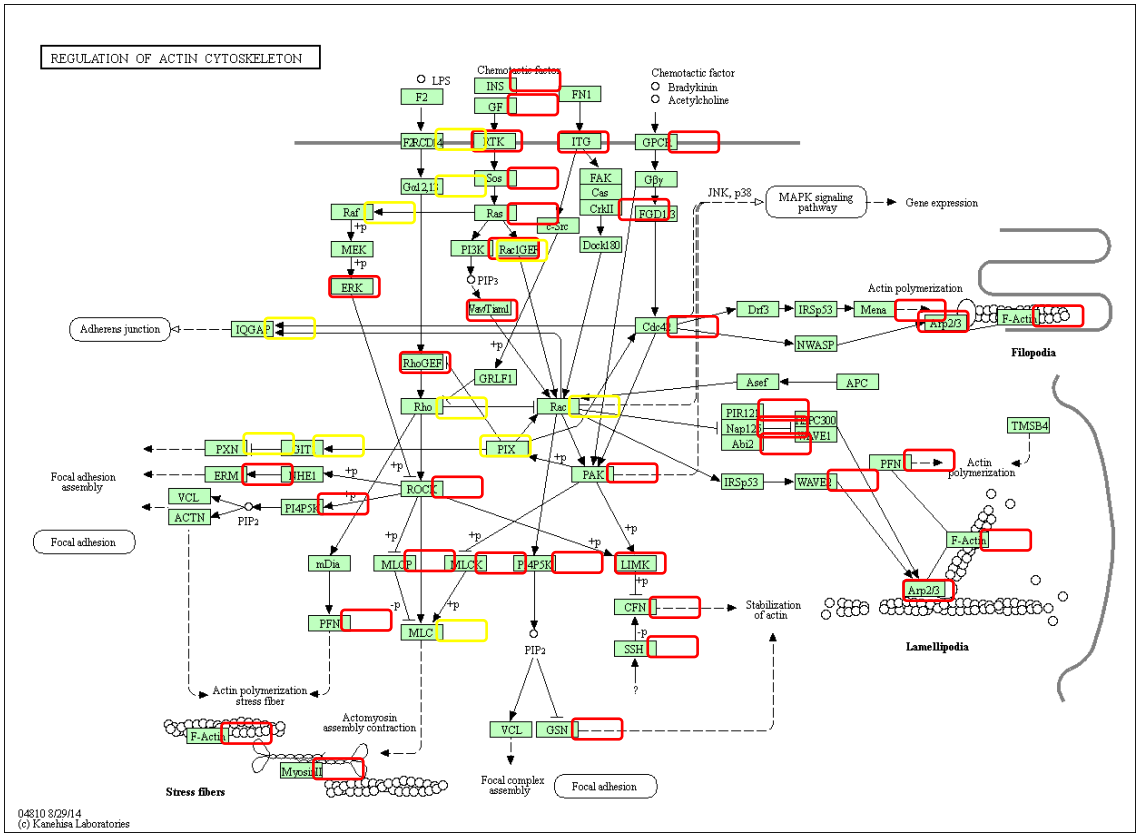


Figure 5.17: KEGG map of the regulation of actin cytoskeleton pathway. Reorganisation of the actin cytoskeleton is a key event in platelet activation and adhesion, and therefore this pathway was of interest with regard to platelet function. Genes circled in red are predicted targets for multiple miRNA, and genes circled in yellow are predicted targets of single miRNA.

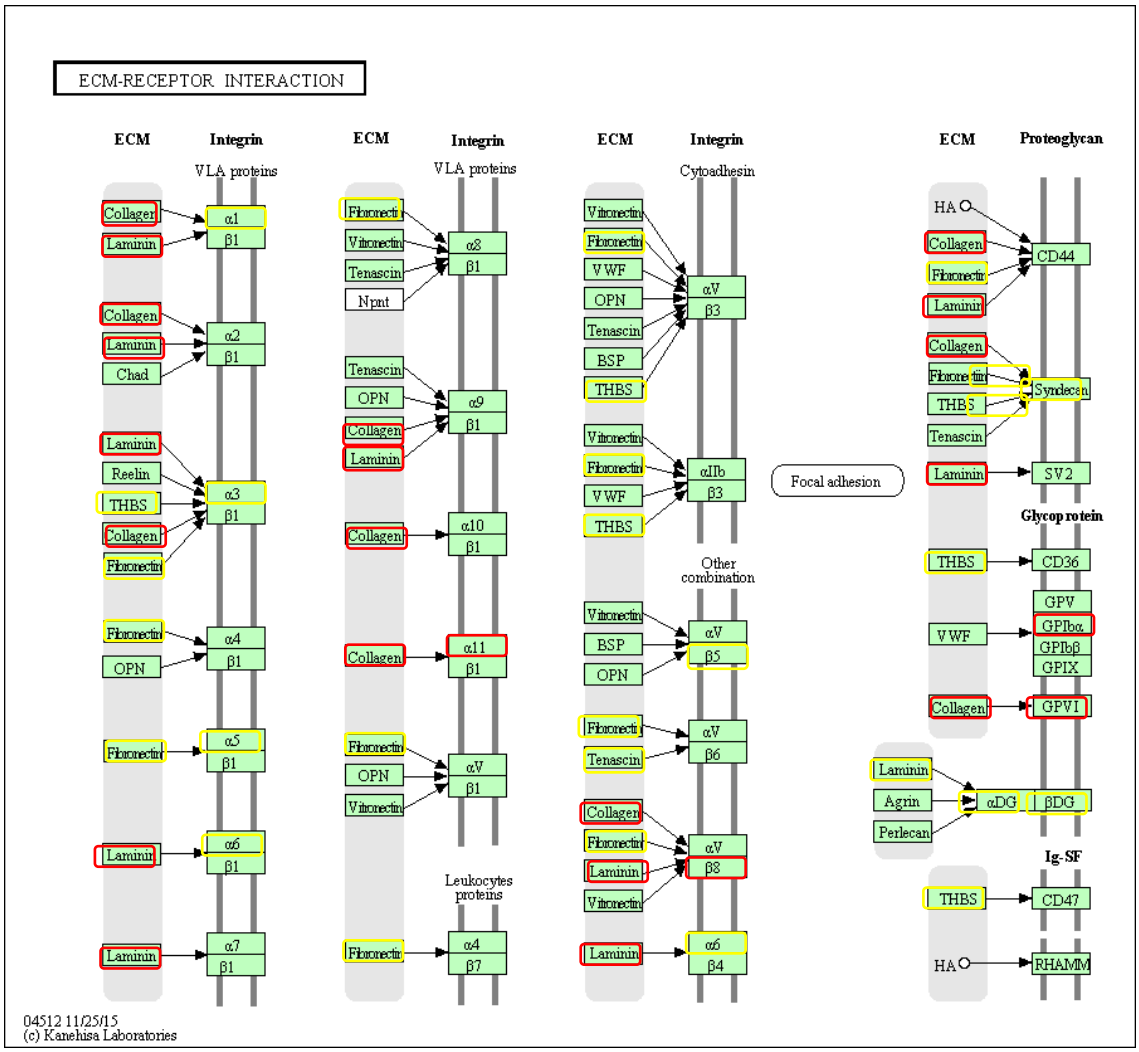


Figure 5.18: KEGG map of the ECM – receptor interaction pathway. Platelets express 5 different integrin's, which facilitate platelet adhesion to ECM proteins including collagen, laminin and fibronectin, amongst others, during platelet adhesion. This pathway was therefore chosen as it was relevant to platelet adhesion functions. Genes circled in red are predicted targets for multiple miRNA, and genes circled in yellow are predicted targets of single miRNA.

5.2.6.3.1 miRNA targets of the Wnt signalling pathway

The Wnt signalling pathway involved the largest number of differentially regulated miRNA post DI, as shown previously in Table 5.5. The protein biomarkers Axin1 and DKK1 were also differentially expressed post DI. Their gene targets can be regulated by multiple miRNA, which, together can affect gene expression and protein expression. We used online databases to determine additional miRNA which could target Axin1 and DKK1 (Figure 5.19). The differentially expressed miRNA are shown with an asterisk above them, while other potential targets do not have an asterisk. The combined action of multiple down or upregulated miRNA could have affected the gene and subsequent protein expression of Axin1 and DKK1.

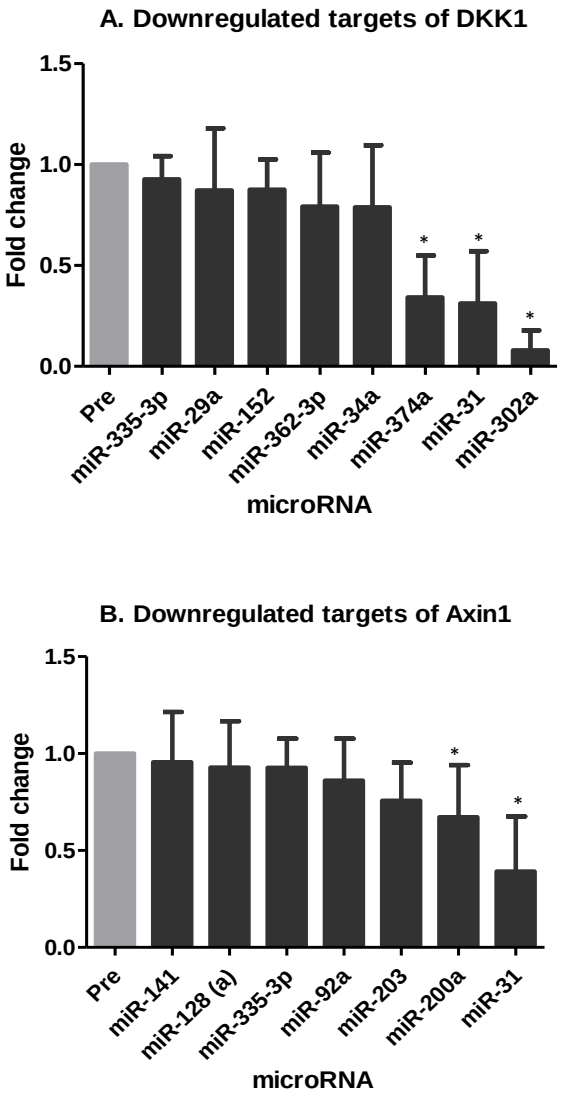


Figure 5.19: Potential miRNA targets of genes involved in the Wnt signalling pathway. All data are expressed mean ±SD. Graph A shows miRNA which are potential targets of DKK1 and graph B shows downregulated miRNA which are potential targets of Axin-1.

5.3 Discussion

Despite the strong links between physical inactivity and CVD risk (Prasadi and Das 2009), there remains a relative scarcity of experimental investigation into the effects of physical inactivity in healthy subjects, making it difficult to quantify the cellular and molecular effects of physical inactivity. Using the DI method, this study allowed us a unique opportunity to investigate the effects of physical inactivity on platelet function.

Exposure to microgravity induces modification of all physiological systems, and in particular the cardiovascular system. DI is characterised by enforced physical inactivity (Grigor'ev *et al.,* 2004). During DI, the muscle atrophy, bone mass loss, cardiovascular deconditioning and other alterations mimic the adaptation observed in astronauts. CV deconditioning is a state whereby the CV system does not react efficiently to challenge, distinguished by a reduced capability for exercise, increased resting heart rate, orthostatic intolerance and tachycardia (Coupe *et al.,* 2009). Initial reactions to DI occur in the first 12 hours and are caused by immediate modifications in body fluid distribution and a removal of support structure (Naviaslova *et al.,* 2011). Primary responses include haemodynamic and water-electrolyte changes (Larina *et al.*, 2011).

We aimed to investigate the effects of physical inactivity on the cellular and molecular aspects of platelet function using a number of techniques. The main finding of this chapter was that physical inactivity by way of DI induced subtle / modest changes in platelet function and platelet biomarkers in healthy men. The degree of changes in parameters of platelet function was found to vary among individuals. These changes were characterised by a rapid onset. Twenty-four hours of normal physical activity appeared sufficient to reverse signs of cardiovascular impairment and to restore plasma volume and platelet activation markers to near basal levels. Evaluation of platelet poor plasma protein and platelet microRNA expression before and after the DI resulted in identification of 15 proteins, which were differentially expressed (DE) after the DI and 22 miRNA, which were DE expressed after the dry immersion.

5.3.1 Characteristics of the study population

The study included a group of 12 French healthy male volunteers with a mean age of 31.75 ± 4.38 years and mean BMI of 23.48 ± 1.54 kg/m^2 (Table 5.2). Aerobic fitness measured by VO_2 max had an average of 38 ml/kg/min. During immersion, the subjects remained in the supine position for all activities, except for hygiene procedures, which were performed in a lateral position. Overall, the 3-day DI was well tolerated by the study group with no drop-outs. However, all subjects experienced moderate back pain at the beginning, as shown in the VAS scale (Appendix C). Analysis of cortisol levels in DI studies usually indicates an increase on the first day of DI, reflecting emotional stress (Naviaslova *et al.,* 2011).

5.3.2 Effect of physical inactivity on physiological characteristics

Figure 5.2 displays changes in physiological parameters during the DI. Body weight decreased by approximately 1kg at the end of the DI. Other studies have reported weight loss of approximately 1kg at the end of a 3-day DI (Iwase *et al.,* 2000) and 2kg at the end of a 7 day DI (Larina *et al.,* 2008; Naviaslova *et al.,* 2011). Grigorev and Shulzhenko (1979) state that the weight loss is due to increased fluid loss. A reduction in lean body mass but an increase in adipose tissue was shown by BIA analysis in a 5-day DI in 14 healthy males (Noskov *et al.,* 2014).

We observed an increase in systolic and diastolic blood pressure after the DI, with decreases in both parameters after the recovery period, suggesting a quick cardiovascular adaptation upon return to normal activity. Most studies have not found significant changes in resting blood pressure after 3-7 days of DI (Iwase *et al.,* 2000, Bravyi *et al.,* 2008). Other authors have described 15-20% increases in diastolic BP after 7 days (Vil-Viliams and Shulzhenko, 1980). There was a significant increase in Heart Rate (HR) post DI and a significant decrease between the post and recovery stages (Figure 5.2). This data agrees with other studies which reported increased HR after DI (Pavy-Le Tron *et al.,* 2007; Coupe *et al.,* 2013).

5.3.3 Effect of physical inactivity on haematological parameters

Figure 5.3 highlights changes in red and white blood cell parameters before, after and at the recovery time point of the DI. Both spaceflight and its proxies result in an initial plasma volume decrease (approximately 10-15% on the first day) after which it remains stable (Navasiolava *et al.,* 2010; Larina *et al.,* 2008). We noted a 14 % decrease in plasma volume post DI and a 4% increase at the recovery time point. Blood viscosity usually increases due to the decrease in plasma volume. With the reduction of plasma volume in space, corresponding decreases in RBC is required to maintain blood viscosity (Watenpaugh *et al.,* 2016). However, hemoconcentration was reflected by an increase in RBC post DI. There were also significant increases in HGB and HCT after the immersion, although these values remained within the normal limits for healthy individuals. These findings were expected; as lower plasma volume would result in increased concentrations of RBCs. In general, real and simulated microgravity results in changes to physical properties of RBCs (Naviaslova *et al.,* 2011)

Other groups have reported similar results after both simulated and real microgravity experiments. Both a 7-day DI (Ivanova *et al.,* 2011) and 5-day DI (Ivanova *et al.,* 2013) resulted in an increase in RBCs and altered morphological composition of red blood in healthy males. Navasiolava *et al.,* (2010) reported a significant increase in RBC, HGB and HCT after seven days of DI, which returned to normal after recovery. Unlike our study, they did not observe a significant difference in WBC. Bedendeeva *et al.,* (2009) noted a 40% increase in leukocytes after DI. Overall, most haematological indices appear to increase after DI.

5.3.4 Effect of physical inactivity on platelet indices

Similar to chapters 3 and 4, we investigated the effect of DI on all platelet indices as primary indicators of platelet activation and function. This is highlighted in Figure 5.4. There was an increase in platelet count (PLT) between pre and post time points, probably reflecting the loss of plasma volume during the DI. There was a significant decrease in PLT between post and recovery suggesting that platelet count returned to pre-immersion levels once subjects began to resume ambulatory activity. Navasiolava *et al.,* (2010) found no significant difference in PLT after 7 days of DI. Other studies have indicated a significant increase in PLT after 7 day DI (Kirichenko *et al.,* 1985, 1988).

There were no changes in MPV, PDW or PLCR after three days of DI. There was a significant increase in plateletcrit from pre to post and a significant decrease in plateletcrit from post to recovery. Plateletcrit appears to be one of the more sensitive platelet indice markers, as not only is it reflecting changes in this intervention, it also reflected changes in the exercise study. Overall, the combined increases in PLT and PCT platelet could be a reflection of the activation status of platelets after the DI.

5.3.5 Effect of physical inactivity on platelet function

The effect of physical inactivity on platelet function was quantified using the Impact R cone and plate analyser and results are shown in Figure 5.5. We observed a significant increase in platelet adhesion (SC) from pre to post immersion suggesting stronger platelet-surface interactions in response to physical inactivity and DI. Accordingly, there was a significant decrease in SC from post to recovery indicating platelet adhesion levels had returned to their basal state. There was a significant increase in AS from pre to post suggesting that PI results in elevated platelet aggregation with increased activation of the αIIbβ3 receptor and increased affinity for fibrinogen binding. Platelet aggregation decreased slightly from post to recovery. The reduction in blood volume as a consequence of reduced total body water in the body during microgravity has been suggested as a factor for thrombotic tendencies, which could have been a determinant of increased platelet activation in this study (Watenpaugh, 2001). Figure 5.6 highlights the changes (increases) in platelet adhesion and aggregation observed using the Impact R pre and post DI and following one day of recovery.

Physical inactivity can cause endothelial dysfunction (Moyna and Thompson, 2004; Di Francesco-Marino 2009) which can affect platelet function. The decrease in shear stress forces during physical inactivity and DI can negatively impair both endothelial and platelet function. Laminar shear stress exerts an atheroprotective effect on the endothelium (Pan, 2009) causing a release of vasoactive substances such as nitric oxide (NO) and prostacyclin, which negatively regulate platelet activation. In contrast, regions of low or oscillatory shear stress, such as that induced by DI, results in the procoagulant phenotype of endothelial cells (ECs) and are associated with atherosclerotic plaque development, attracting platelets and enhancing the thrombotic potential. Increase in soluble von Willebrand factor (vWF), tissue factor (TF) expression and activity and endothelial microparticle production can act as mediators to induce platelet activation under disturbed flow, inferring that altered shear stress may not activate platelets directly (Ruggeri *et al.,* 2009; Yin *et al.*, 2016).

Haemoconcentration, body fluid redistribution, hypodynamia experienced during DI may increase the risk of thrombosis and could also have contributed to enhanced platelet adhesion and aggregation observed after the DI (Naviaslova *et al.,* 2011). The increase in blood viscosity after 24 hours of DI could also have been a contributory factor to the platelet hyperaggregability (Ivanov *et al.,* 1983). This could be tested by the use of a viscometer and in parallel with platelet function tests could provide informative data (Kim *et al.,* 2000). Interestingly, Kuzichkin *et al.,* (2010) observed an increase in plasma fibrinogen concentration after short-term space flights and 7-day DI. Increased availability of plasma fibrinogen could influence platelet aggregation and assessment of fibrinogen levels and αIIbβ3 activation levels by flow cytometry would provide an insight into the increased platelet aggregation post DI (Vij, 2009).

A major symptom of CV deconditioning is the effect of DI on maximal oxygen consumption (VO_2 max). A decline in the adaptive ability to exercise has been reported after just one day of DI (Beliaev 1981) and in 3-7 days of DI VO_2 max falls by 10-18% (Anashkin and Beliav, 1982; Sonkin *et al.,* 1996; Vinogradova *et al.,* 2002 (NASA, 2012) and this could subsequently affect platelet function as work in chapter 4 showed that subjects with a lower CRF had a tendency for increased platelet reactivity.

To our knowledge, there have been no studies on the effect of DI on platelet function in humans to date. Some research on other experimental physical inactivity models has been carried out on platelet function. Arinell *et al.,* (2013) examined the effect of 60 days' head down bed rest (HDBR) on platelet activation markers P-Selectin and PDGF in 15 healthy females. While expecting platelet activation to be elevated, both platelet activation markers decreased during HDBR and remained at lower levels for 8 days in the recovery period.

5.3.6 Effect of physical inactivity on platelet VASP phosphorylation

A commonly used assay to assess platelet function is the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (Shumacher *et al.,* 2007). VASP is an intracellular regulator of actin dynamics in platelets and plays a key role in regulating platelet adhesion and aggregation. VASP is phosphorylated by cAMP and cGMP-regulated protein kinases on three phosphorylation sites (Ser157, Ser239 and threonine 278). VASP phosphorylation is paralleled with the inhibition of platelet activation, inhibition of αIIbβ3 and a restriction of VASP to bind to F-actin (Wentworth *et al.,* 2006). Consequently, decreased VASP phosphorylation can result in platelet hyperreactivity.

We examined the impact of physical inactivity on platelet reactivity and the VASP phosphorylation status. The results of the VASP test are reported as a percentage value of the platelet reactivity index (PRI) whereby increases in the PRI reflect elevated $P2Y_{12}$ mediated platelet reactivity (Gaglia *et al.,* 2011). Values obtained for PRI before and after DI in our subjects varied between 65 -95%. Normal PRI values for healthy individuals not on clopidogrel treatment are between 70-95% (Bagoly *et al.,* 2013) or between 69 -100% (Siller-Matula *et al.,* 2008) as non-treated patients exhibit high PRI results. After DI, while we observed a minor non-significant increase in the average PRI (5%) suggesting a reduction in VASP phosphorylation and increase in platelet activation. This is shown in Figure 5.8. However, there were individual fluctuations with some subjects showing greater degrees of changes.

As the VASP/$P2Y_{12}$ assay is primarily used to test responses to effectiveness of clopidogrel treatments, there is a scarcity of studies examining the relationship between obesity/physical inactivity and platelet VASP phosphorylation in healthy subjects. However, obesity is linked to elevated PRI levels (Gremmel *et al.,* 2013; Pankert *et al.,* 2014). Russo *et al.,* (2007) reported that VASP phosphorylation was significantly lower in obese subjects compared to healthy controls after platelets were treated with cyclic nucleotide analogs. This suggests that obesity, and possibly physical inactivity/sedentariness reduces the ability of cyclic nucleotides to inhibit platelet adhesion and aggregation resulting in platelet hyperactivity.

Furthermore, endothelial prostacyclin and NO inhibit platelet function. Their actions are mediated by platelet adenylyl and guanylyl cyclases which produce cAMP and cGMP to phosphorylate a number of proteins including VASP (Smolenski *et al.,* 2012). Reduced NO production in endothelial cells resulted in vascular inflammation and decreased phosphorylation of VASP at the Ser239 site in (Cheng *et al.,* 2014). A reduction in NO bioavailability would have downstream effects on platelet VASP phosphorylation, encouraging platelet adhesion and aggregation.

Smoking also induces a loss of sensitivity to prostacyclin and NO generated by the endothelium and has been characterised by elevated platelet αIIbβ3 levels and release of platelet microparticles (Pamukcu *et al.,* 2011). Assinger *et al.,* (2010) showed that VASP phosphorylation at basal levels on the Ser239 residue was significantly reduced in smokers. In response to picomolar and nanomolar concentrations of PGE_1, smokers still had reduced VASP phosphorylation, which was linked with elevated P-selectin expression. However, at maximal PGE_1 concentrations (hugely exceeding concentrations that can be achieved *in vivo*) they noted no difference between smokers and non-smokers. Similarly, using the VASP/$P2Y_{12}$ kit, PRI (which uses maximal effective doses of PGE_1) was virtually identical between the smokers and the non-smokers, suggesting that the assessment of VASP phosphorylation in the presence of submaximal quantities of PGE_1 could be more beneficial. Physical inactivity could produce similar responses and require the same level of investigation, as we only observed small decreases in VASP phosphorylation. Also, other platelet signalling pathways could have contributed to the elevated platelet adhesion and aggregation.

5.3.7 Effect of physical inactivity on protein biomarker expression

We also examined the effect of physical inactivity on platelet poor plasma (PPP) based protein biomarkers. The Proseek® Multiplex assay is a sensitive and specific assay developed for biomarker research which enabled the screening of 157 protein biomarkers related to CVD and inflammation using only 1µl of sample. The Proseek® assays use PEA technology, where samples are treated with matched antibody pairs tagged with DNA reporter molecules. Once the antibodies bind to their respective antigens, the corresponding DNA primers form an amplicon that is quantified by real time PCR, generating a measurable fluorescent signal which correlates with protein abundance (Figure 5.20) Problems with cross-reactivity are practically abolished in PEA assays as only matched DNA reported pairs are amplified during real-time PCR.

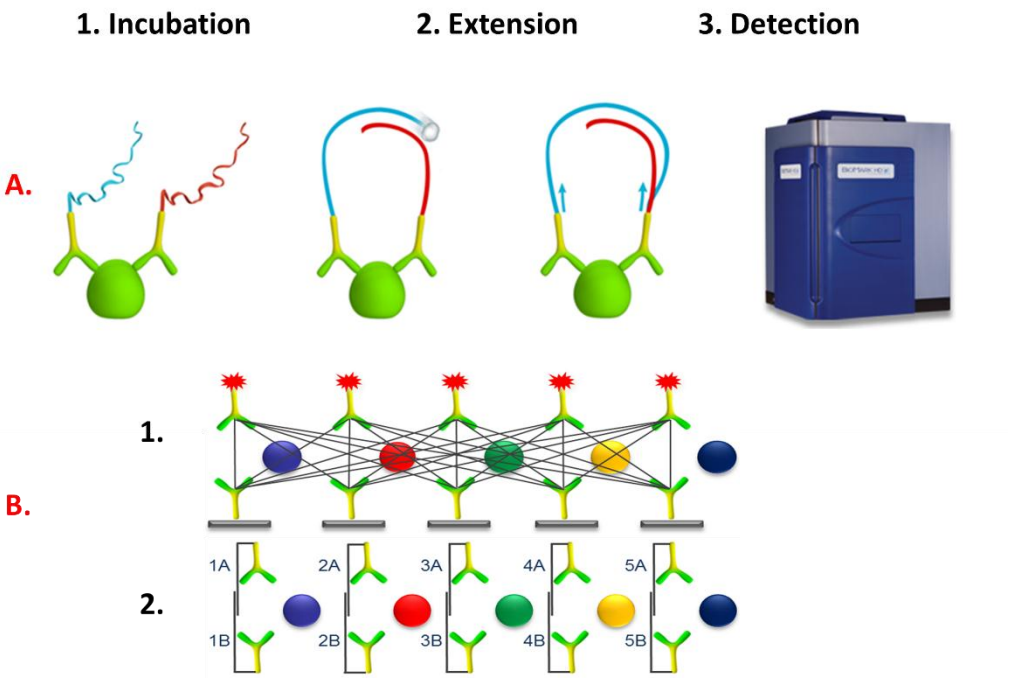


Figure 5.20: Novel Proseek Proximity Extension Assay. Part A shows (1) Proseek probes (DNA oligo-labelled antibodies) bind in proximity to target proteins (2) Only correct matched sequences hybridise and extension and creation of real-time PCR amplicons occur and (3) Proseek amplification and detection by real-time PCR on the BioMark HD system. Part B (1) represents conventional immunoassays where cross reactivity occurs due to unspecific binding of antibodies, thereby limiting the degree of multiplexing and (2) unique DNA oligo sequences report only matched DNA-pairs (e.g. 1A+1B) and cross reactive events are not identified.

The expression profiles of these proteins were analysed at pre, post and recovery. 131 out of 157 of the proteins were detected in all samples. We expected that a number of inflammatory and CVD proteins would be differentially expressed after the DI. As displayed in Table 5.3, the expression levels of 15 proteins differed significantly between different stages of the DI. For the purpose of this discussion, we focused on some key differentially expressed proteins related to platelet function. On the CVD panel, key proteins affected by physical inactivity included Heat shock protein 27 (HSP27), Lectin-like oxidised LDL receptor (LOX-1), NF-Kappa-B essential modulator (NEMO), Proto-oncogene tyrosine protein kinase (SRC) and Dickkopf-related protein (DKK1). The expression levels of these proteins are displayed in Figure 5.9.

Numerous environmental and physiological stresses such as inflammation cause the expression of heat shock proteins (HSPs) found in several cell types (Batulan *et al.,* 2016). HSPs have been identified in atherosclerosis (Majid *et al.,* 2012) and post exercise (Periard *et al.,* 2012). HSP-27 was significantly elevated after DI suggesting a stress response to the effects of acute physical inactivity. HSP27 has been proposed as a key player in actin polymerisation during platelet shape change (Zhu *et al.,* 1994; Polanowska-Grabowska and Gear, 2000). Studies by Tokuda *et al.,* (2015) show that HSP27 is released from human platelets upon collagen activation, and this release is associated with the acceleration of platelet aggregation. Kageyama *et al.,* (2013) suggest that the GTPase, Rac, controls this collagen-induced HSP27 phosphorylation. Enomoto *et al.,* (2011) also showed that ADP induces phosphorylation of HSP27 with resultant platelet activation markers PDGF and sCD40L release. Elevated HSP-27 post DI indicates that it was not in response to changes in temperature (as they remained consistent) but probably in response to the physiological stress of physical inactivity.

Lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) is a scavenger receptor, which binds to and degrades oxidised low-density lipoprotein (Reiss *et al.,* 2009). High plasma levels of LDL-cholesterol are a significant risk factor for atherosclerosis. LOX-1 is expressed on numerous cells including platelets (Chen *et al.,* 2001) but importantly is expressed in an activation dependent manner. LOX-1 was significantly elevated post DI (Figure 5.9). As LOX-1 recognises and binds to activated platelets, exposure of LOX-1 on the surface of activated platelets might encourage thrombus formation. Furthermore, inhibition of LOX-1 in platelets was shown to prevent platelet aggregation (Chen *et al.,* 2001; Marwali *et al.,* 2007). LOX-1 is associated with obesity and physical inactivity and could represent a marker of platelet activation in response to sedentary behaviour (Yan *et al.,* 2011).

DKK1 was another CVD panel protein that was significantly upregulated post DI. Platelets represent a major source of circulating DKK1, which is an antagonist of the Wnt signalling pathway (Ueland *et al.,* 2009). DKK1 is released from platelet α-granules (Voorzanger-Rousselot *et al.,* 2009) upon activation.

Plasma DKK1 levels are significantly higher in disease states including T2DM (Lattanzio *et al.,* 2014) and atherosclerosis (Ueland *et al.,* 2009). Increased levels of DKK1 were associated with urinary levels of TXB_2 and plasma CD40L, suggesting that DKK1 levels could echo platelet activation. DKK1 can also influence platelet-mediated endothelial cell activation involving the Wnt/β-cat signalling pathway and necrosis factor-KB pathways.

The role of platelets in atherosclerosis is not only related to their prothrombotic properties but also their inflammatory properties (Morrell *et al.,* 2014) and platelets exert inflammatory effects on a scale that exceeds their individual size. A number of proteins on the inflammatory array were also differentially expressed post DI. These are shown in Figure 5.10 and included Axin-1, Interleukin-6 (IL-6) and Sir2 like protein (SIRT2). Axin-1 is a key member of the Wnt signalling pathway, acting as a scaffold protein and a negative regulator of the Wnt signalling pathway (Kilikuchi, 1999; Pronobis *et al.,* 2015). As Wnt signalling negatively regulates platelet function and modulates the major platelet receptor αIIbβ3, (Steele *et al.,* 2009), an increase in Axin-1 levels could also have contributed to platelet adhesion and aggregation levels in this study.

IL-6, a pleiotropic inflammatory cytokine directly involved in the inflammatory cascade and linked to the pathogenesis of many chronic diseases was significantly increased after the DI. IL-6 has been adversely linked with sedentary time in a large study of >500 participants aged ~63 years and at high risk for T2DM (Henson *et al.,* 2013) and appears to be elevated after physical inactivity in our study. While platelets do not express IL-6 (Marino *et al.*, 2013), its production from other cells can affect platelet activation, as Il-6 activates platelets *in vitro* (Oleksowicz *et al.,* 1994; Soslau *et al.,* 1997; Bester, 2016). IL-6 is also involved in the regulation of haematopoiesis. IL-6 is also a potent thrombopoietic factor promoting maturation of human megakaryocytes *in vitro* (Ishibashi *et al.,* 1989). IL-6 appears to affect platelet function in a manner that predisposes the vasculature to thrombosis development. IL-6 has been suggested as a mediator of platelet hyperactivity, thrombocytosis and increased thrombus development in conditions of experimental colitis (Senchenkova *et al.,* 2013) and perhaps influenced platelet activation during the course of this study.

The effect of DI on protein expression has mainly revolved around muscle and urine samples (Pastushkova *et al.,* 2014). However, a serum proteome study by Pakharukova *et al.,* (2009) described significant increases in apolipoprotein CIII indicating an elevation in blood triglycerides and cholesterol after DI. Additionally, Pastushkova *et al.,* (2011) demonstrated increases in fibrinogen levels and apolipoprotein CI of blood plasma proteome during a 5-

day DI. Increases in fibrinogen levels can occur during acute inflammation, stress and alterations of physical activity (Gomez-Marcos *et al.,* 2013). The propensities of total plasma cholesterol, LDL and triglyceride concentrations to increase after DI were also observed by Markin *et al.,* (2008). Another 3 day DI experiment on male subjects measured lipid peroxidation products and antioxidant activity in blood serum. Results showed in increase in lipid peroxidation products after the DI and the index of antioxidant activity (tocopherol content) was distinctly reduced. This suggested a pronounced stress reaction after exposure to DI (Zhuravleva *et al.,* 2012). Most of these changes, similar to our study, were characterised by both a rapid development and quick restoration after return to normal conditions.

Evidently, a number of inflammatory and CVD proteins were differentially expressed after our DI, suggesting that extensive physical inactivity affects both immune and platelet function. However, we cannot state that PPP is reflective of changes purely in the platelet proteome as there will inevitably be some protein from other cell types including RBC and WBCs. Future work on the platelet proteome and proteins identified here would be essential.

5.3.8 Effect of physical inactivity on platelet poor plasma microvesicles

Microvesicles (MVs) are constitutively produced from the cell surface, but their generation can be elevated by activation or apoptosis (Loyer *et al.,* 2014). Exosomes have a size range of 30-100nm in diameter while microparticles represent the larger class of MVs (100nm-1µm). Activated platelets release MVs, rendering MVs as useful biomarkers of platelet activation (Heijnen *et al.,* 1999; Nunes-Franca *et al.,* 2015). Platelet MVs levels are altered in pathological states including obesity and atherosclerosis (Pap *et al.,* 2009), and also after physical activity (Sossdorf *et al.,* 2011; Chaar *et al.,* 2011). We investigated the effect of physical inactivity on the number and size of MVs in PPP. Nanoparticle tracking analysis enabled the use of small sample volumes.

Figure 5.11 summarises the changes in microvesicles in response to DI. There was no change in average MV size after DI. We found non-significant increases in overall MV concentration. For separate analysis of MV subpopulations, MVs were divided into three distinct categories; Exosomes (30-100nm), microparticles (100-255nm), and large microparticles (>255nm). There were non-significant increases in exosome and MP concentrations post-immersion.

Interestingly, there was a modest, non-significant increase in larger MP concentration after the DI, which could suggest the generation of larger and potentially more procoagulant MPs. Different activation pathways regulate both the quantity and type of MV subpopulation in platelets (Aatonen *et al.,* 2014), thus governing their different molecular profiles and facilitating tailor-made participation in intercellular communication.

Other studies have shown that endothelial MP levels increased on the third day of a seven-day DI experiment (Navasiolava *et al.,* 2010). Reduced daily physical activity (<5000 steps) in healthy males was significantly associated with elevated EMPs (Boyle *et al.,* 2013). As DI results in reduced stroke volume and cardiac output, it was suggested that an endothelial dysfunction to NO and deterioration in hemodynamic conditions could contribute to increases in EMPs. The changes in endothelial vasodilatory capacity could also have resulted in platelet hyperreactivity and increased MV levels in our study.

Platelet-derived MVs represent the most abundant MV source (70-90%) released into blood circulation, and we aimed to provide an estimate of overall levels and sizes. However, as previous data in this chapter suggested increased platelet activation post DI, we expected to see a larger increase in the quantity of microvesicles produced post DI. Concerning the exact origin of the microvesicles, it would be necessary to stain them with a fluorophore linked antibody, specific for surface markers on certain cell types, e.g. CD42b to bind to GP1b on platelets. This would have resulted in more accurate reflection of platelet specific MVs.

5.3.9 Effect of physical inactivity on platelet microRNA (miRNA)

Experimental results outlined in this chapter so far have demonstrated that physical inactivity using the DI model, has an impact on platelet phenotype and health. This was exposed through quantification of platelet activation/adhesion/aggregation markers and changes in expression of PPP protein biomarkers. To further understand the significance of physical inactivity in the regulation of platelet function, and the molecular mechanisms that underpin and fine tune platelet function, we hypothesised that miRNA play a large role in the regulation of platelet function. In brief, miRNA are short (18-24) nucleotide long non-coding RNA molecules. They regulate gene expression by hybridising to the 3’ UTR of mRNA, causing mRNA degradation or translational inhibition, depending on the complementarity between the strands (Ambros *et al.,* 2001).

The existence and functionality of miRNA pathway in the anucleate human platelet has recently been described in the literature (Landry *et al.,* 2009). miRNA constitute 80% of all small RNAs in platelets (Ple *et al.,* 2012) and, even though only small RNA yields were possible in this study, research by Teruel-Montoya *et al.,* (2014) comparing RNA and miRNA by cell type showed that despite a low RNA yield, platelets expressed relatively high quantities of miRNA compared to their nucleated counterparts.

Various components of the miRNA processing components including Dicer, Argonaute 2 (Ago2) have been reported in platelets (Landry *et al.,* 2009). In order to validate the expression of the miRNA processing components in platelets, western blots to detect protein expression of Drosha, Dicer and Ago2 were performed in leukocyte-depleted platelet lysates. Dicer and Ago2 were identified by western blot analysis and, in line with research from the literature, we were unable to identify the nuclear processing component drosha in the platelet. This is demonstrated in Figure 5.12. The lack of the nuclear protein, Drosha, is consistent with the anucleate nature of platelets. Therefore, concerning the origin of platelet miRNA, the likelihood that they are derived from pri-miRNA transcripts within platelets is highly improbable, as Drosha is necessary for miRNA maturation. Indeed, the pre-miRNA and mature miRNA are derived from megakaryocytes. Novel research has shown that platelets contain a limited number of pre-miRNA, and whether these are converted into mature miRNA remains an area of debate.

There exists a miRNA profile or "miRNome" characteristic to individual cell types and diseases states (Leidinger *et al.,* 2014). To determine if the miRNome for platelets was altered by physical inactivity, platelet miRNA profiles were examined pre and post immersion. The miRNA profile was determined by RT-qPCR. For a complete miRNA profile, two panels (A and B panel) of 384 targets per panel were used, allowing accurate quantification of 754 human miRNAs. The A card contains miRNA which have been well published tending to be functionally defined and broadly expressed. The B card contains miRNA targets which are generally narrowly expressed and/or expressed at low levels and not as well functionally defined.

5.3.9.1 Number of miRNA detected in platelets

The average number of miRNA detected on the A panel was 281 before the DI, and 278 after the DI, with the B card average at 155 and 160 pre and post DI respectively (Figure 5.12, B). Therefore, platelet RNA in our samples expressed a total average of 436 miRNA pre and 438 post DI. This is similar to the 492 platelet miRNA identified in a profiling study by Ple *et al.,* (2012), and 544 platelet miRNA in another comparative study by Teruel Montoya *et al.,* (2014). The Applied Biosystems expression suite software was used to analyse the data. The global measure of miRNA expression was used as the normalising factor. Normalization of the data is employed to adjust the data to remove any bias/ change in expression not arising from the biological conditions under examination. Global normalization is based on the assumption that although specific miRNA may alter expression across samples, as a result of experimental condition or stimulus, the overall expression pattern of miRNA is invariable and thus can be used as a normalizing factor. This method has been shown to outperform other methods such as use of endogenous small nuclear RNA in terms of better reduction of technical variance and more accurate appreciation of biological changes (Mestdagh *et al.,* 2009).

5.3.9.2 Identification of abundantly expressed platelet miRNA

Figure 5.13 shows the most highly expressed miRNA on both the A card across the 8 subjects. A small number of highly expressed miRNAs have been characterised in human platelets. miR-223 has been identified as the most highly expressed platelet miRNA (Gatsiou *et al.,* Edelstein and Bray, 201l; Halkein and Windt, 2013) and has roles in thrombopoeisis, cell proliferation and megakaryocyte differentiation (Laffont *et al.,* 2013). miRNA-223 is thought to mediate regulation of the $P2Y_{12}$ platelet ADP receptor expression, as the 3'UTR of $P2Y_{12}$ mRNA has been identified as complementary to the miR-223 seed region (Landry *et al.,* 2009; Shi *et al.,* 2015). Similarly, in this study, miR-223 was the most highly expressed miRNA on the A panel across all samples, followed by miR-126, the second most highly expressed miRNA in platelets, and which plays central roles in vascular inflammation (Fish *et al.,* 2008; Gatsiou *et al.,* 2012). Downregulation of miR-126 in megakaryocytopoiesis has been suggested to unblock target genes involved in differentiation (Nagella *et al.,* 2011). Of late, miR-223, miR-126, and other abundant platelet miRNAs have showed significant correlations with the platelet VASP assay (Kaudewitz *et al.,* 2016).

As expected, miRNA on the B card were less highly expressed than A card miRNA, shown by the presence of light red and some blue colours on the heat map in Figure 5.14. miR-720, a highly expressed B panel miRNA, has also been identified as an abundant platelet miRNA (Nagalla *et al.,* 2011). Some abundant miRNA on the B card are denoted with an asterisk beside their name such as miR-126*, miR-223*, miR-106b*, miR-222* and miR-15b*. These represent miRNAs which arise from two opposite arms of the same hairpin structure (5' and 3') with the same name, however the miRNA which is expressed in significantly lower levels in the cell is specified by the asterisk (Stakos *et al.,* 2013). Nevertheless, these miRNAs still exert effects on the cell. Other abundant miRNA on the B card have previously been associated with platelet function; miR-409-3p is significantly downregulated in patients with sickle cell disease who exhibit platelet hyperreactivity (Jain *et al.,* 2013). Overall, miRNA on the A card were considerably more abundantly expressed than miRNA on the B card.

5.3.9.3 Identification of miRNA affected by physical inactivity

After the data had been analysed using the default settings, the relative quantification (RQ) values for each condition (pre and post DI) were extrapolated in order to identify some of the most differentially expressed miRNA between the two time points. Therefore, using the pre DI samples as a baseline reference, we were able to identify changes in the post DI miRNA expression relative to the pre time point. Most reported miR expression fold-changes are small (~1.5–2-fold changes) (Daniels *et al.,* 2014). We identified 22 miRNA which were significantly up or downregulated. These miRNA from each card are shown in Figure 5.15. Some of these miRNA have previously been associated with platelet biology with some quite novel miRNA which could be potentially involved in platelet biology.

Downregulation of miRNA has been associated with mRNA translation and subsequent production of proteins relating to platelet activation (Edelstein *et al.,* 2013). On the A panel, 7 miRNA were significantly downregulated after the DI, shown in Figure 5.15 (B). Downregulation of miR-143 has been implicated in platelets of patients with T2DM compared to healthy and age-/sex-matched controls (Elgheznawy *et al.,* 2015), with subsequently elevated platelet adhesion and aggregation thought to be as a result of reduced NO and prostacyclin production.

The miR-200 family is well established in miRNA biology. miR-200a was significantly downregulated after the DI and while miR-200a and miR-200b only differ by a single nucleotide, they are predicted to share mRNA targets (Howe *et al.,* 2012). Indeed, miR-200b has been described to target the PRKAR2B gene that plays a role in the suppression of further platelet activation and aggregation. Although miR-200b did not make the shortlist for this experiment, it was downregulated after DI, indicating its involvement in platelet activation (Nagalla *et al.,* 2011).

Both miR-500 and miR-888 were also significantly downregulated after the DI. Neither have previously been described in human platelets, however the translation of the fibrinogen receptor subunits (αIIb and β_3) were reported to be regulated by miR-500 in a dicer knockdown model of murine megakaryocytes, resulting in increased surface expression of the respective integrins and subsequent platelet aggregation (Rowley *et al.,* 2016). This miRNA could represent a new marker of physical inactivity in platelets. Another downregulated miRNA, miR-203, has been predicted to regulated SOCS1 gene and was differentially expressed in patients with essential thrombocythemia compared to healthy controls (Navarro *et al.,* 2016).

On the B panel, there were 3 downregulated miRNA post immersion; miR-145*, miR-15a* and miR-374a* shown in Figure 5.15 D. miR-145 is a highly expressed platelet miRNA (Edelstein *et al.,* 2011) and its differential expression has been linked to thrombocytosis and megakaryocyte dysplasia (Banchi *et al.,* 2016). miR-145/145a have been shown to stimulate megakaryopoieses (Starczynowski *et al.,* 2010). miR-15a* has also been linked with platelet physiology. Yu *et al.,* (2014) identified miR-15a as one miRNA downregulated during apheresis platelet storage suggesting a role in apoptosis. miR-374a family has been implicated in CVD whereby leukocyte miR-374 was significantly lower in CVD patients compared to healthy controls (Ward *et al.,* 2013).

Other studies have shown changes in the platelet miRNA profile in response to various stimuli. Tanriverdi *et al.,* (2006) investigated the effect of thrombin induced aggregation on the platelet miRNA profile. Expression of 9 miRNAs were downregulated with thrombin activation between 0.76 fold to 0.53 fold, similar expression changes to the downregulated miRNA in our study. Nagalla *et al.,* (2011) examined whether platelet miRNA levels were associated with reactivity and phenotype in 19 healthy donors and demonstrated that 74 miRNAs were differentially expressed (DE) when stimulated with epinephrine and also that the miRNA profiles were correlated with the platelet responses to epinephrine.

Among the upregulated miRNA on the A panel shown in Figure 5.15 part (A), were miR-484 and miR-299-5p. miR-484 is a highly expressed miRNA in megakaryocytes and platelets (Hussein *et al.,* 2009; Edelstein *et al.,* 2013; Pan *et al.,* 2014). It appears to regulate haematopoiesis (Hunter *et al.,* 2008; Petriv *et al.,* 2010) and is implicated in the effects of the pathogen reduction system on platelet function (Osman *et al.,* 2015). miR-299-5p was the one of the more upregulated miRNAs and has also been linked with megakaryocyte biology. Tenedini *et al.,* (2010) reported that over expression of miR-299-5p in CD34+ cells caused a (1.5 fold) increase in CD41a expression of megakaryocytic colonies in collagen-based assays and of MKC forms with polynucleotide elements. Interestingly, the same cells treated with an anti-299-5p exhibited decreases in CFU-mk. MiR-30b has also been described in platelet biology (Edelestein *et al.,* 2013).

Figure 5.15D shows upregulated miRNA on the B panel. These included miR-744*, miR-24-2*, miR-190b, miR-34a* and miR-106b*, all of which have been identified in human platelets, except for miR-744 (Nagalla *et al.,* 2011; Pan *et al.,* 2014; Willeit *et al.,* 2013). miR-34a, one of the most highly upregulated miRNA, is highly expressed in platelets (Gatsiou 2012; Edelstein *et al.,* 2013) and is involved in megakaryocyte differentiation, whereby elevated levels of miR-34a appear to enhance megakaryopoieses. Higher levels of miR-34a have been observed to increase megakaryocyte colony formation from $CD34^+$ cells (Navarro *et al.,* 2009).

There is limited research investigating the effect of microgravity on miRNA expression (Heather *et al.,* 2006). This is the first study to quantify changes in miRNA in human cells (*ex vivo*, uncultured cells) in a simulated microgravity environment. Mangala (2011) examined the effects of simulated microgravity on the miRNA profile of human lymphoblastic cells using a high aspect ratio vessel (bioreactor), either in the rotating condition to model microgravity in space or in static condition as a control. Expression of several miRNA were altered in the simulated microgravity condition including miR-150, miR-34a, miR-423-5p, miR-22, and miR-222, some of which overlap with our study. Recently, Forty-two miRNAs from cultured human blood lymphocytes from 12 healthy subjects were differentially expressed in microgravity stimulated cells compared to static cells, with resultant mRNA gene targets involved in inflammatory and apoptotic responses (Girardi *et al.,* 2014).

As platelets do not express the miRNA nuclear machinery Drosha and DGCR8 (Landry *et al.,* 2009; Zampetaki and Mayr, 2015), the *de novo* synthesis of new miRNA in platelet is negligible. Upregulation of miRNA in response to physical inactivity could be derived from the processing of pre-miRNA to mature miRNA or as a reflection of increased levels miRNA in their megakaryocyte precursor. Recent research has shown that platelets have higher levels of pre-miRNA than other blood cells (Pan *et al.,* 2014).

As platelets can release microvesicles-containing miRNA upon activation, downregulated miRNA in this study may reflect this process. These findings suggest that platelet miRNAs might reflect quantitatively platelet activation *in vivo* and, as such, might have a great potential as biomarkers of physical inactivity. Importantly, the cellular bearing of the majority of miRNA-mRNA interactions is a fine-tuning of protein output and not huge repression of expression (Baek *et al.,* 2008). Notably, as little as a 20% decrease in miRNA levels can alter cellular phenotypes (Alimonti *et al.,* 2008).

5.3.9.4 Bioinformatics of differentially expressed DI regulated miRNA

To determine which genes may be affected by the DI regulated miRNA shown in Figures 5.15, their putative targets were downloaded from online computational methods for miRNA-mRNA interactions including Targetscan, miRANDA and microRNAorg. Complementarity between the seed region of the miRNA and the 3'UTR of an mRNA is the underlying method used for *in silico* prediction of targets used in Targetscan. The results of the three databases were collectively used to predict the roles of DI regulated miRNA in the regulation of gene expression. DAVID bioinformatics software was then employed to highlight the involvement of these *in silico* predicted genes in pathways of interest from the Kyoto Encyclopaedia of Genes and Genomes (KEGG). DAVID bioinformatics resources consist of an integrated biological knowledge base and analytical tools aimed at extracting meaning from large protein or gene lists. The first stage is to upload putative targets containing any number of gene identifiers (e.g. official gene symbol). Following this, analysis is carried out taking advantage of the vast pathway miming tools such as KEGG biological pathways.

The fold change, number of conserved targets, and number of pathways the predicted genes are linked to are included in Table 5.4 for each miRNA. Table 5.5 shows the miRNA potentially involved in the pathways of interest and whether these specific miRNAs were up or downregulated with DI.

The pathway maps downloaded using DAVID from KEGG were used to show the role of predicted targets in pathways of interest. These pathways are shown in Figures 5.16 -5.18. Genes within the pathways of interest that were targeted by a single miRNA are circled in yellow, while those potentially targeted by two or more miRNA are circled in red. Pathways were chosen based on their involvement in platelet function and signalling, specifically in adhesion and aggregation, but also their inflammatory potential. Included in these were the Wnt signalling, regulation of actin cytoskeleton, ECM interaction and Toll-like receptor pathways, of which the Wnt signalling pathway appeared repeatedly in both the up and downregulated miRNA targets. Following this, the regulation of actin cytoskeleton pathway was the second most frequent pathway found to be associated with the DI regulated miRNAs.

A number of differentially expressed miRNA post DI targeted genes involved in the actin cytoskeleton (Figure 5.17). The shape change required for platelet adhesion, from a discoid to spread shape, involves extensive reorganisation of the actin cytoskeleton (Aslan *et al.,* 2011). The miR-143/145 cluster controls the expression and function of various components of the cytoskeleton in smooth muscle cells (Xin *et al.,* 2009) and can switch the phenotype of SMCs (Rangrez *et al.,* 2011). As both miR-143 and miR-145 were downregulated after the DI, this could have contributed to an upregulation of mRNA involved in the actin cytoskeleton and subsequently assisted in platelet adhesive processes. miR-199a also had mRNA targets involved in this pathway, including F-actin and α-actin.

Some of the downregulated miRNA were involved in the ECM-receptor interaction pathway (Figure 5.18). Platelets express 5 different integrin's which facilitate platelet adhesion to ECM proteins including collagen, laminin and fibronectin. miR-143 had targets involved in this pathway including collagen, and receptors α1, α6 and β8. In comparison, miR-199a targets included fibronectin and integrin subunit α3. Targets of miR-302a (downregulated) included integrin subunit β3 and α2, suggesting an overexpression of these mRNA, and thus the potential for an increased capability of platelets to adhere to ECM proteins.

Relevant to earlier findings in this chapter was the recurrence of the Wnt signalling pathway (Figure 5.16), involving targets of the differentially expressed miRNA, as two protein biomarkers (DKK1 and Axin-1), that were altered by the DI are also involved in the Wnt pathway. The Wnt family consists of 19 glycoproteins which play key roles in diverse biological processes including the regulation of adult homeostasis (Macauley *et al.,* 2013).

Wnt ligands propagate signals through several receptors, activating one of three different signalling pathways; the Canonical/ Wnt-β-catenin pathway, the Planar Cell Polarity pathway and the Wnt/Ca^{2+} pathway, to mediate effects on gene transcription and cell adhesion.

The literature has described not only a role for mainly the Wnt-β-catenin (Steele *et al.*, 2009), but also the non-canonical Wnt signalling pathways in platelet function (Kim *et al.*, 2011). Responsiveness of platelets toward Wnt ligands was recognised when a recombinant Wnt3a ligand was shown to inhibit platelet adhesion, shape change, dense granule secretion and inhibiting activation of αIIbβ3 resulting in decreased platelet adhesion to fibrinogen and subsequently reduced aggregation (Steele *et al.*, 2009).

Focusing on the β-catenin signalling pathway, Wnts bind to a platelet surface receptor complex composed of the lipoprotein receptor related protein 5/6 (LRP5/6) and the frizzled (Fzd) receptor. This signal is propagated to the cytoplasmic protein, dishevelled (Dvl), where downstream effectors control the stability of β-catenin (β-cat). When Wnt is absent, β-cat is phosphorylated by a destruction complex of casein kinase 1 (CK1), glycogen synthase kinase 3β (GSK3β), Axin-1, FRAT-1 and adenomatous polyposis coli (APC), which mark β-cat for degradation by ubiquitination and then proteasomal degradation. Oppositely, in the presence of Wnt, Dvl negatively regulates the phosphorylation of β-cat preventing its degradation.

Central to the Wnt/β-catenin pathway is the cytosolic level of β-cat, whose disassembly from the destruction complex in the cytoplasm enables nuclear transcriptional activation of genes. However, as platelets are anucleate, stabilised β-cat cannot have a nuclear transcriptional role. β-cat also forms direct associations between cadherins (CDHs) and α-catenin, the latter of which associates with the actin cytoskeleton. CDH6 has been associated with platelet adhesion and aggregation (Heuberger and Birchmeier *et al.*, 2010; Dunne 2012). Thus, the amount of functional β-cat would impact platelet adhesion and aggregation (Kumari and Dash 2013). Steele *et al.*, (2012) reported that Wnt3a modulates platelet function by the regulation of small GTPase activity and in particular four central GTPase proteins (Rap1, Cdc42, Rac1 and RhoA) that play key roles in platelet activation.

Axin-1, a key mediator of the Wnt/ β-cat pathway, was identified as one of the proteins that was upregulated after the DI. Axin-1 can scaffold the β-cat destruction complex to encourage β-cat degradation and inhibitor of Wnt signal transduction (Song *et al.,* 2014). Interestingly, two downregulated miRNAs, miR-203 and miR-200a were identified as potential targets of Axin-1. Additionally, a number of other miRNA predicted to target Axin-1 were also downregulated as shown in Figure 5.19B. The simultaneous downregulation of multiple miRNA targeting Axin-1 could have resulted in an increase in Axin-1 mRNA expression, and subsequent protein production. Increased Axin-1 levels can reduce Wnt signalling in platelets with elevated platelet adhesion and aggregation as a result.

In the ECM, a number of proteins bind to Wnts, preventing their interaction with either Fzd or LRP 5/6, to inhibit Wnt signalling, one of which comprise of Dickkopf proteins (DKKs). Platelet derived DKK1 is a major Wnt antagonist that binds to low-density lipoprotein receptor-related protein 5/6 (LRP5/6) to inhibit signal transduction from occurring (Macaulay *et al.*, 2013). DKK1 was one of the significantly upregulated proteins identified from the biomarker panel post-DI and we therefore sought to identify miRNA (in addition to the DE expressed miRNA) that could be targeting DKK1. These are displayed in Figure 5. 19A. Multiple miRNA can target DKK1. microRNA.org listed miR-302a and miR-374a as possible regulators of DKK1 when viewing target sites of conserved miRNA. A number of these miRNA were downregulated in our study, suggesting that a simultaneous downregulation of multiple miRNA targeting DKK1 may act together therefore increasing DKK1 transcript and ultimately, protein levels. In particular, miR-302a was the most downregulated miRNA, which also targeted DKK1. An increase in DKK1 could have contributed to a negative regulation of the Wnt signalling pathway and ultimately, elevated platelet adhesion levels.

Guo *et al.,* (2015) demonstrated that platelet-sourced DKK1 was the prominent Wnt antagonist which contributed to suppression of Wnt/β-catenin in alveolar epithelial cells in acute lung inflammation. Zhang *et al.,* (2011) found that DKK1 was regulated by miRNA control of miR-335-3p in osteoblast lineage cells, and Xu *et al.,* (2015) reported that a downregulation of miR-152 contributes to increased DKK1 expression in multiple myeloma. Similarly, these miRNAs were downregulated in our study, shown in Figure 5.19A.

Evidence of physical activity-specific microRNA signatures (Kangas *et al.*, 2013; Bye *et al.*, 2012 and Altana *et al.*, 2015), have seeded the notion that there must also be physical inactivity-specific miRNA profiles. Epigenetic variation could be a potential mechanism allowing for independent or perhaps synergistic effects of physical inactivity on platelet function. Hibler *et al.*, (2015) recently described indications for epigenetic variation (by miRNA expression) as a link between physical activity and sedentary lifestyle. An epigenetic adaptation to habitual exercise has previously been described (Ling *et al.*, 2014; Pareja-Galeano *et al.*, 2014).

Similarly, we hypothesised that an epigenetic adaptation to physical inactivity is also present. Inactivity could induce epigenetic changes in megakaryocytes to generate unhealthy platelets phenotypes with a direct change in platelet reactivity and platelet miRNA could act as a marker of this epigenetic drift. Importantly, we have identified changes in the platelet miRNA profile in response to physical inactivity. Investigating miRNA-mRNA and miRNA-protein interactions would provide more knowledge in this area.

While not the main focus of this chapter, we also assessed other aspects of the effect of DI on platelet inflammatory function, specifically on toll-like receptor gene expression during the DI. This can be found in Appendix C.

5.4 Limitations

Although the nature of DI studies provides an extremely controlled environment, some limitations became apparent. The current DI study used a random subject population and, while this is beneficial as subjects are their own control, some baseline measurements varied amongst subjects. Measurements during the baseline period were obtained whilst subjects were ambulatory. However, they were confined to the clinic and so these physical activity measurements may not reflect free-living conditions. There were twelve research teams working on the same study and this meant that volunteers had to follow a strict and perhaps stressful regime in terms of test measurements (as depicted in the VAS pain scale measurement). Psychological stress and time out of baths could have contributed to the observed baseline/differences post DI. While a lot of time and effort goes into the timing of each test and the order they are in, it may be seen as a limitation also.

5.5 Summary and conclusion

In summary, physical inactivity had a modest effect on platelet function over three days. Except for plateletcrit and platelet count, the remainder of the platelet indice markers did not change significantly. There were significant increases in platelet adhesion, aggregation, and an elevation of PRI after the DI. This study has identified 15 proteins whose expression trends could be of importance for developing 'biosignatures' of physical inactivity. We also identified a set of 'physical inactivity' related miRNA which have potential targets involved in pathways associated with platelet function. It is evident from this study and literature findings that the identification of unique signatures of several miRNA, rather than a single miRNA in isolation may enhance diagnostic accuracy (Meder *et al.*, 2011).

The canonical Wnt signalling pathway may signify a novel endogenous mechanism for regulating platelet activity in response to physical inactivity. Investigation around the Wnt negative regulators Axin-1 and DKK1, in combination with miRNA involved in this pathway, could be an interesting avenue, as a both the protein and miRNA profiles indicated involvement in this pathway. In conclusion, the results suggest an inclination in healthy males of an unhealthier/pro-thrombotic platelet profile in response to physical inactivity. This could be indicative of the changes seen in sedentary individuals. Collectively, our results provide evidence for the early and robust deleterious impact of reduced daily activity on platelet function and phenotype, highlighting the vulnerability of the vasculature to a sedentary lifestyle. These results involving the measurement of various aspects of platelet function are novel and provide an interesting insight into the potential mechanisms behind physical inactivity-induced platelet dysfunction.

Chapter 6: Overall Summary and Future Perspectives

6.1 Overall Summary

Efforts in coping with CVD require further understanding of its etiology and the risk factors behind it in order to develop cost-effective preventive strategies (primordial, primary, secondary and tertiary) to prevent and manage it. CVD risk factors can be classed as modifiable or non-modifiable. Modifiable risk factors included smoking, diabetes, unhealthy diet, cholesterol, physical inactivity (sedentary lifestyle and low cardiorespiratory fitness) and overweight and obesity. Risk factors for CVD track from childhood into adulthood and are strong predictors of subclinical disease in early adulthood (Juhola *et al.,* 2011). Up to 80% of CVD may be prevented if modifiable risk factors are evaded (Mc Neal *et al.,* 2010; WHO). This thesis focused on the risk factors of physical inactivity, overweight/obesity and low cardiorespiratory fitness.

Platelets are small, anucleate cells, which are released by bone marrow megakaryocyte precursor cells into blood circulation (Laffont *et al.,* 2013; McFadyen and Kaplan, 2015). They travel as resting discoid fragments in circulation, however, elaborate morphological properties allow shape changes to occur when they come in contact with an injured blood vessel. This enables platelets to perform their main physiological function to prevent blood loss in primary haemostasis by the formation of a 'platelet plug' through processes of adhesion, activation and aggregation (Cimmino and Golino 2013).

Platelets play a central role in CVD, both in the pathogenesis of atherosclerosis and in the development of acute thrombotic events (Maiwand *et al.,* 2015). It has been recognised that regular exercise may reduce the risk of major vascular thrombotic events and protect against CVD (Blair and Norris, 2009). Differences in known factors explain a large percentage of the inverse relationship between physical activity and CVD risk (Mora *et al.,* 2007; Kwasniewska *et al.,* 2016). However, over 40% of the inverse association remains unexplained. Although the beneficial effects of regular exercise on blood lipids and blood pressure have been well accepted, research focusing on platelet function has only recently gained greater attention.

In contrast to the accumulating evidence supporting the benefits of regular exercise, relatively little is acknowledged about the deleterious mechanisms underlying the physiological, cellular and molecular responses to physical inactivity, specifically with regard to platelet function. Small non-coding RNAs such miRNAs have been shown to play important post-transcriptional regulatory roles in cells. The role of microRNA (miRNA) in regulating platelet function in is a recent fertile area of investigation (Landry *et al.,* 2009; Kaudevitz *et al.,* 2016). Platelets are highly reflective of physiological and lifestyle changes, making them extremely sensitive biomarkers of human health.

The purpose of this thesis was to build on previous knowledge surrounding the involvement of platelets in physiological health and overweight/obesity, their profile in physical activity and inactivity and the mechanisms that regulate their activity in response to such stimuli. The overall hypothesis, therefore, was that both physical inactivity and overweight/obesity adversely impact platelet function. Furthermore, miRNA expression could influence and modulate the platelet function response to physical inactivity. Experimental work designed to test this hypothesis was divided across three chapters.

In **Chapter Three**, a cross-sectional study approach was employed to evaluate the relationship between platelet function and physiological health. Whilst it is known that platelet function and platelet indices (markers of platelet activation) are altered in pathological states such as CVD, only a minority of studies have solely examined the relationship between overall physiological health and platelet function in healthy subjects (Santilli *et al.,* 201; Goshal, 2014).

This study explored the feasibility of platelet indices and whole blood platelet function measurements, as useful, non-invasive initial biomarkers of early/subclinical CVD risk and lifestyle parameters in a nationally representative population of 155 adults. The most interesting results from this chapter showed that higher platelet counts and plateletcrit were associated with less favourable cardiovascular risk profiles, in particular with measures of overweight. Importantly, platelet adhesion was significantly increased in male and female adults who had a BMI classed as overweight compared to those with a healthy weight BMI. The elevated PLT, PCT and SC levels observed in overweight subjects indicate a more reactive platelet profile and could imply the occurrence of subclinical CVD symptoms.

Following on from Chapter 3, instead of taking basal single point measurements, we felt that perhaps stressing the cardiovascular system using *in vivo* instead of *in vitro* or *ex vivo* stress models, by prescribed exhaustive exercise may provide much more valuable and conclusive evidence in relation to the body's response, and in particular the platelets adaptive response, to stress. Therefore, research in **Chapter Four** extended our research using a 'human laboratory' approach, evaluating the effect of controlled aerobic exercise, as a cardiovascular stressor, on platelet function in male adolescents (n=30) and adults (n=16) of varying cardiorespiratory fitness levels (CRF). Inconclusive information is available on the effect of acute exercise on platelet function in adults, with almost no available studies centring on platelet function in healthy adolescents and with respect to acute exercise. (Heber and Volf, 2015). In this study, subjects performed the gold standard test for aerobic fitness, the VO_2 max test. Blood samples were drawn before and after exercise and platelet indices and function were evaluated, again using the Impact-R Cone and Plate analyser. Subjects were categorised into groups depending on their CRF.

It was observed that acute exhaustive exercise resulted in increased platelet function in adults and adolescents, and that this increase was more pronounced in moderate to low fit adolescents and the moderately fit adults. The same effect of exercise in the high fit adults and adolescents was not observed, possibly due to adaptive mechanisms from long-term habitual physical activity and exercise. Our results support past studies highlighting the different effect of CRF on platelet function in adults with novel findings in an adolescent population. Those with a low CRF level are at increased risk of thrombotic events following maximal exercise. These findings highlight the need to develop a strategy to increase activity amongst adolescents and adults to combat the early development of subclinical CVD.

A low cardiorespiratory fitness is associated with physical inactivity (Wei *et al.,* 2000). Physical inactivity has major health effects globally, with approximately 3.2 million deaths each year attributable to inadequate physical activity. Evidence has shown that physical inactivity and sedentary behaviour have direct effects on CVD risk factors such as obesity and hypertension (Prentice and Jebb, 2004). Moreover, in contrast to the accumulating evidence supporting the benefits of regular exercise, relatively little is understood about the deleterious mechanisms underlying the physiological, cellular and molecular responses to PI, specifically with regard to platelet function.

The European Space Agency (ESA), employs ground-based models of microgravity (the condition in which people or objects appear to be weightless), including dry water immersion (DI), to study the effects of spaceflight on human physiology in a precisely controlled environment. DI involves immersing a subject in a bath of thermoneutral water covered by a waterproof fabric (Coupe *et al.,* 2013). Several factors act simultaneously on the human body during immersion, including hydrostatic compression, supportlessness and extensive physical inactivity. Hypokinesia and hypodynamia are the major characteristics of physical inactivity induced by dry immersion. Hypodynamia involves a reduction in postural muscle load, whereas hypokinesia is a decline in motor activity. For these reasons, DI has been well accepted as a valuable tool to study physical inactivity (Widlansky, 2010). Subjecting twelve healthy men to three days of DI presented a unique opportunity to analyse the specific effects of physical inactivity on platelet and related biomarkers. Therefore, in **Chapter Five,** we examined the effect of three days of acute physical inactivity on platelet function.

Platelet function was assessed at three time points throughout this study; pre immersion, post immersion, and after one day of recovery. This chapter was essentially divided into two parts. In the first, we assessed platelet function using the Impact R and flow cytometric assessment of intra-platelet VASP phosphorylation, a marker of basal platelet activation. Platelet indices and haematological characteristics were also examined. We found that physical inactivity (by way of DI) resulted in significant increases in platelet adhesion and aggregation post DI, suggesting a more pro-thrombotic platelet profile post DI. After DI, while we observed a minor increase in the average PRI (5%) suggesting a reduction in VASP phosphorylation and increase in platelet activation. However, as the VASP phosphorylation changes were minimal, other (thrombin/collagen) platelet signalling pathways could have contributed to the elevated platelet adhesion and aggregation.

We also examined the effect of physical inactivity on platelet poor plasma (PPP) based protein biomarkers using the novel The Proseek® Multiplex assay. We observed changes in the expression levels of 15 proteins between different stages of the DI. In particular, increases in the Wnt signalling pathway proteins DKK1 and AXIN1 were observed. As Wnt signalling negatively regulates platelet function and modulates the major platelet receptor αIIbβ3, (Steele *et al.,* 2009), an increase in DKK1 and AXIN1 levels could also have contributed to platelet adhesion and aggregation levels post DI in this study.

Experimental results outlined to this point in Chapter five, demonstrated that physical inactivity, using the DI model, has an impact on platelet phenotype and health. This was exposed through quantification of platelet activation/adhesion/aggregation markers and changes in expression of PPP protein biomarkers. To further understand the significance of physical inactivity in the regulation of platelet function, and the molecular mechanisms that underpin and fine tune platelet function, we hypothesised that miRNA play a role in the regulation of platelet function. This part constituted the second half of the chapter.

To determine if the miRNA signature or 'miRNome' for platelets was altered by physical inactivity, platelet miRNA profiles were performed pre and post immersion. To date, there are no reports of literature characterising platelet miRNA with regard to physical inactivity/ lifestyle. We generated a list of differentially expressed miRNA (downregulated and upregulated) from comparisons of profiles at pre to post immersion. Some of these miRNA have previously associated with platelet biology, in addition to novel miRNA, which could be potentially involved in platelet biology. miR-143 was significantly downregulated after the DI. Downregulation of miR-143 has been implicated in platelets of patients with T2DM compared to healthy and age-/sex-matched controls (Elgheznawy *et al.*, 2015), with subsequently elevated platelet adhesion and aggregation thought to be as a result of reduced NO and prostacyclin production – this also could have been a factor in our study.

Bioinformatics analysis was performed on the most differentially expressed miRNA post DI. This included downloading of in silico predicted targets of each miRNA from the online algorithm Targetscan. These putative targets were then entered into DAVID bioinformatics database, which mapped their involvement in pathways of interest. Pathways were chosen based on their involvement in platelet function and cell signalling, specifically in adhesion and aggregation. Included in these were the Wnt signalling pathway, regulation of actin cytoskeleton and ECM interaction, of which the Wnt signalling pathway appeared repeatedly in both the up and downregulated miRNA targets. Relevant to earlier findings in this chapter was the recurrence of the Wnt signalling pathway, involving targets of the differentially expressed miRNA, as two protein biomarkers (DKK1 and AXIN1), which were affected by the DI are also involved in the Wnt pathway.

This study has identified 15 proteins whose expression trends could be of importance for developing 'biosignatures' of physical inactivity. We also identified a set of 'physical inactivity' related miRNA, which have potential targets involved in pathways associated with platelet function. It is evident from this study and literature findings that the identification of unique signatures of several miRNA, rather than a single miRNA in isolation may enhance diagnostic accuracy (Meder *et al.,* 2011). The canonical Wnt signalling pathway may signify a novel endogenous mechanism for regulating platelet activity in response to physical inactivity. Investigation around the Wnt negative regulators Axin-1 and DKK1, in combination with miRNA involved in this pathway, could be an interesting avenue, as a both the protein and miRNA profiles indicated involvement in this pathway.

Our results from this chapter suggest an inclination in healthy males toward an unhealthier/pro-thrombotic platelet profile in response to physical inactivity. This could be indicative of the changes seen in sedentary individuals. Collectively, our results provide evidence for the early and robust detrimental impact of reduced daily activity and/or a low cardiorespiratory fitness on platelet function and phenotype, highlighting the vulnerability of the vasculature to a sedentary lifestyle. These results involve the measurement of various aspects of platelet function are novel and provide an interesting insight into the potential mechanisms behind physical inactivity-induced platelet dysfunction.

6.2 Future research avenues

The studies conducted in this thesis reflect the first comprehensive attempt to evaluate the relationship between platelet function and physical activity, physical inactivity and overweight. While exploratory in nature, we have also enhanced the knowledge in this area. Naturally, several questions remain unanswered and so further studies are warranted.

The search for simple biomarkers that allow for early identification of subclinical/CVD risk is ongoing. Platelets can reflect changes in unhealthy lifestyle patterns. The Impact-R test is a relatively inexpensive test that can reliably detect changes in platelet adhesion and could be employed for CVD risk evaluation amongst subjects who are asymptomatic. Platelet indices and function markers should be further tested in larger populations to determine their reliability as surrogate markers for evaluating physiological health and to test during either pharmacological and lifestyle interventions.

Exercise-related studies in this thesis examined the effect of maximal exercise on platelet function. For subjects with a low CRF, a true VO_2 max can be difficult to attain and therefore, submaximal exercise tests could be more useful. Future research should also contemplate the optimal amount and intensity of warm up exercise that could reduce negative exercise induced changes in the thrombotic state seen post exercise in low fit subjects. Warm ups prior to strenuous exercise in low fit subjects may attenuate these changes and a role for an active cool down may be required (Wang *et al.*, 2006).

A relatively low dose of exercise has been shown to be sufficient to normalise platelet function in low fit females (Heber *et al.*, 2016). Larger studies incorporating exercise interventions at low doses over a lengthy period of time and examining more extensive aspects of platelet function in low fit subjects (such as flow cytometric evaluation of platelet activation markers) would develop this knowledge.

MedEx (Medical Exercise) is a unique chronic rehabilitation service ran by Dublin City University, which delivers exercise-based programmes to individuals with chronic disease, including CVD. The prescription of anti-platelet therapy is frequently used to treat CVD patients. However, the other residual risks (oxidative stress, inflammation etc.), which occur due to associations between CVD risk factors, are not eliminated efficiently by these therapies. In this sense, physical activity has been emphasised as it promotes favourable physiological adaptations, which may attenuate the cardiovascular risk factors and residual

risks. Regular exercise could also impact platelet function in CVD patients. Exercise interventions in these populations could be beneficial in terms of reducing antiplatelet therapy dosage or combining antiplatelet therapy with exercise (Martins *et al.,* 2016), i.e. prescriptive exercise medicine as adjuvant/adjacent management strategy/therapy.

Unlike the platelet transcriptome and proteome, the investigation of epigenetic processes is almost a completely unexplored area in platelet biology as analysis of these mechanisms require DNA (Freson, Izzi and Van Geet, 2012). Platelets have functionally active mitochondria (Antony *et al.,* 2012). Like nuclear DNA, mitochondrial DNA (mtDNA) can also be methylated, moderating control of mitochondrial gene expression. Understanding epigenetic regulation of mitochondrial genes in platelets may prove crucial to understanding their implication in CVD development. Novel research by Baccarelli and Byun (2015) showed that CVD patients had significantly higher platelet mtDNA methylation than healthy individuals in MT-CO1, MT-C02, MT-CO3, and MT-TL1, genes involved in ATP synthesis. These results suggest that DNA methylation in platelet mitochondria could be a potential contributor to CVD development through the regulation of platelet function. Furthermore, miRNA have recently been linked with platelet mitochondrial health in stored platelets (Dahiya *et al.,* 2016)

While the nuclear miRNA processing components Drosha and DCGR8 have not been identified in human platelets due to their anucleate nature, platelets contain the machinery to process pre-miRNA to mature miRNA (Landry *et al.,* 2009). Platelets contain higher levels of pre-miRNA than other blood cells (Pan *et al.,* 2013). Although not examined in this thesis, we acknowledge that the maturation of pre-miRNA could have contributed to upregulated miRNA after the dry immersion. It was likely that pre-miRNA could have been cleaved into mature miRNA, thereby increasing mature miRNA numbers. Future studies should therefore clarify the possible processing/levels of pre and mature miRNA in platelets to determine their contribution to the regulation of gene expression. This may be a more focused and efficient method of monitoring platelet function.

Targeting the levels of other biogenesis molecules in the miRNA pathway would also be an interesting avenue of platelet miRNA biology. Recently, Elgheznawy *et al.,* (2015) showed that, Dicer was decreased in patients with TD2M compared to healthy controls, whereas interestingly, Argonaute 2 levels did not differ. Experiments investigating the levels of miRNA processing machinery such as Dicer and Argonaute 2 in physically active and sedentary populations would be of major interest.

Platelet microvesicles are released upon platelet activation. Microvesicles can carry genetic information such as mRNA and miRNA. In this respect, perhaps the most intriguing feature regarding platelet miRNA is their extracellular function. miRNA can be packaged and delivered to distant cells in the form of platelet microvesicles, fulfilling novel processes of gene regulation in target cells (Diehl *et al.*, 2012). Downregulation of platelet miRNA upon platelet activation could be as a result of miRNA packaging in platelet MVs. Comparing the microRNA spectrum between platelets, platelet microparticle and platelet exosome miRNA content could provide interesting answers.

Long-term lifestyle choices such as physical inactivity and obesity may incur epigenetic changes in megakaryocytes. Thus, platelet miRNA could reflect these epigenetic changes, holding substantial diagnostic potential of both health and disease (Figure 6.1). Epigenetic changes in the megakaryocyte genome such as methylation of genes determining PLT or changes in histone acetylation with aging have been suggested to play an important role in platelet function (Daly, 2011). Prescribed exercise could induce epigenetic changes in megakaryocytes to produce a healthier phenotype of platelets with a direct change in platelet reactivity. New molecular techniques such as Bisulfite sequencing would be useful to assess the methylation state of megakaryocyte DNA, with subsequent detection of miRNA in platelets.

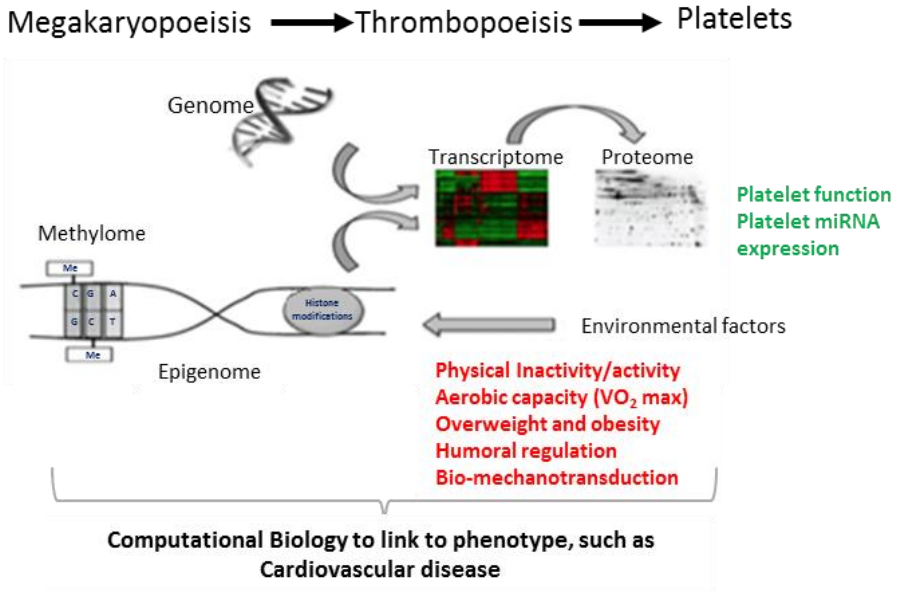


Figure 6.1: Model linking epigenomics and genomics to transcriptomics and proteomics. Environmental factors such as physical activity/overweight can lead to stable changes in the epigenome which alter CVD disease susceptibility. Platelet miRNA may reflect long-term changes in the megakaryocyte genome such as DNA methylation changes. *(Adapted from Freson et al., 2012).*

Concluding thoughts

To conclude, it is evident that lifestyle factors such as physical (in) activity and overweight do impact platelet function in apparently healthy subjects. Work from the three chapters of this thesis have collectively demonstrated that platelets are indeed reflective of physiological and lifestyle changes, making them sensitive biomarkers of human health. Platelets represent a tangible link to physiological and pathological changes within the body. Research continuing from this thesis, in the areas discussed above, will no doubt contribute to a greater mechanistic understanding of the relationship between cardiovascular health, lifestyle factors and platelet biology.

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Appendix

This appendix is composed of additional and or preliminary data for each of the results chapters. This data was not essential to test the thesis hypothesis but is rather included in the appendix to demonstrate the breadth of the studies performed and potential avenues for future work. It also contains supplementary data for each chapter, such as questionnaires or report forms for exercise protocols. The appendix is split into three parts – Appendix A contains information related to Chapter 3, Appendix B contains information related to Chapter four and Appendix C contains information related to Chapter 5.

Appendix A

FAT LAB GENERAL QUESTIONAIRE

1. **Gender:** Male □ Female □
2. **Date of birth** _____
3. **Is English your first language?** Yes □ No □
4. **To which racial or ethnic group(s) do you most identify?**
   Irish □ Irish traveller □ Any other white background □ African □
   Any other black background □ Chinese □ Any other Asian background □ Other □
5. **Do you smoke cigarettes?** Yes □ (How many per day) ____ Used to □ No □
6. **How often do you have a drink containing alcohol?** Never □ Monthly, or less □
   2-4 times a month □ 2-3 times a week □ 4 or more/week □
7. Are you taking any medication? Yes □ (Name medication)_____ No □
8. **Have you taken any NSAIDS or aspirin medication in the last 10 days?**
   Yes □ No □
9. **Do you exercise? Yes □ No □ If yes, how many times per week? _____**
10. **Have you performed strenuous exercise in the last 24 hours? _____**
11. **In general, would you say your health is?** Excellent □ Good □ Fair □ Poor □
12. **Do you have any physically limiting conditions?** Yes □ No □ If yes specify details _____
13. **Have you ever been advised by a physician to avoid any type of exercise or lifting any weight?** Yes □ No □ _____
14. **How physically fit would you consider yourself?** Unfit □ Fairly fit □ Fit □ Very Fit □
15. **Do you (or does someone in your family) have a cardiac condition or have you ever had cancer?** Yes □ No □ If yes, Details: _____If yes to either, when were you diagnosed? ____
16. **What time do you usually go to sleep and wake up? _____**
17. **Do you work during the day time or at night? _____**
18. **At what part of the day do you consider yourself most active? _____**
19. **What have you eaten today and what time? _____**
20. **Do you prefer savoury or sweet foods? _____**
21. **How many calories do you think you consume per day? _____**
22. **How many calories do you think you 'burn' each day? _____**
23. **What do you estimate your weight and height to be? _____**

PARTICIPANT INFORMATION LEAFLET

Title of study: To screen platelet function in the General Irish Population.

Names of Researchers: Ms. Laura Twomey, Dr. Ronan Murphy.

Location: Science Gallery, Trinity College, Dublin.

Introduction:

The purpose of this project is to profile platelet reactivity in a cohort of the Irish population. Platelets are tiny fragments of larger cells which circulate within the blood stream. Their main function is to aid in primary haemostasis - blood clotting. They complete this by sticking to an injured vessel wall and then sticking to each other to form a small clot. Platelet activity can vary amongst individuals of varying health status/fitness/age and is increasingly being noted as a marker of vascular health.

Exclusion from participation: *You cannot participate in this study if you are:*

1. Under the age of 18
2. Know you are, or think you might be, infected with Hepatitis B or C
3. Know you are, or think you might be, infected with HIV (the AIDS virus) have, or have had a sexual partner who is infected with hepatitis or HIV.
4. Are, or may be pregnant.

Procedures:

In order to obtain results I understand that the following procedures need to take place:

- I am required to complete a general questionnaire for FAT to complete the TANITA measurement before the platelet test.
- A single, small (4.5ml) blood sample will be taken for this test. This will take about five minutes.
- This blood sample will be processed on the cone and plate analyser to determine platelet function and the blood counter to determine platelet count.

Why is blood sampling required? Platelet activity can only be measured in blood samples. An instrument called a cone and plate analyser will help to measure your platelet activity.

Benefits:

This test will be extremely informative and will provide you with a better understanding of how blood cells work in the body. You will be provided with your own full platelet function profile screen readout which includes platelet number, platelet activity - adhesion and aggregation.

Risks

There are no major risks involved. Blood will be taken by a qualified phlebotomist. You may feel a slight discomfort when the sample is drawn, a very small needle size is used to reduce this. A tiny bruise could develop where the sample took place. There is a small risk of fainting, participant can choose to sit or lie down for the test.

Confidentiality:

Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone outside the study group. Data will be stored in password protected files at Science Gallery Trinity College.

The research may be used for a PhD thesis, published in scientific journals and / or presented and discussed at scientific meetings, without revealing any of my personal details.

Compensation: *This study is covered by standard institutional indemnity insurance. Nothing in this document restricts or curtails your rights.*

Voluntary Participation: *This research is voluntary and you may decide to withdraw at any stage.*

Stopping the study: *I understand that the investigators may withdraw my participation in the study at any time without my consent.*

Permission: This study has ethical approval from TCD and DCU ethics.

Further information: Please contact Dr. Ronan Murphy or Ms. Laura Twomey who can be contacted at 017008824 or laura.twomey2@mail.dcu.ie for any further information you require.

FAT LAB GENERAL CONSENT FORM

Letter of Consent to partake in experiments during the FAT LAB exhibition at the Science Gallery, Trinity College Dublin, May 16th – June 29th 2014.

Research Team: Science Gallery Trinity College Dublin (TCD), Jennifer Fortune, Department of Physiotherapy, School of Medicine, Trinity Biosciences Institute,TCD: Suzanne Doyle, Joanne Lysaght and Jacintha O'Sullivan, Department of Surgery, School of Medicine,TCD: David Hevey, Department of Psychology,TCD: Helen Roche and Aoibheann Mc Morrow, Institute of Food and Health UCD: Laura Twomey, Ronan Murphy, Johann Issartel, School of Health & Human Performance, Faculty of Science and Health, DCU.

You have received this consent form because you have indicated that you wish to take part in experiments conducted during the FAT LAB exhibition at the Science Gallery, Trinity College Dublin. FAT LAB is delivered by Science Gallery in partnership with Trinity Biosciences Institute, Trinity College Dublin.

The experiments explore different aspects of the theme of FAT, using simple computerized tests, questionnaires and scale ratings, fitness tests and measurement of various physiological parameters. These hands-on, real data collecting experiments are connecting with research groups working in the area of nutrition, obesity and food and health research - in Trinity Biomedical Sciences Institute, TCD; School of Medicine, TCD; Trinity College Institute of Neuroscience and School of Psychology; Institute of Food and Health, Conway Institute, UCD; Obesity Research Group, St Vincent's University Hospital, School of Health and Human Performance, Dublin City University. These will include research currently being carried out in the areas of sensory motor perception and coordination, changing physiological parameters in response to activity levels, energy expenditure, fitness, obesity and cognition, and on the type of food we eat as well as body image and perception of obesity. The experiments will explore these relationships across the lifespan - young adults (18 years and up), middle aged and older people.

If you wish to take part in these experiments, please read this consent form carefully and confirm your consent by signing the form.

Signing this consent form does not yet represent agreement to participate in any of the specific experiments but allows us to collect and retain some background information about you and to assign you a unique Participant ID Code which you will need in order to volunteer for any of the individual experiments. As you walk around the gallery you will see that there are a variety of different experiments being conducted. A mediator will be available at each experiment station to explain what experiments are being conducted and to answer any questions you may have.

You are under no obligation to take part but if you wish to volunteer for a particular experiment you may then be asked to fill out a separate consent form, specific to that experiment, and to provide your Participant ID Code. *Please note research results take time to process and we will not be able to give you specific individual feedback after every experiment. Feedback or results cannot be emailed to any participants due to the anonymous nature of the study.*

TERMS OF CONSENT

I hereby give my consent to participate in the experiments carried out during the FAT LAB exhibition. The consent I provide pertains to experiments running as part of this specific exhibition only and not to any other experiments conducted by members of the research team outside the exhibition. (in the case of which there will be a separate consent from the researcher for any participation in experiments outside of the exhibition).

Once I have signed this consent I understand that

I will be assigned a Participant ID Code and become a member of the FAT LAB participant panel

As a member of the FAT LAB participant panel I consent to provide demographic information to the researchers involved, and answer questions relating to: *sex; age; education; gender; relationship status; family circumstances; employment status; recreational habits, general health and lifestyle.* I understand that I can decline to provide some or all of the information requested. I understand that this demographic information will never be made available to anyone other than members of the research team or their assistants. The demographic information I have provided and the data collected from me will be stored anonymously under my ID code only. I understand that my ID code cannot be linked to my name or other identifiable information in any way. (*The Freedom of Information Act does not apply to data stored anonymously).* I also understand that any data obtained through my participation in a research study will be treated as confidential and processed only in accordance with the Data Protection Acts, and that they will be used only for the purposes of research.

Signing this consent form does not represent agreement to participate in any of the experiments being conducted at the FAT LAB exhibition. If you wish to participate in an experiment you may be asked to read a separate information sheet and to sign a separate consent form.

I may withdraw my participation at any time during any study;I will not be requested to participate in any study that could be foreseen to be detrimental to a person's well-being, under normal circumstances, and that every study that I will be invited to participate in will have received prior approval from the relevant ethics committees within Trinity College Dublin. (*You will be informed of the general nature of any study before participating, and further explanation of the aims of the study can be provided after you have completed your participation, at which time any further questions may be raised*).

Please note that in the case of any measured results that concern you, please consult your GP.

I, the undersigned, give my informed consent to take part in the FAT LAB experiments running at the Science Gallery, Trinity College Dublin.

Full Name: _____ Signed: _____ Date:

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person legally competent to give consent. Participants must be over 18 years of age for data to be used.

NAME OF PARTICIPANT, PARENT OR GUARDIAN:
…….....

SIGNATURE: ……...

RELATION TO PARTICIPANT:……

Statement of Investigator's Responsibility: I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Contact emails for researchers:

Jennifer Fortune (fortunej@tcd.ie), Suzanne Doyle (doyles4@tcd.ie), Joanne Lysaght (jlysaght@tcd.ie), David Hevey (heveydt@tcd.ie), Aoibheann Mc Morrow (aoibheann.mcmorrow@ucdconnect.ie),Laura Twomey (twomey.laura@gmail.com), Johann Issartel (johann.issartel@dcu.ie), Helen Roche (helen.roche@ucd.ie), Ronan Murphy (ronan.murphy@dcu.ie)

Relationship between platelet and haematological parameters

The main finding was that platelet count and plateletcrit were significantly associated with platelet adhesion as shown in Figure A1. There were no significant correlations between platelet volume indices (MPV, PDW and PLCR) and any blood cell measurement (Table A1). WBC was significantly correlated to PCT and PLT (Table A2).

		Platelet parameter					
		PLT	**MPV**	**PDW**	**PLCR**	**PCT**	**SC**
Male	**PLT**	1					
	MPV	-.224	1				
	PDW	-.173	.970*	1			
	PLCR	-.181	.995*	.980*	1		
	PCT	.919*	.167	.222	.167	1	
	SC	.416*	-.036	-.025	-.024	.470*	1
	AS	.081	.150	.172	.187	.033	.368*
Female	**PLT**	1					
	MPV	-.361*	1				
	PDW	-.345*	.947*	1			
	PLCR	-.344*	.993*	.965*	1		
	PCT	.897*	.091	.002	.021	1	
	SC	.418*	-.142	-.188	-.188	.387*	1
	AS	.262*	.059	.041	.059	.273*	.316*

Table A1: Correlation between platelet indices and platelet function. Values are r squared values. PLT - Platelet count, PDW - Platelet distribution width, MPV - Mean platelet volume, PLCR - Platelet large cell ratio, PCT - Plateletcrit, SC - Surface coverage, AS - Aggregate size. *P <0.05. Partial correlation adjusting for age.

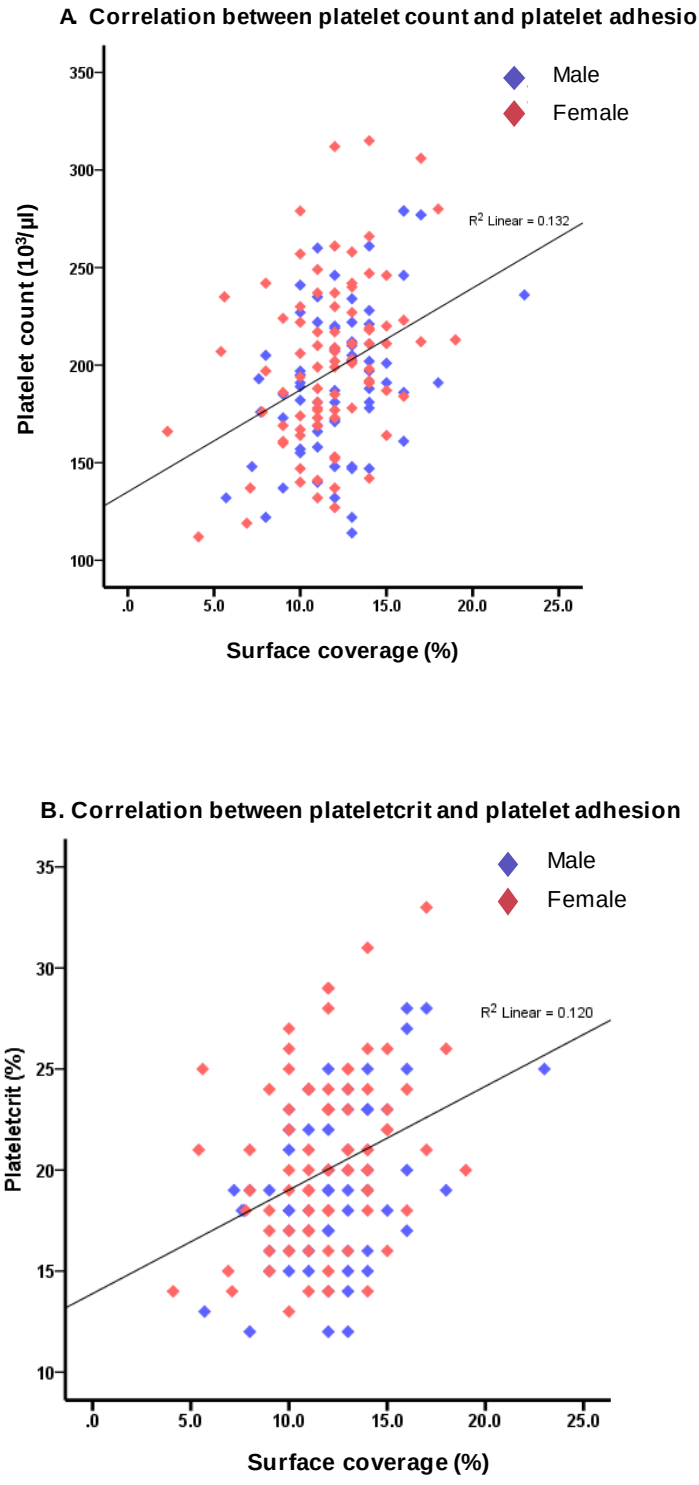


Figure A1: Correlation between platelet indices and platelet function measurements. Left: Scatterplot depicting relationship between PLT and platelet adhesion in males and females. Right: Scatterplot showing the relationship between PCT and platelet adhesion in males and females.

Table A2: Correlation between platelet and haematological parameters. PLT - Platelet count, PDW - Platelet distribution width, MPV - Mean platelet volume, PLCR - Platelet large cell ratio,

PCT – Plateletcrit, SC - Surface coverage, AS – Aggregate size. WBC – White blood cell count, MCV – Mean corpuscular volume, RDW – Red cell distribution width. *P<0.05. Partial correlation adjusting for age.

Haematological parameters				
		WBC	**MCV**	**RDW**
Female				
	PLT	.331*	-.276*	-.193
	PCT	.354*	-.338*	.033
	SC	.230*	.134	.108
	AS	.190	.154	.111
Male				
	PLT	.332*	-.044	.021
	PCT	.337*	-.061	.043
	SC	.138	.184	.268*
	AS	.178	.042	.282*

Relationship between haematological parameters and measures of obesity/overweight

To examine the relationships between red blood cell and white blood cell parameters and various parameters of overweight and obesity, correlation analysis was performed between these variables (Table A3, A4 and A5).

Table A3: Correlation between RBC, WBC and BMI. WBC - White blood cell count, RBC - Red blood cell count, HGB - Hemoglobin, HCT - Hematocrit, MCV - Mean corpuscular volume, RDW - Red cell distribution width. Partial correlation accounting for age.

Haematological parameter							
		WBC	**RBC**	**HCT**	**HGB**	**MCV**	**RDW**
BMI	**Male**	.095	.153	.226	.193	.035	.097
	Female	.192	.129	.050	.079	.048	·093

Table A4: **Correlations between waist circumference and hematological parameters.** WBC - White blood cell count, RBC - Red blood cell count, HGB - Hemoglobin, HCT - Hematocrit, MCV - Mean corpuscular volume, RDW - Red cell distribution width. *P<0.05. Partial correlation adjusting for age.

		Haematological parameter					
		WBC	**RBC**	**HCT**	**HGB**	**MCV**	**RDW**
WC	**Male**	.072	.205	.192	.152	.153	.150
	Female	.268*	.270*	.146	.179	.187	.028

Table A5: Relationship between BIA body composition measurements and haematological parameters in females. WBC - White blood cell count, RBC - Red blood cell count, RDW - Red cell distribution width. BF % - Body fat percent, VAT - Visceral adipose tissue, FM - Fat mass, FFM - Fat free mass, MM - muscle mass, TBW % - Total body water percent, *P<0.05. Partial correlation adjusting for age. There was no correlation between HCT, HGB or MCV and any BIA measurement.

	BIA body composition measurement					
Female	**BF**	**VF**	**FM**	**FFM**	**MM**	**TBW**
WBC	.269*	.168	.184	-.028	-.051	-.284*
RBC	.239*	.211	.264*	.053	.036	-.237*
RDW	.005	.028	.020	.134	.306*	.024

Variation in platelet function according to smoking status

Based on the questionnaire responses, subjects were categorized as current, previous or nonsmokers. This enabled comparisons of platelet parameters between groups shown in Figure A2. There was no difference in MPV between groups. PDW was slightly higher in current smokers in females compared to non-smokers.

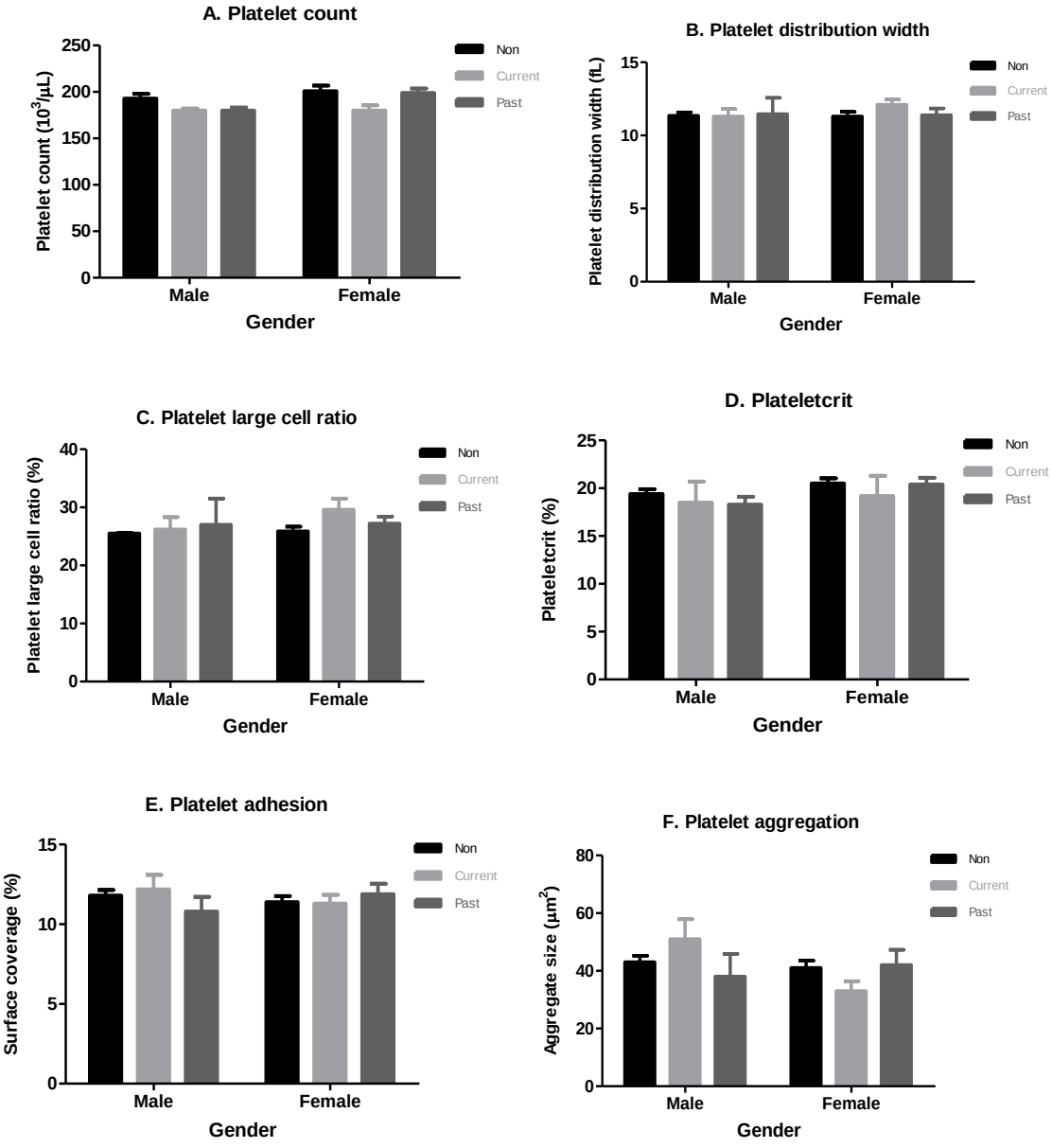


Figure A2: Comparison of platelet parameters between smokers, non-smokers and past smokers. Graphs represent mean ± SEM. One way ANCOVA adjusting for age.

Variation in platelet function according to Ethnicity

Variation in platelet indices according to ethnicity, a non-modifiable risk factor for CVD was examined. Subjects provided their ethnicity upon questionnaire completion. For comparison purposes, subjects were divided into three ethnic groups: Irish, Any other white background (AOWB) and Other (comprising Asian, middle eastern and Indian nationalities) shown in Figure A3.

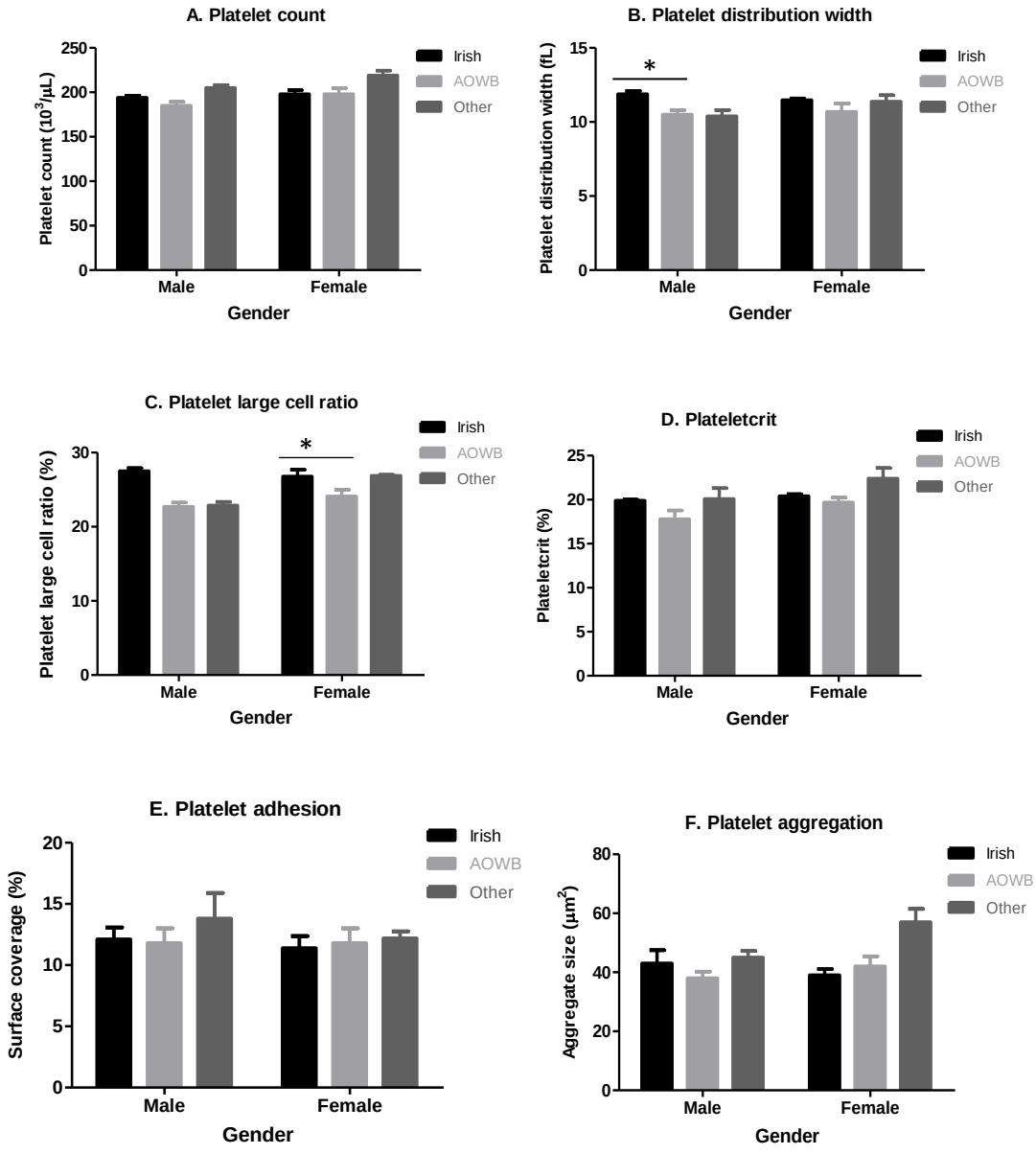


Figure A3: Comparison of platelet parameters according to ethnicity. Graphs represent mean ± SEM. One way ANCOVA adjusting for age. *P< 0.05.

Variation in platelet function and haematological characteristics according to Age

To assess the relationship between age, platelet parameters and haematological parameters, correlation analysis was performed, as shown in Tables A6 and Table A7. The main findings were that platelet count and plateletcrit decrease with aging, and platelet aggregation is significantly associated with aging in females only. To compare variances in platelet parameters between different age categories, subjects were divided into four groups based on their age shown in Figure A4. Main findings include a significant difference in male PLT between the 50-60 year and 60 + year age brackets. No changes in PDW or MPV were observed.

Table A6: Correlation between age and platelet indices. PLT - Platelet count, PDW - Platelet distribution width, MPV - Mean platelet volume, PLCR - Platelet large cell ratio, PCT – Plateletcrit, SC - Surface coverage, AS – Aggregate size. * P<0.05. Partial correlation adjusting for BMI.

		Platelet parameter						
		Plt	**MPV**	**PDW**	**PLCR**	**PCT**	**SC**	**AS**
Age	**Male**	-.279	.003	.027	.010	-.237	.017	.149
	Female	-.115	.076	.099	.087	-.111	.090	.293*

Table A7: Relationship between age and leukocyte/erythrocyte parameters. WBC - White blood cell, RBC – Red blood cell, MCV – Mean corpuscular volume, RDW - Red cell distribution width. *P< 0.05. Partial correlation adjusting for BMI.

		Blood cell parameter			
		WBC	**RBC**	**MCV**	**RDW**
Age	**Male**	-.274*	-.394*	.379*	.125
	Female	-.262*	-.170	.083	.260*

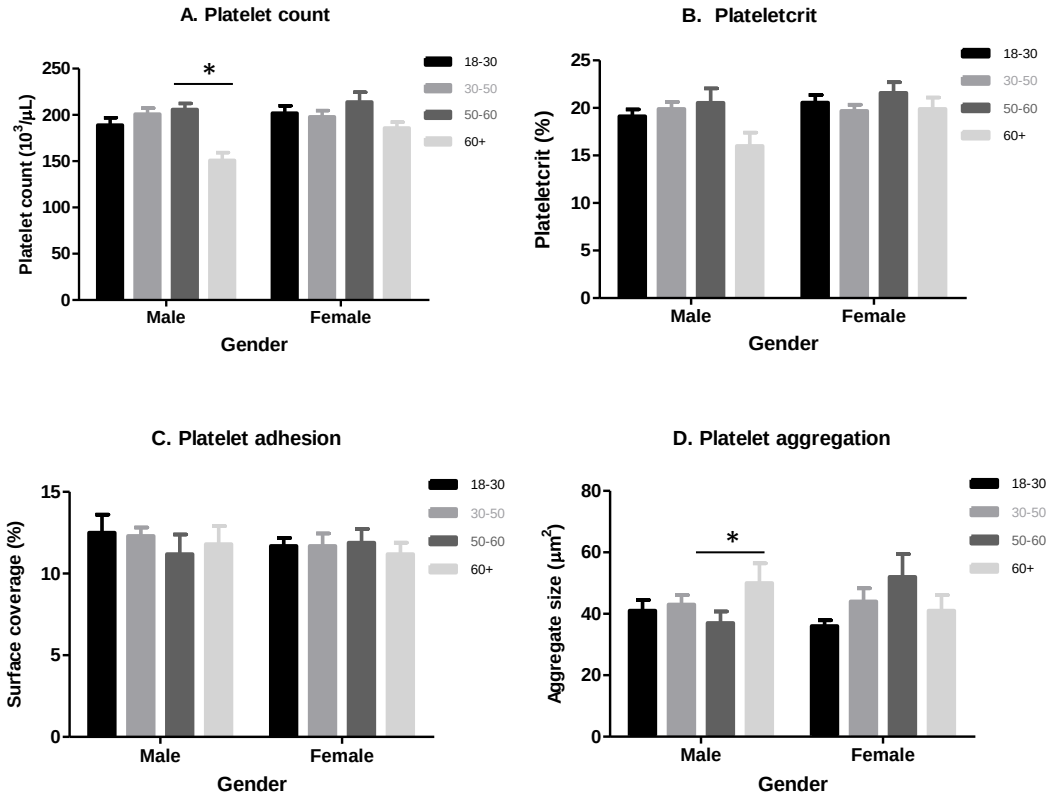


Figure A4: Age related changes in platelet parameters. Graphs represent mean ± SEM variances in A – Platelet count, B – Plateletcrit, C – Platelet adhesion and D – Platelet aggregation. *P< 0.05. One-way ANOVA and independent *t*-test.

Variation in platelet function according to blood pressure and resting heart rate

A cohort of subjects chose to receive blood pressure (BP) measurements. Subjects were categorised into groups based on their systolic and diastolic blood pressure outlined in Table A8. Correlation analysis was performed to elucidate the relationships between BP, RHR and platelet parameters (Table A9). There was no correlation between BP and platelet parameters in males or females. The most pertinent result was the positive correlation between aggregate size and diastolic blood pressure in the prehypertensive (PHT) group. There was a positive correlation between BP and RBC parameters in males, shown in Table A9.

Table: A8: Blood pressure classes. Numbers are percentages of the subject population in each category. PHT – Prehypertensive, Stage 1 HT – Stage one hypertension.

Blood Pressure					
	Systolic			Diastolic	
	Male	Female		Male	Female
Normal (<120)	60 %	72%	Normal (<80)	76%	74%
PHT (120-139)	24%	19.5%	PHT (80-90)	18%	20%
Stage 1 HT (140 +)	16%	7.5%	Stage 1 HT (90+)	6%	6%

Table A9: Correlation between blood pressure, resting heart rate, and platelet parameters. PLT - Platelet count, PDW - Platelet distribution width, PCT – Plateletcrit, SC - Surface coverage, AS – Aggregate size. DBP – diastolic blood pressure. PHT - prehypertensive. *P <0.05. Partial correlation adjusting for age and BMI.

Platelet parameter					
		PLT	**PCT**	**SC**	**AS**
SBP					
	Male (all)	.032	.095	.108	-.070
	Female (all)	.101	.157	.026	-.006
DBP					
	Male (all)	-.003	.014	-.028	.042
	Female (all)	.052	.061	-.025	.084
	Female (PHT)	.293	.246	.368	.684*
RHR					
	Male	.061	.061	-.067	-.095
	Female	.250*	.298*	.044	-.069

Table A10: Correlation between haematological indices, RHR and blood pressure in males. WBC – White blood cell count, RBC – Red blood cell count, HCT – Haematocrit, HGB – Haemoglobin, SBP – Systolic BP, DBP – Diastolic BP. *P<0.05. Partial correlation adjusting for age and BMI.

Haematological parameter				
		RBC	HCT	HGB
Male				
	SBP	.512*	.507*	.496*
	DBP	.291*	.300*	.244
	RHR	.138	.115	.157

Relationship between platelet parameters and blood cells

In peripheral blood, there is ample interplay between RBCs, WBCs and platelets (Ho, 2004) and altered levels of blood cells and their morphology have been associated with CVD (Maajid 2013; Cetin *et al.,* 2014;). Platelet adhesion and aggregate size, as assessed by the Impact-R system is influenced by platelet indices, RBC and WBC (Shenkman et al, 2000; Peerschke *et al.,* 2007). In this view, we investigated the associations between the various indices of each blood cell to interpret the multicellular contribution to thrombogenesis and CVD risk.

As shown in Table A1, there was no correlation between PDW, MPV and PLCR with either platelet adhesion or aggregation. To support this finding, PLCR was not associated with platelet aggregation in a study by De Luca *et al.,* (2010), whilst Beyan *et al.,* (2006) also found no correlation between MPV, PDW and platelet aggregation in healthy subjects. Contrarily, platelet adhesion was significantly positively correlated with PCT and PLT in males and females (Figure 3.1) and in female's, aggregation was significantly correlated to PCT and PLT.

Table A2 highlights the relationship between platelet and haematological parameters. Both PLT and PCT were positively associated with WBC in both genders, in concordance with the MOLI-SANI study (Santimone *et al.,* 2011). This association remained significant when age, BMI, and blood pressure were adjusted for with partial correlations. Furthermore, WBC was positively associated with platelet adhesion in males and females, and this correlation was significant in females, highlighting the link between platelets and inflammation. Adhesive mechanisms involving the interaction between platelets and WBC include the binding of P-selectin on platelets to P-selectin glycoprotein ligand -1 (PSGL-1) on leukocytes. PSGL-1 prompts a signalling response to activate leukocytes by inducing a conformational change of the β-2 integrin. PSGL-1 binding to monocytes results in the formation of platelet-monocyte aggregates and has the potential to prompt inflammation by the cyclooxegenase-2 (COX-2) pathway (Lam *et al.,* 2015). This interaction between activated blood cells could explain the correlation between WBC and PLT suggesting that platelets regulate other immune cells and mediate a broad range of physiological responses. To highlight the importance of the WBC/PLT interactions, a novel biomarker, the platelet to lymphocyte ratio (PLR) has recently been suggested as a combined biomarker of CVD risk (Song *et al.,* 2016).

The association between RBC count and morphological features with platelet parameters was also studied. We found no associations between RBC, HCT, HGB and any platelet parameter. Correspondingly, a cross sectional study of 215 healthy volunteers showed no correlation between PCT and HCT or between MPV and MCV (Wiwanitkit, 2004). Significant correlations between distribution widths, PDW and RDW were noted in two separate cross sectional studies (Wiwanitkit 2004; Saouli *et al.*, 2007) suggesting that anisocytosis (unequal sized cells) of RBCs and platelets could co-occur. This association was not observed in this study, but there did appear to be an association between MCV and PLT/PCT in females (Table A2). Iron deficiency is associated with reduced MCV, increased RDW and increased PLT, which could explain this observation, as females tend to have lower iron reserves than males (Rushton *et al.*, 2001; Akan *et al.*, 2000).

Lower HCT levels have been associated with abnormal platelet adhesion (Varon *et al.*, 1997; Kenet *et al.*, 1998). HCT was not correlated to platelet adhesion. This was also the case in other studies investigating variability in platelet function in healthy volunteers (Neeves et al, 2013). This was likely due to the fact that HCT and PLT counts in this study were within the normal ranges of (.035 - .050 g/dL) and (150,000 – 450,000 x 10^3/µL) respectively. RDW was associated with platelet function and was positively correlated to adhesion and aggregation in males only. RBC's are known to encourage platelets toward the vessel wall (Chestnut and Han, 2013) which can affect platelet adhesion and aggregation. This result indicated that a larger range of RBC size was linked to increased platelet adhesion and aggregation in males.

Tables A3-A5 displays the association between RBCS, WBCs and measures of body composition. In this study, WBC was positively correlated with fat mass, body fat %, and visceral fat in females only (Charles *et al.*, 2012) and RBC was positively correlated with fat mass in females, suggesting the simultaneous increase in all blood cells as body composition worsens, reaching an inflammatory state. Elevated WBC has been associated with obesity and inflammation (Dixon and O'Brien 2006).

Smoking

Cigarette smoking has long been identified as a major modifiable risk factor for CVD. Platelet activation appears to be one of the main dynamics by which smoking mediates the pathogenesis of CVD (Ichiki *et al.*, 1996). Platelet hyper-activation has been associated with smoking status in CVD patients. (Pamukcu *et al.*, 2011) However, there is a lack of studies

evaluating all platelet indices parameters, to acquire a global view of platelet function in healthy subjects. We therefore elucidated the variance between platelet indices and function in smokers, non-smokers and past smokers in this study.

The majority of participants were non-smokers, with only 8% and 8.5% of males and females' current smokers. A recent cross sectional study of over 500 healthy males demonstrated elevated PLT in smokers compared to non-smokers, and reduced PCT in the smoker group (Ghahremanfard *et al.,* 2015). Similarly, PCT was reduced in smokers but in contrast, Figure A2 indicates that PLT was reduced in smokers and past smokers compared to non-smokers, with minimal differences. This is in agreement with work by Varol *et al.,* (2013) who observed that PLT was significantly lower in the smoker group compared to control group across gender.

Smoking can induce both long-term and short-term effects on platelets. A recent study showed that even brief smoking can alter PLT and cause damage to the endothelium. One bout of smoking increased platelet MP concentrations and percentage of MP's exposing the platelet activation marker CD62P in healthy subjects, suggesting stress induced release of MP's from smoking (Mobarrez *et al.,* 2014. This study showed that chronic male chronic smokers had slightly elevated platelet adhesion compared to non-smokers. They also displayed increased platelet aggregate size (51 μm^2 compared to 42 μm^2). This result is in accordance with a recent study examining the effect of cyclooxygenase-1 (COX-1) mediated platelet function in healthy males whereby collagen induced platelet aggregation was higher in smokers than non-smokers (Loke *et al.,* 2014).

Ethnicity

Ethnicity is a significant non-modifiable risk factor for developing CVD. In a population based study of different ethnic groups in Canada, Europeans had the highest incidence of atherosclerosis compared to South Asians and Chinese (Anand *et al.,* 2000). Subjects in our population were primarily of an Irish descent (70%) with 21% of subjects categorised as any other white background (AOWB) and 5% categorised as other. Interestingly, there were variances observed amongst platelet indices and platelet function measurements shown in Figure A3. Of the platelet indices, PLCR was highest in those of an Irish descent compared to other nationalities and was significantly higher in Irish males compared to males from any other white background (AOWB). An initial study on platelet counts in different racial groups by Saxena *at al.* (1987) identified a discrepancy in PLT between healthy white, black, and latin-american people. Black women had significantly increased PLT than white

women. Ethnic variations have further been referred to by Segal and Moliturno (2006) whereby a cross sectional study of 12,000 Americans showed that PLT was highest in non-Hispanic blacks compared to other ethnicities.

Figure A3 compares platelet function between ethnic groups. Our study showed that platelet aggregation and adhesion was highest in the "other" category compared to Irish and AOWB, however as the number of subjects in that category was small, it is difficult to draw conclusions. To support this finding, variation in platelet function has been illustrated in other studies, where other ethnic groups have displayed enhanced platelet aggregation compared to Caucasians, in particular platelet aggregation in response to PAR4-AP which activates platelets through the PAR4 thrombin receptor (Edelstein *et al.,* 2013).

Age

Data collected in this study also included information on non-modifiable CVD risk factors such as ethnicity and age. Aging and platelet function is a novel area of research.

Meta-analysis of over 40,000 subjects showed a strong inverse relationship between PLT and age (Biino *et al.,* 2011; Biino et al., 2013) whereby PLT decreased by 35% in males and 25% in females when compared to early childhood. Similar results have been noted in population based studies in France and the US (Segal and Moliterno 2006, Troussard, 2014). Correspondingly, our smaller cross sectional study also highlighted an inverse relationship between age and PLT in males and females (Table A6). However, the strength of these correlations were weak and not significant, likely due to the population size and the fact that majority of participants were between 20-30 years. The relationship between age and PLT does not appear to be linear as it remains stable during middle age (25-60) and decreases in old age (60+). Zhang *et al.,* (2015) also showed that age-related changes are very noticeable in very old age where PLT is diminished by 21% and 22 % in subjects (> 81) years compared to 18-30 year olds. There was no association between MPV, PDW or PLCR with age. Likewise, other studies have shown that the link between platelet volume indices and age yield contrasting results. Zhang *et al.,* (2015).

Regarding the mechanisms responsible for the age-dependent decline in PLT, it has been postulated that reduced hematopoietic stem cell (HSC) reserves could be an important factor, as there is a reduction of HSC reserve with aging. Epigenetic changes in the megakaryocyte genome such as hypomethylation of genes determining PLT or changes in histone acetylation with aging have been suggested to play an important role (Daly, 2011). Further investigation is required to detect these epigenetic mechanisms underpinning age-related change.

The variation in platelet function and age in Figure A4 point to a change in platelet phenotype with aging. The enduring thought was that platelet activity increases with age. Research on age-related changes in platelet function to date have deduced that aging results in increased platelet aggregation (Gleerup, 1995, Knight, 1997; Bastyr *et al.*, 1990). Use of modern flow based techniques (PFA-100) found that older subjects (> 40 years) had platelet CTs shorter than those seen in younger subjects and that females had longer CTs than males (Cho *et al.*, 2008). Contrastingly, Sestito *et al.*, (1999) found no correlation between age and platelet CT in a separate group of healthy individuals.

Kent *et al.*, (2010) used the Dynamic Platelet Function Assay (parallel plate flow chambers coated with human vWF) to measure platelet activation, adhesion and translocation on vWF in over 100 healthy subjects (aged 18-82, but only 3 older than 65) Platelet behaviour on vWF was significantly altered with ageing (Cowman *et al.*, 2015). Furthermore, these changes were more pronounced in women compared to men.

Similarly, we observed alterations in platelet function with aging (A3) using the flow based Impact-R assay. Platelet adhesion showed little association with age whereas there was a small positive significant association between platelet aggregation and age in females only. Stratified by age groups, platelet aggregation appeared stable until 60 years in males, after which it increased by approximately 20% compared to the younger age groups. A similar trend followed for females, however the peak in aggregation was observed in the 50-60-year-old age group compared to the younger categories. These results could be linked to the possible influence of sex hormones testosterone and estrogen. Testosterone has been linked to increased platelet TxA_2 receptors and increased platelet aggregation in response to arachidonic acid (Ajayi *et al.*, 1995; Campelo *et al.*, 2012). These age-related changes in platelet function may contribute to the augmented prevalence of thrombotic events seen with aging.

Platelet function changes with aging is most likely caused by changes in hematopoietic tissue, blood composition and vascular health (Jones *et al.*, 2016) or biochemical alterations in the platelet resulting in differential mRNA expression (Simon *et al.*, 2014), and increases in oxidative stress (Dayal *et al.*, 2013). However, a strong illustration of the extensive effects of aging is demonstrated by the age-related change in platelet mRNA and microRNAs, the molecular genetic mechanisms controlling the modifications (Simon *et al.*, 2014). Analysis of platelet RNA from healthy male and female subjects showed that 129 mRNA and 15 miRNAs were differentially expressed with age. The inverse correlation between these mRNA-miRNA pairs suggest that miRNA may regulate mRNA levels in aging. Further analysis of these miRNA revealed their role in platelet function pathways such as cytoskeletal organisation and vesicle transport. A new area of research will be how these changes in miRNA link to changes in the platelet proteome during ageing. This study showed that platelet indices varied with age and in particular PLT and PCT declined with aging in both men and women. Due to the cross sectional nature of this study, it is difficult to interpret the relationship between platelet indices and age and longitudinal studies are required.

Cardiovascular measures – hypertension and resting heart rate

Hypertension and RHR are major risk factors for CVD and increased blood pressure in individuals younger than 50 years old is linked to greater cardiovascular risk (Lee et a. 2012). Subjects were categorised into groups based on norms for age and sex and blood pressure (Thompson *et al.*, 2008). Hypertension is defined as a resting/consistently elevated systolic blood pressure equal to or above 140 mmHg and/or a diastolic blood pressure equal to or above 90 mm Hg. The majority of subjects in this cohort had normal systolic and diastolic BP, however, there was a substantial number of subjects who were categorised as pre-hypertensive with regard to both SBP and DBP (Table A8). Pre-hypertension, a systolic BP of 120-39 mmHg and/or a diastolic BP of 80-90 mmHg is the precursor to clinical hypertension and is associated with greater risks of CVD (Zhang and Li, 2011; Vasan *et al.*, 2001). Platelet dysfunction in hypertension is a potential mechanism of enhanced cardiovascular morbidity as some studies indicating that different parameters of platelet activation are returned to normal functioning levels with treatment for high BP (Yazici *et al.*, 2009).

We examined the relationship between BP, RHR and platelet parameters by correlation analysis (Table A9). There was no association between platelet function and SBP/DBP in

males or females, however when selectively examining the relationship in the pre-hypertensive group, there was a significant positive relation between platelet aggregation and DBP, Yazici *et al.,* (2009) have reported increased MPV and PCT values in pre-hypertensive subjects and more importantly, that 12 weeks of lifestyle modifications can reduce MPV to the level of normotensive subjects. This was not observed in this study, possibly due to the low number of subjects in the pre-hypertensive group. Increased platelet volume indices have been associated with the Framingham risk score for CVD (Maluf *et al.*, 2016). In hypertension, exposure of blood vessels to high pressure generates an alteration between the vasoconstrictors and vasodilator balance (Lip, 2003) subsequently initiating platelet activation and possibly promoting platelet aggregation. Increases in body composition measures in females with PHT could also be responsible for the elevated platelet indices and platelet aggregation values.

RHR has been identified as a predictor of cardiovascular mortality independent of CVD risk factors in the general population and in CVD patients (Mensink and Hoffmeister, 1997, Palatini, 2004, Custodis et al., 2013). In a recent meta-analysis of 46 studies and over one million patients from a general population, those with a RHR of over 80 bpm had a considerably increased risk of cardiovascular mortality (Zhang *et al.,* 2016). In general, a lower RHR indicates an increased heart function proficiency and overall cardiovascular fitness.

Table A9 displays correlations between RHR and selected platelet parameters. There was no association between RHR and platelet indices or function in males but a significant positive relationship between PLT and RHR and PCT and RHR in females, suggesting that a higher RHR was associated with increased platelet activation. This correlation was attenuated when adjusting for BMI, indicating that it was overweight measures, not RHR, were affecting platelet indices. Besides overweight, the shear stress experienced by blood vessels in subjects with a high RHR could be another mechanism for enhanced platelet activity as high shear stress can encourage platelet activation (Huang *et al.,* 2013). Only one study has investigated the relationship between RHR and the platelet indice MPV in the context of patients with obstructive sleep apnoea (OSA). Patients without considerable CVD risk factors also did not have enhanced MPV levels (Akyüz *et al.,* 2014). Pathophysiological mechanisms concerning RHR, platelet function and CVD include increased endothelial shear stress, a deficiency of NO, platelet aggregation and subsequently, the initiation of atherosclerosis (Arnold *et al.,* 2008).

Reference ranges for body composition and anthropometric measurements

1. Visceral adipose tissue (VAT)
   - Healthy 1-12
   - Unhealthy 12-50

2. Total body water percentage
   - Females: 45 to 60%
   - Males: 50 to 65%

3. Body fat percentage (see below) (From Thompson *et al.*, 2009)

ACSM Body Composition (% Body Fat) For Men and Women

Male	AGE				
Fitness Category	20-29	30-39	40-49	50-59	60+
Essential Fat	2 - 5	2 - 5	2 - 5	2 - 5	2 - 5
Excellent	7.1 - 9.3	11.3 - 13.8	13.6 - 16.2	15.3 - 17.8	15.3 - 18.3
Good	9.4 - 14	13.9 - 17.4	16.3 - 19.5	17.9 - 21.2	18.4 - 21.9
Average	14.1 - 17.5	17.5 - 20.4	19.6 - 22.4	21.3 - 24	22 - 25
Below Average	17.4 - 22.5	20.5 - 24.1	22.5 - 26	24.1 - 27.4	25 - 28.4
Poor	>22.4	>24.2	>26.1	>27.5	>28.5

Female	AGE				
Fitness Category	20-29	30-39	40-49	50-59	60+
Essential Fat	10 - 13	10 - 13	10 - 13	10 - 13	10 - 13
Excellent	14.5 - 17	15.5 - 17.9	18.5 - 21.2	21.6 - 24.9	21.1 - 25
Good	17.1 - 20.5	18 - 21.5	21.3 - 24.8	25 - 28.4	25.1 - 29.2
Average	20.6 - 23.6	21.6 - 24.8	24.9 - 28	28.5 - 31.5	29.3 - 32.4
Below Average	23.7 - 27.6	24.9 - 29.2	28.1 - 32	31.6 - 35.5	32.5 - 36.5
Poor	>27.7	>29.3	>32.1	>35.6	>36.6

4. Bone mass (see below)

Women: Average of estimated bone mass

Weight (lb)		
Less than 110 lb	110lb - 165 lb	165 lb and up
4.3 lb	5.3 lb	6.5 lb

Weight (kg)		
Less than 50 kg	50 kg - 75 kg	75 kg and up
1.95 kg	2.40 kg	2.95 kg

Men: Average of estimated bone mass

Weight (lb)		
Less than 143 lb	143 lb - 209 lb	209 lb and up
5.9 lb	7.3 lb	8.1 lb

Weight (kg)		
Less than 65 kg	65 kg - 95 kg	95 kg and up
2.66 kg	3.29 kg	3.69 kg

5. Fat free mass, muscle mass and BMR.
   No ranges for these are provided by the Tanita BIA analyser. BMR is dependent on calorie intake and weight and ranges were not provided.

Appendix B

School of Health and Human Performance

Dublin City University

Glasnevin

18 May 2015

Dear Parent,

As part of the ongoing study to compare vascular health in low and high fit teenagers in the health and human performance department of DCU, we are looking at platelet function in a group of high fit adolescents.

Your child will have a tablespoon of blood taken, before and after an exercise test which will last no longer than 15 minutes. They do not need to fast and should eat their normal breakfast on the day of the fitness test.

If you have any questions, please feel free to contact me on 0861219423 or twomey.laura@gmail.com

Yours Sincerely,

Laura Twomey

Body Composition - Data Collection Sheet

Name: _____

ID Code: _____

School: _____

Fitness Category: _____

Date of Birth: _____/_____/_____

Medical Conditions:

Cardiovascular Disease □

Pulmonary Disease □

Diabetes □

Asthma □

Other Medical conditions:

_____

_____

Height: _____ cm

Weight: _____kg

Waist circumference: _____ cm

Hip Circumference: _____cm

Adolescents Informed Consent Form

Research Study Title

Comparison of Cardiovascular Disease Risk Factors and Vascular Health in Low fit, Moderately Fit and High Fit Irish Teenagers

Principle Investigator

Prof. Niall M. Moyna, Centre for Preventive Medicine, School of Health and Human Performance

II. Confirmation of particular requirements as highlighted in the Plain Language Statement

Participant – please complete the following (Circle Yes or No for each question)

I have read the Plain Language Statement (or had it read to me) Yes ☐ No ☐
I understand the information provided Yes ☐ No ☐
I have had an opportunity to ask questions and discuss this study Yes ☐ No ☐
I have received satisfactory answers to all my questions Yes ☐ No ☐

III. Confirmation that involvement in the Research Study is voluntary

Your child may withdraw from the Research Study at any point.

IV. Advice as to arrangements to be made to protect confidentiality of data, including that confidentiality of information provided is subject to legal limitations

Your child's and other personal information will not be revealed, published or used in further studies. Your child will be assigned an ID number under which all personal information will be stored in a secure locked cabinet and saved in a password protected file in a computer at DCU. The named investigators will have access to the data. Data will be shredded after 5 years by Prof. Moyna.

Confidentiality is insured, but you must be aware that confidentiality of information provided can only be protected within the limitations of the law. It is possible for data to be subject to subpoena, freedom of information claim or mandated reporting by some professions.

V. Any other relevant information

If your child is in a dependent relationship with any of the researchers their involvement in the project will not affect ongoing assessment/grades/management or treatment of health at DCU.

VI. Signature:

I have read and understood the information in this form. My questions and concerns have been answered by the researchers, and I have a copy of this consent form. Therefore, I consent to allow my child to take part in this research project

Participants Signature: _____

Name in Block Capitals: _____

Witness: _____ **Date:**

Adolescents Assent Form

Study Title: Comparison of Cardiovascular Disease Risk Factors and Vascular Health in Low fit, Moderately Fit and High Fit Irish Teenagers

- My physical education teacher has talked to me about being part of a research study.
- I have been told that researchers from Dublin City University (DCU) will visit my school and measure by fitness using a bleep test
- I have been told that I may be selected at random to visit the Vascular Research Unit at DCU to undergo additional tests. I will also wear a small device called an accelerometer on my hip for 7 days to record my physical activity levels
- The visit to DCU will take place in the morning and will last for about 3 hours.
- I will not eat any food from 10 pm the previous evening. I will be allowed to drink water.
- I will not do any exercise that makes me tired the day before the bleep test in school or the tests in DCU.
- I will have about 2 tablespoons of blood taken from a vein in my arm. Drawing blood may cause a slight pain where the needle is inserted and may leave a bruise on my arm that will clear up in a few days
- A special ultrasound machine will take a picture of an artery in my neck. This will take about 15 minutes.
- The health of a blood vessel in my arm will also be measured using the ultrasound machine. The first step will involve blocking the blood flow in my arm for 5 minutes using a blood pressure cuff and then taking a picture of my blood vessel when the cuff in released. The second step involves spraying a chemical under my tongue and taking a picture image 3 minutes later. This test will take about 45 minutes,
- Stopping the flow of blood in my arm for 5 minutes may feel a little uncomfortable. The chemical that is sprayed under my tongue may cause a headache that may last for 5-10 minutes
- I will run on treadmill to see how fit I am. During the test I will wear a nose clip on my nose and a mouthpiece in my mouth.
- I will be allowed to stop any of the tests whenever I want.
- I may feel tired or be out of breath when I am running on the treadmill and my legs may feel tired
- If I wish, I can stop doing the tests at any time.
- If I wish, I may choose not to take part in any of the tests.
- I know that the people in DCU, my physical education teacher and my parents/guardian will not be upset with me if I decide not to take part in this study, or if I wish to stop taking part in the study.

SIGNED: _____DATE: _____

(Participant's name)

SIGNED: _____DATE: _____

(Witness name)

VO_2 max Data Collection Sheet

RHR: _____ bpm **Resting BP: ____/____**

Date of Test: ____/____/_____ **Time of Test: _____**

Tester's Name: _____ Polar HR Start time: ___

Stage	Time (mins)	**Speed/Slope**	**RPE-O**	**HR (bpm)**

Maximal Data:

HR max: _____ bpm, RPE-O max: _____, Absolute VO_{2max}: _____ l/min, Relative VO_{2max}:_____ ml/kg/min

INFORMED CONSENT FORM (Adults)

PROJECT TITLE: The effect of acute exercise on platelet function

PRINCIPAL INVESTIGATORS: Ms. Laura Twomey, Dr. Ronan Murphy

In order to obtain results, I understand that the following procedures need to take place:

1. I am required to complete a general questionnaire for the study.
2. Different anthropometric tests will be completed, including TANITA, waist and hip circumferences, skinfolds.
3. I am required to complete a maximal aerobic test for this study.
4. Two small (4.5ml) blood samples will be taken for this test, one pre V02 and one post VO_2 giving a total of 9mls.
5. The blood samples will be processed on the cone and plate analyser to determine platelet function and the blood counter to determine platelet count.
6. Flow cytometry will be used to assess platelet VASP phosphorylation and microparticle levels.

Why is blood sampling required? Platelet activity can only be measured in blood samples. An instrument called a cone and plate analyser will help to measure your platelet activity.

Assurance of Confidentiality

I understand that my confidentiality will be protected insofar as this is permitted by Irish law and that the research may be published in scientific journals and / or presented and discussed at scientific meetings, without revealing any of my personal details.

DECLARATION:

I have read, or had read to me, the information leaflet for this project and I understand the contents. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study. I understand that I may withdraw from the study at any time and I have received a copy of this agreement

PARTICIPANT'S NAME/ID: ……

CONTACT DETAILS: ……

PARTICIPANT'S SIGNATURE:……**Date:……...**

Statement of investigator's responsibility: I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

INVESTIGATOR'S SIGNATURE:……Date:…….

PAR-Q and YOU Questionnaire

Physical Activity Readiness
Questionnaire - PAR-Q
(revised 2002)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO		
☐	☐	1.	**Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?**
☐	☐	2.	**Do you feel pain in your chest when you do physical activity?**
☐	☐	3.	**In the past month, have you had chest pain when you were not doing physical activity?**
☐	☐	4.	**Do you lose your balance because of dizziness or do you ever lose consciousness?**
☐	☐	5.	**Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?**
☐	☐	6.	**Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?**
☐	☐	7.	**Do you know of any other reason why you should not do physical activity?**

If you answered YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- start becoming much more physically active – begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal – this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever – wait until you feel better; or
- if you are or may be pregnant – talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME _____

SIGNATURE _____ DATE _____

SIGNATURE OF PARENT or GUARDIAN (for participants under the age of majority) _____ WITNESS _____

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.

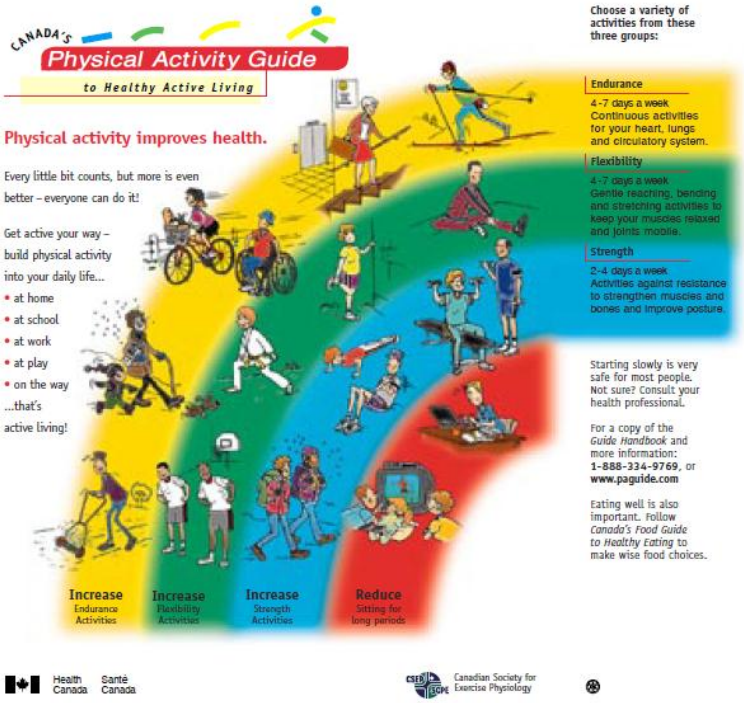
Supported by: Health Canada Santé Canada

continued on other side...

...continued from other side

PAR-Q & YOU

Physical Activity Readiness Questionnaire - PAR-Q (revised 2002)



Choose a variety of activities from these three groups:

Endurance
4-7 days a week
Continuous activities for your heart, lungs and circulatory system.

Flexibility
4-7 days a week
Gentle reaching, bending and stretching activities to keep your muscles relaxed and joints mobile.

Strength
2-4 days a week
Activities against resistance to strengthen muscles and bones and improve posture.

Starting slowly is very safe for most people. Not sure? Consult your health professional.

For a copy of the *Guide Handbook* and more information: **1-888-334-9769**, or **www.paguide.com**

Eating well is also important. Follow *Canada's Food Guide to Healthy Eating* to make wise food choices.

Get Active Your Way, Every Day – For Life!

Scientists say accumulate 60 minutes of physical activity every day to stay healthy or improve your health. As you progress to moderate activities you can cut down to 30 minutes, 4 days a week. Add-up your activities in periods of at least 10 minutes each. Start slowly... and build up.

Time needed depends on effort

Very Light Effort	Light Effort *60 minutes*	Moderate Effort *30-60 minutes*	Vigorous Effort *20-30 minutes*	Maximum Effort
• Strolling • Dusting	• Light walking • Volleyball • Easy gardening • Stretching	• Brisk walking • Biking • Raking leaves • Swimming • Dancing • Water aerobics	• Aerobics • Jogging • Hockey • Basketball • Fast swimming • Fast dancing	• Sprinting • Racing

Range needed to stay healthy

You Can Do It – Getting started is easier than you think

Physical activity doesn t have to be very hard. Build physical activities into your daily routine.

- Walk whenever you can – get off the bus early, use the stairs instead of the elevator.
- Reduce inactivity for long periods, like watching TV.
- Get up from the couch and stretch and bend for a few minutes every hour.
- Play actively with your kids.
- Choose to walk, wheel or cycle for short trips.
- Start with a 10 minute walk – gradually increase the time.
- Find out about walking and cycling paths nearby and use them.
- Observe a physical activity class to see if you want to try it.
- Try one class to start – you don t have to make a long-term commitment.
- Do the activities you are doing now, more often.

Benefits of regular activity:	Health risks of inactivity:
• better health • improved fitness • better posture and balance • better self-esteem • weight control • stronger muscles and bones • feeling more energetic • relaxation and reduced stress • continued independent living in later life	• premature death • heart disease • obesity • high blood pressure • adult-onset diabetes • osteoporosis • stroke • depression • colon cancer

Health Canada Santé Canada

Canadian Society for Exercise Physiology

Source: Canada's Physical Activity Guide to Healthy Active Living, Health Canada, 1998 http://www.hc-sc.gc.ca/hppb/paguide/pdf/guideEng.pdf

FITNESS AND HEALTH PROFESSIONALS MAY BE INTERESTED IN THE INFORMATION BELOW:

The following companion forms are available for doctors' use by contacting the Canadian Society for Exercise Physiology (address below):

The **Physical Activity Readiness Medical Examination (PARmed-X)** – to be used by doctors with people who answer YES to one or more questions on the PAR-Q.

The **Physical Activity Readiness Medical Examination for Pregnancy (PARmed-X for Pregnancy)** – to be used by doctors with pregnant patients who wish to become more active.

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Thomas, S., Reading, J., Shephard, R.J. (1992). Revision of the Physical Activity Readiness Questionnaire (PAR-Q). **Can. J. Spt. Sci.** 17:4 338-345.

For more information, please contact the:

Canadian Society for Exercise Physiology
202-185 Somerset Street West
Ottawa, ON K2P 0J2
Tel. 1-877-651-3755 • FAX (613) 234-3565
Online: www.csep.ca

The original PAR-Q was developed by the British Columbia Ministry of Health. It has been revised by an Expert Advisory Committee of the Canadian Society for Exercise Physiology chaired by Dr. N. Gledhill (2002).

Disponible en français sous le titre «Questionnaire sur l'aptitude à l'activité physique - Q-AAP (revisé 2002)».

Supported by: Health Canada Santé Canada

Global Physical Activity Questionnaire (GPAQ)

I am going to ask you about the time you spend doing different types of physical activity in a typical week. Please answer these questions even if you do not consider yourself to be a physically active person.

1. Does your work involve vigorous-intensity activity that causes large increases in breathing or heart rate like *[carrying or lifting heavy loads, digging or construction work*] for at least 10 minutes continuously?
2. In a typical week, on how many days do you do vigorous-intensity activities as part of your work?
3. How much time do you spend doing vigorous-intensity activities at work on a typical day?
4. Does your work involve moderate-intensity activity that causes small increases in breathing or heart rate such as brisk walking *[or carrying light loads*] for at least 10 minutes continuously?
5. In a typical week, on how many days do you do moderate-intensity activities as part of your work?
6. How much time do you spend doing moderate-intensity activities at work on a typical day?

The next questions exclude the physical activities at work that you have already mentioned. For example, to work, for shopping, to market, to place of worship.

1. Do you walk or use a bicycle (*pedal cycle*) for at least 10 minutes continuously to get to and from places?
2. In a typical week, on how many days do you walk or bicycle for at least 10 minutes continuously to get to and from places?
3. How much time do you spend walking or bicycling for travel on a typical day?

Now I would like to ask you about sports, fitness and recreational activities (leisure

1. Do you do any vigorous-intensity sports, fitness or recreational (*leisure*) activities that cause large increases in breathing or heart rate like [*running or football,]* for at least 10 minutes continuously?
2. In a typical week, on how many days do you do vigorous-intensity sports, fitness or recreational (*leisure*) activities?
3. How much time do you spend doing vigorous-intensity sports, fitness or recreational activities on a typical day?
4. Do you do any moderate-intensity sports, fitness or recreational *(leisure*) activities that causes a small increase in breathing or heart rate such as brisk walking, (*cycling, swimming, volleyball*) for at least 10 minutes continuously?
5. In a typical week, on how many days do you do moderate-intensity sports, fitness or recreational (*leisure*) activities?
6. How much time do you spend doing moderate-intensity sports, fitness or recreational (*leisure*) activities on a typical day?

The following question is about sitting or reclining at work, at home, getting to and from places, or with friends including time spent [sitting at a desk, sitting with friends, travelling in car, bus, train, reading, playing cards or watching television], but do not include time spent sleeping.

1. How much time do you usually spend sitting or reclining on a typical day? (Hours/Minutes)

Effect of acute exercise on haematological parameters

We simultaneously assessed the changes in white and red blood cell haematological parameters in conjunction with the platelet function parameters. Table A11 and A12 display the changes in these parameters after acute exercise. There were significant changes in WBC count in all cohorts and significant changes in RBC parameters in the HF adolescents and MF and HF adults.

Table A11: Effect of acute strenuous exercise on RBC and WBC characteristics in adolescents. Values are mean ± SEM. RBC – Red blood cell count, WBC – White blood cell count, HCT – Haematocrit, HGB – Haemoglobin, MCV – Mean corpuscular volume, RDW – Red cell distribution width. *P <0 .05. Paired samples t-test.

	Adolescent cohort		
Group	**Time point**		
MLF	**Pre**	**Post**	**P value**
WBC (10^3/µl)	5.64 ± 0.41	10.20 ± 0.21	*
RBC (10^6/µl)	4.98 ± 0.07	5.06 ± 0.10	.238
HCT (%)	44.37 ± 0.61	45.33 ± 0.86	.138
HGB (g/dl)	14.84 ± 0.21	15.22 ± 0.31	.112
MCV (fl)	88.51 ± 0.92	88.83 ± 0.98	.911
RDW (%)	13.08 ± 0.21	13.09 ± .18	.162
HF			
WBC (10^3/µl)	6.15 ± 0.45	9.83 ± 0.82	*
RBC (10^6/µl)	4.86 ± 0.12	5.06 ± 0.12	*
HCT (%)	42.64 ± 0.97	44.5 ± 0.95	*
HGB (g/dl)	14.19 ± 0.34	14.87 ± 0.34	*
MCV (fl)	87.84 ± 0.93	88.26 ± 0.89	.070
RDW (%)	13.35 ± 0.17	13.41 ± 0.17	.336

Table A12: Effect of acute strenuous exercise on RBC and WBC characteristics in adults. RBC – Red blood cell count, WBC – White blood cell count, HCT – Haematocrit, HGB – Haemoglobin, MCV – Mean corpuscular volume, RDW – Red cell distribution width. *P <0 .05. Paired samples t-test.

Adult cohort			
Group	**Time point**		
	Pre	**Post**	**P value**
MF			
WBC (10^3/µl)	7.95 ± .76	11.9 ± 1.2	*
RBC (10^6/µl)	4.72 ± .09	4.85 ± .13	.051
HCT (%)	43.35 ± .71	43.75 ± 1.13	*
HGB (g/dl)	14.1 ± .31	14.54 ± .38	.050
MCV (fl)	89.40 ± 0.90	90.21 ± 0.86	*
RDW (%)	12.81 ± 0.25	12.98 ± 0.28	.086
HF			
WBC (10^3/µl)	4.61 ± .11	4.81 ± .09	*
RBC (10^6/µl)	5.98 ± .37	9.4 ± .58	*
HCT (%)	42.7 ± .89	45.13 ± 1.9	*
HGB (g/dl)	14.2 ± .32	14.86 ± .31	*
MCV (fl)	92.70 ± 1.19	93.90 ± 1.36	*
RDW (%)	12.73 ± 0.24	13.10 ± .31	.097

Effect of exercise on haematological parameters

We simultaneously measured the effect of acute vigorous exercise on other haematological parameters. According to Cadroy *et al.*, (2002) the heightened thrombotic tendency may be related to the observed increased concentration of circulating blood cells and coagulation factors such as fibrinogen and vWF. Tables A11 and A12 show the change in RBC and WBC in adolescents and adults after exercise. In adolescents, there was a significant increase in WBC in both HF and MLF subjects. The adult cohort followed a similar trend. Elevated leukocyte levels after exercise has previously been reported (Risoy *et al.*, 2003) and is largely due to demargination (neutrophils in tissues rapidly entering into peripheral circulation) caused by increased blood flow, exercise- induced increases in epinephrine/cortisol levels and an acute inflammatory response (Kratz *et al.*, 2002).

The concurrent increase in PLT and WBC post exercise points to a possible interaction between these blood cells, and the possibility of increased platelet-leukocyte aggregates (PLAs). Studies in adults have shown increased platelet P-Selectin after exercise and formation of PLAs. Indeed, numerous subtypes of leukocytes have shown increased interactions with platelets after acute exercise including platelet-neutrophil (Aldemir and Cilic, 2005), platelet-granulocyte and platelet lymphocyte interactions (Hilberg *et al.,* 2008).

Changes in HCT occur quickly during and after exercise. There was an expected rise in HCT in all groups, likely due to a decrease in plasma volume and fluid loss from sweating. Erythropoiesis (production of RBCs) is also a common process during exercise. Huskins (2016) report that RBCs significantly contribute to thrombin generation in whole blood and therefore an increase in RBC post exercise may affect platelet activation. RBC increased after acute exercise in all groups, as depicted in Tables A11 and A12. Cadroy *et al.,* (2002) reported similar findings in HCT and blood cell counts after exercise in healthy males.

Appendix C

Measurement of Pain and Length outside Baths during the DI

Table A13: Visual assessment of pain during the DI. The VAS scale is an ease of use measurement of pain intensity. The scale was completed by the subjects each day during the DI. Using a ruler, the subject marked a point on the line to best represent their pain intensity. The distance from "pain free" to their mark is measured in millimetres (mm) and provides their VAS score (1-100).

	Day					
	DI 1	DI 2		DI 3 (post)		R+0 (recovery)
Subject	**pm**	**am**	**pm**	**am**	**pm**	**am**
A	ND	ND	0	0	50	55
B	ND	ND	68	35	75	76
C	55	72	68	35	50	54
D	35	60	70	70	80	68
E	24	30	53	70	60	52
F	25	53	44	45	40	50
G		85	59	69	58	55
H	20	72	78	79	88	54
I	28	0	0	0	0	0
J	0	0	0	0	0	0
K	24	50	34	18	11	15
L	24	23	19	22	15	10

Table A14: Length outside baths during the three days of dry immersion. For certain experimental procedures, hygiene reasons and other tests, subjects were removed from the baths for short periods of time and remained horizontal with the use of bed/trolleys. This table shows the day, subject and time (minutes) in which the subject was out of the baths.

		Subject											
Day		**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
DI1	Wash												
	Weigh t												
	Sheets	5		23	12	13	3	19	10	2			
	Tests	14	25	24	13	10	6	13	11	10	8	19	11
	total	**19**	**25**	**47**	**25**	**23**	**9**	**32**	**21**	**12**	**8**	**19**	**11**
DI2	Wash	43	25	26	25	19	19	18	27	15	17	25	32
	Weigh t	3	3	3		3	3	5	5	2	2	3	3
	Sheets	5	5	5	32			5	7			7	
	Tests			8					15				
	total	**51**	**33**	**42**	**57**	**22**	**22**	**28**	**54**	**17**	**19**	**35**	**35**
DI3	Wash	17	41	23	16	34	24	30	30	25	27	16	19
	Weigh t	8	3	3	4	3	3	3	3	3	3	3	2
	Sheets	5	10										
	Tests	202	218	219	212	216	195	182	180	196	187	191	173
	total	**232**	**272**	**245**	**232**	**253**	**222**	**215**	**213**	**224**	**217**	**210**	**194**
R+1	Weigh t	3	4	3	3	2	2	3	3	3	3	2	2
Tota l	mins	**305**	**334**	**337**	**317**	**300**	**255**	**278**	**291**	**256**	**247**	**266**	**242**

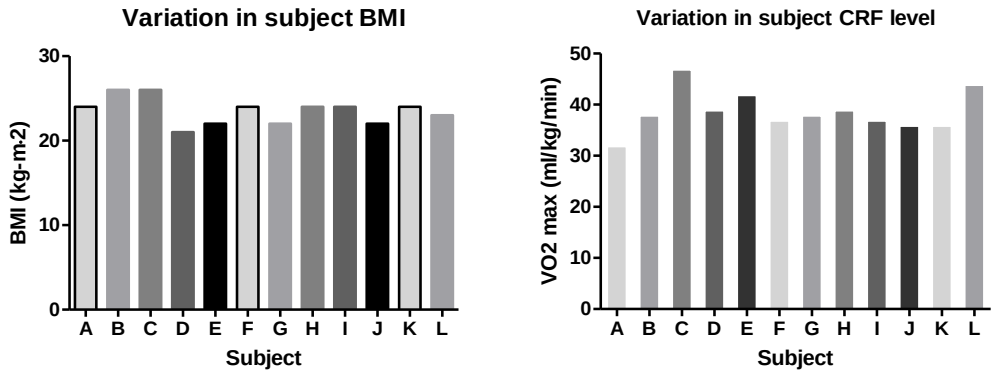

Figure A5: Cardiorespiratory fitness and BMI of each subject in the dry immersion study.

Effect of dry immersion on plasma protein biomarkers

Below are protein biomarkers which increased after DI and may be involved in platelet activation/function but which were also statistically significant and were not discussed as such in chapter 5.

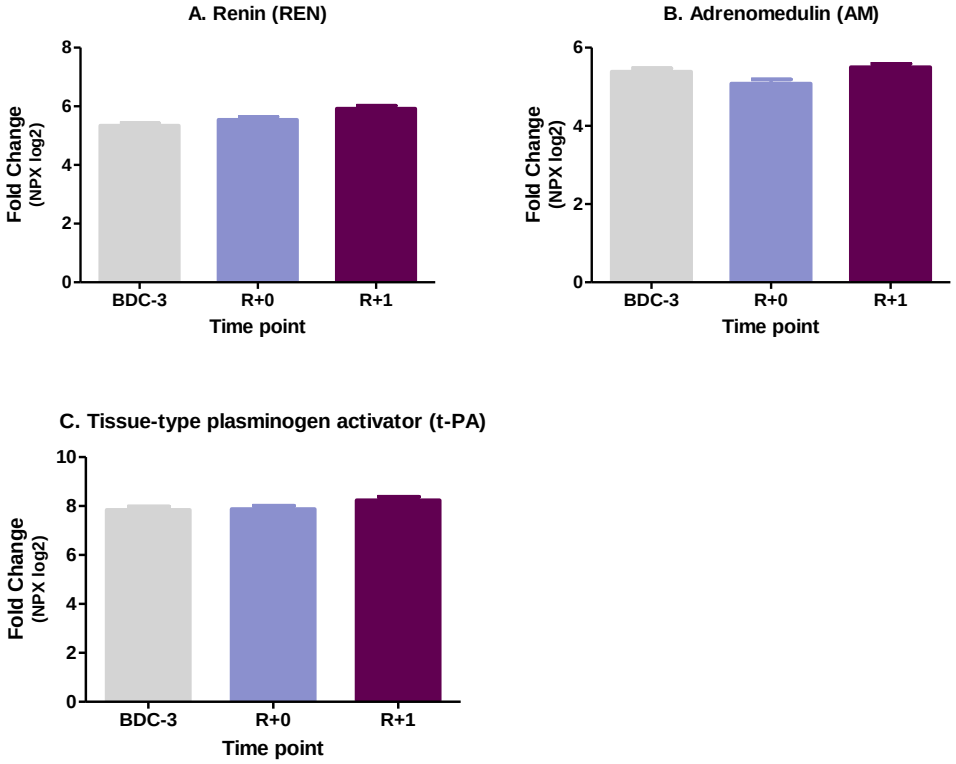

Figure A6: The effect of physical inactivity on TLR family gene expression. Graph A shows changes in Renin, Graph B shows changes in Adrenomedulin and Graph C shows changes in Tissue-type plasminogen activator. All data is expressed as mean ± SEM. Paired samples t-test and repeated measures ANOVA.

Renin (REN) was increased after DI, and unlike most of the other proteins, was elevated further at the recovery time point. Renin is involved in the body's renin-angiotensin system (RAS) which mediates plasma sodium concentration and arterial blood pressure. Angiotensins 1-9 have been shown to regulate platelet function in mice (Mogielnicki *et al.*, 2003) and have been reported to inhibit platelet aggregation (Fraga-Silva *et al.*, 2008). Adrenomedullin (AM) is a vasodilative peptide hormone which exerts significant positive influences on the cardiovascular system. AM also increases platelet cAMP levels. Plasma AM was significantly decreased post DI, suggesting the vasodilatory properties of the cardiovascular system were compromised. Tissue plasminogen activator (Tpa) was increased after the DI and further increased at the recovery period and is involved in the plasminogen activator system and the breakdown of blood clots.

mRNA TLR expression (gene expression)

Platelets contain several toll-like receptors (TLR) which can be activated by TLR ligands (Cognasse *et al.,* 2005). To further assess if physical inactivity had an impact on platelet inflammatory function, transcript expressions of TLR1-10 were quantified in total RNA extracted from platelets in ten subjects pre and post immersion. This is shown in Figure A7.

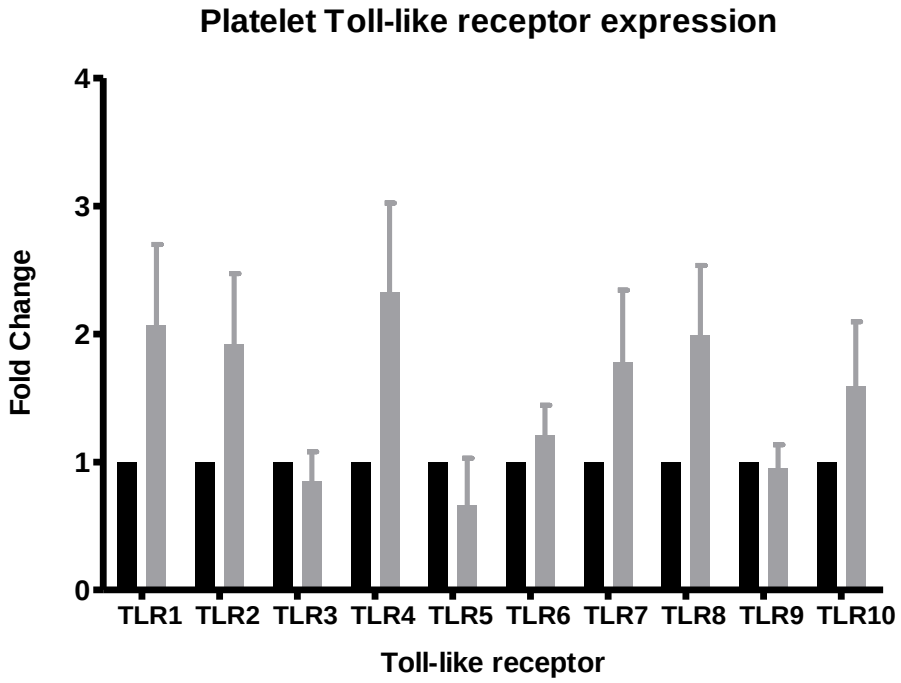

Figure A7: The effect of physical inactivity on TLR family gene expression. All data is expressed as mean ± SEM.

Platelets express a collection of pattern recognition receptors called toll-like receptors (TLRs) that identify molecular motifs called pathogen associated molecular patterns (PAMPs) and initiate immune responses (Cognasse *et al.,* 2015). Platelets express functional TLR 1-9, whilst TLR2 stimulation in platelets by bacteria through the activation of the PI3K signalling pathway induce a proinflammatory response (Blair *et al.,* 2009). Platelet TLR9 activation has been associated with thrombosis and oxidative stress, and is found within the previously mentioned novel T-granules in platelets (Thon *et al.,* 2012). Profiling of platelet TLR expression pre and post dry immersion showed an overall increase in TLR expression post immersion. However, this was largely variable amongst the individual subjects.

Table A15: Real-Time Ready Gene expression targets

mRNA target	Forward (F) Reverse (R)	Sequence
GAPDH	F	AGCCACATCGCTCAGACAC
	R	GCCCAATACGACCAAATCC
TLR1	F	TTGGATTTGTCCCACAACAA
	R	CCAAGTGCTTGAGGTTCACA
TLR2	F	CTCTCGGTGTCGGAATGTC
	R	AGGATCAGCAGGAACAGAGC
TLR3	F	GCGCTAAAAAGTGAAGAACTGG
	R	CCAATTGCGTGAAAACACC
TLR4	F	CAAGATGCCCCTTCCATTT
	R	TCCTTAGGAATTAGCCACTAGACTTT
TLR5	F	GACACAATCTCGGCTGACTG
	R	TGTCAGGAACATGAACATCAATC
TLR6	F	TGAAACAGTCTCTTTTGAGTAAATGC
	R	TCCATTTGGGAAAGCAGAGT
TLR7	F	GCCCCCAAGATGGTTTAAG
	R	GCATCCCCAATTTCTTTGG
TLR8	F	CAGTCCTGGGGATCAAAGAG
	R	TGCAGTGACATCTGAAACACAA
TLR9	F	CGCTACTGGTGCTATCCAGA
	R	AGCCCAGGGAGGAGCTAAG
TLR10	F	AACCTCCTTTTTCAACTCCAGA
	R	TTATGGCATAGAATCAAAACTCTCA

Table A16: Biomarkers in Proseek Multiplex CVD II Panel

2,4-dienoyl-CoA reductase, mitochondrial (DECR1)	Natriuretic peptides B (BNP)
ADM (ADM)	NF-Kappa-B essential modulator (NEMO)
Agouti-related protein (AGRP)	Osteoclast-associated immunoglobulin-like receptor (hOSCAR)
Alpha-L-iduronidase (IDUA)	P-selectin glycoprotein ligand 1 (PSGL-1)
Angiopoietin-1 (ANG-1)	Pappalysin-1 (PAPPA)
Angiopoietin-1 receptor (TIE2)	Pentraxin-related protein PTX3 (PTX3)
Angiotensin-converting enzyme 2 (ACE2)	Placenta growth factor (PlGF)
Bone morphogenetic protein 6 (BMP-6)	Platelet-derived growth factor subunit B (PDGF subunit B)
Brother of CDO (Protein BOC)	Poly [ADP-ribose] polymerase 1 (PARP-1)
C-C motif chemokine 17 (CCL17)	Polymeric immunoglobulin receptor (PIgR)
C-C motif chemokine 3 (CCL3)	Pro-interleukin-16 (IL16)
C-X-C motif chemokine 1 (CXCL1)	Programmed cell death 1 ligand 2 (PD-L2)
Carbonic anhydrase 5A, mitochondrial (CA5A)	Proheparin-binding EGF-like growth factor (HB-EGF)
Carcinoembryonic antigenrelated cell adhesion molecule 8 (CEACAM8)	Prolargin (PRELP)
Cathepsin L1 (CTSL1)	Prostasin (PRSS8)
CD40 ligand (CD40-L)	Protein AMBP (AMBP)
Chymotrypsin C (CTRC)	Protein-glutamine gamma-glutamyltransferase 2 (TGM2)
Decorin (DCN)	Proteinase-activated receptor 1 (PAR-1)
Dickkopf-related protein 1 (Dkk-1)	Proto-oncogene tyrosine-protein kinase Src (SRC)
Fatty acid-binding protein, intestinal (FABP2)	Receptor for advanced glycosylation end products (RAGE)
Fibroblast growth factor 21 (FGF-21)	Renin (REN)
Fibroblast growth factor 23 (FGF-23)	Serine protease 27 (PRSS27)
Follistatin (FS)	Serine/threonine-protein kinase 4 (STK4)
Galectin-9 (Gal-9)	Serpin A12 (SERPINA12)
Gastric intrinsic factor (GIF)	SLAM family member 5 (CD84)
Gastrotropin (GT)	SLAM family member 7 (SLAMF7)
Growth hormone (GH)	Sortilin (SORT1)
Growth/differentiation factor 2 (GDF-2)	Spondin-2 (SPON2)
Heat shock 27 kDa protein (HSP 27)	Stem cell factor (SCF)
Heme oxygenase 1 (HO-1)	Superoxide dismutase [Mn], mitochondrial (SOD2)
Hydroxyacid oxidase 1 (HAOX1)	T-cell surface glycoprotein CD4 (CD4)
Interleukin-1 receptor antagonist protein (IL-1ra)	Thrombomodulin TM
Interleukin-1 receptor-like 2 (IL1RL2)	Thrombospondin-2 (THBS2)
Interleukin-17D (IL-17D)	Tissue factor (TF)
Interleukin-18 (IL-18)	TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2)
Interleukin-27 (IL-27)	Tumor necrosis factor receptor superfamily member 10A (TNFRSF10A)
Interleukin-4 receptor subunit alpha (IL-4RA)	Tumor necrosis factor receptor superfamily member 11A (TNFRSF11A)
Interleukin-6 (IL-6)	Tumor necrosis factor receptor superfamily member 13B (TNFRSF13B)
Kidney injury molecule 1 (KIM-1)	Tyrosine-protein kinase Mer (MERTK)
Lactoylglutathione lyase (GLO1)	V-set and immunoglobulin domain-containing protein 2 (VSIG2)
Lectin-like oxidized LDL receptor 1 (LOX-1)	Vascular endothelial growth factor D (VEGF-D
Leptin (LEP)	
Lipoprotein lipase (LPL)	
Low affinity immunoglobulin gamma Fc region receptor II-b (IgG Fc receptor II-b)	
Lymphotactin (XCL1)	
Macrophage receptor MARCO (MARCO)	
Matrix metalloproteinase-12 (MMP-12)	
Matrix metalloproteinase-7 (MMP-7)	
Melusin (ITGB1BP2)	

Table A17: Biomarkers in Proseek Multiplex Inflammation Panel

Adenosine Deaminase (ADA)	Interleukin-22 receptor subunit alpha-1 (IL-22 RA1)
Artemin (ARTN)	Interleukin-24 (IL-24)
Axin-1 (AXIN1)	Interleukin-33 (IL-33)
Beta-nerve growth factor (Beta-NGF)	Interleukin-4 (IL-4)
Brain-derived neurotrophic factor (BDNF)	Interleukin-5 (IL-5)
C-C motif chemokine 19 (CCL19)	Interleukin-6 (IL-6)
C-C motif chemokine 20 (CCL20)	Interleukin-7 (IL-7)
C-C motif chemokine 23 (CCL23)	Interleukin-8 (IL-8)
C-C motif chemokine 25 (CCL25)	Latency-associated peptide transforming growth factor beta-1 (LAP TGF-beta-1)
C-C motif chemokine 28 (CCL28)	Leukemia inhibitory factor (LIF)
C-C motif chemokine 3 (CCL3)	Leukemia inhibitory factor receptor (LIF-R)
C-C motif chemokine 4 (CCL4)	Macrophage colony-stimulating factor 1 (CSF-1)
C-X-C motif chemokine 1 (CXCL1)	Matrix metalloproteinase-1 (MMP-1)
C-X-C motif chemokine 10 (CXCL10)	Matrix metalloproteinase-10 (MMP-10)
C-X-C motif chemokine 11 (CXCL11)	Monocyte chemotactic protein 1 (MCP-1)
C-X-C motif chemokine 5 (CXCL5)	Monocyte chemotactic protein 2 (MCP-2)
C-X-C motif chemokine 6 (CXCL6)	Monocyte chemotactic protein 3 (MCP-3)
C-X-C motif chemokine 9 (CXCL9)	Monocyte chemotactic protein 4 (MCP-4)
Caspase-8 (CASP-8)	Natural killer cell receptor 2B4 (CD244)
CD40L receptor (CD40)	Neurotrophin-3 (NT-3)
CUB domain-containing protein 1 (CDCP1)	Neurturin (NRTN)
Cystatin D (CST5)	Oncostatin-M (OSM)
Eotaxin-1 (CCL11)	Osteoprotegerin (OPG)
Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)	Programmed cell death 1 ligand 1 (PD-L1)
Fibroblast growth factor 19 (FGF-19)	Protein S100-A12 (EN-RAGE)
Fibroblast growth factor 21 (FGF-21)	Signaling lymphocytic activation molecule (SLAMF1)
Fibroblast growth factor 23 (FGF-23)	SIR2-like protein 2 (SIRT2)
Fibroblast growth factor 5 (FGF-5)	STAM-binding protein (STAMPB)
Fms-related tyrosine kinase 3 ligand (Flt3L)	Stem cell factor (SCF)
Fractalkine (CX3CL1)	Sulfotransferase 1A1 (ST1A1)
Glial cell line-derived neurotrophic factor (GDNF)	T cell surface glycoprotein CD6 isoform (CD6)
Hepatocyte growth factor (HGF)	T-cell surface glycoprotein CD5 (CD5)
Interferon gamma (IFN-gamma)	Thymic stromal lymphopoietin (TSLP)
Interleukin-1 alpha (IL-1 alpha)	TNF-beta (TNFB)
Interleukin-10 (IL-10)	TNF-related activation-induced cytokine (TRANCE)
Interleukin-10 receptor subunit alpha (IL-10RA)	TNF-related apoptosis-inducing ligand (TRAIL)
Interleukin-10 receptor subunit beta (IL-10RB)	Transforming growth factor alpha (TGF-alpha)
Interleukin-12 subunit beta (IL-12B)	Tumor necrosis factor (Ligand) superfamily, member 12 (TWEAK)
Interleukin-13 (IL-13)	Tumor necrosis factor (TNF)
Interleukin-15 receptor subunit alpha (IL-15RA)	Tumor necrosis factor ligand superfamily member 14 (TNFSF14)
Interleukin-17A (IL-17A)	Tumor necrosis factor receptor superfamily member 9 (TNFRSF9)
Interleukin-17C (IL-17C)	Urokinase-type plasminogen activator (uPA)
Interleukin-18 (IL-18)	Vascular endothelial growth factor A (VEGF-A)
Interleukin-18 receptor 1 (IL-18R1)	
Interleukin-2 (IL-2)	
Interleukin-2 receptor subunit beta (IL-2RB)	
Interleukin-20 (IL-20)	
Interleukin-20 receptor subunit alpha (IL-20RA)	

Dry Immersion Inclusion and Exclusion Criteria

Inclusion criteria were as follows:

- Healthy male volunteer
- Aged 20 – 45, Height between 158 and 190cm
- Not overweight nor excessively thin with a BMI (weight kg/height m^2) between 20 and 26
- No personal or family record of acute or chronic disease or psychological disturbances which could have affected the physiological data and/or create a risk for the subject during the experiment
- Fitness level assessment required
- if age < 35 years: 35 ml/min./kg < VO_2 max < 60ml/min./kg
- if age > 35 years: 30 ml/min./kg < VO_2 max < 60ml/min./kg
- Active and free from any orthopaedic, musculoskeletal and cardiovascular disorders
- Non-smokers and no alcohol or drug dependence
- Free of any engagement during the study signed consent forms.

Exclusion criteria were as follows:

- Past record of orthostatic intolerance or cardiac rhythm disorders
- Chronic back pain, history of hiatus hernia or gastro-oesophageal reflux
- History of thyroid dysfunction, renal stones, diabetes, migraines,
- Family history of thrombosis or a positive response to the thrombosis screening procedure (anti thrombin III, S-protein, C-protein, factor V Leiden mutation or mutation 20210 of the prothrombin gene)
- Echocardiography: inappropriate thoracic acoustic window
- History of/or active claustrophobia
- History of genetic muscle and bone diseases, bone mineral density: T-score < -1.5 on the hip
- Poor tolerance to blood sampling, having given blood (more than 8ml/kg) in a week period of 8 weeks or less before the experiment
- Subject who, in the judgment of the investigator, was likely to be non-compliant during the study, or unable to cooperate because of a language problem or poor mental development,
- Subject who had received more than 4500 Euros within 12 months for being a research subject.
- Subject already participating or in the exclusion period of a clinical research,

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