Chapter 6

Platelets: Functional Biomarkers of Epigenetic Drift

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Abstract

Cardiovascular disease (CVD) risk factors can be classed as modifiable or non-modifiable. Physical inactivity and obesity represent major behavioural risk factors for the initiation, development and progression of CVD. Platelet dysfunction is pivotal to the aetiology of CVD, a chronic vascular inflammatory condition, which is characterised by a lag time between onset and clinical manifestation. This indicates the role of epigenetic drift, defined by stochastic patterns of gene expression not dependent on dynamic changes in coding DNA. The epigenome, a collection of chemical marks on DNA and histones, is established during embryogenesis and modified by age and lifestyle. Biogenesis and effector function of non-coding RNA, such as microRNA, play a regulatory role in gene expression and thus the epigenetic mechanism. In this chapter, we will focus on the effect of the modifiable risk factors of physical activity/inactivity and overweight/obesity on platelet function, via epigenetic changes in both *megakaryocytopoiesis* and *thrombopoiesis*. We will also discuss the role of acute exercise on platelet function and the impact of cardiorespiratory fitness (CRF) on platelet responses to acute exercise. This chapter will highlight the potential role of platelets as circulating functional biomarkers of epigenetic drift to implement, optimise and monitor CVD preventive management strategies.

Keywords: platelets, epigenetics, microRNA, lifestyle, physical activity, physical inactivity, cardiovascular disease, preventive medicine

1. Introduction

1.1. Epigenetics

Epigenetics describes modifiable changes that occur to genes, *via* chemical modifications and/or varying states of chromatin organisation and structure, which alter the gene expression without altering the DNA sequence itself. Smoking habits, obesity, ageing and physical fitness among others are examples of environmental factors that have been suggested to have a long-term influence on epigenetic changes [1]. Epigenetics may be classed as three distinct but highly interconnected processes; DNA methylation, histone modification and RNA-associated silencing (**Figure 1**). DNA methylation and histone modification alter DNA accessibility for transcriptional machinery and chromatin structure. These changes are heritable and can be passed down between generations through either mitosis or meiosis. DNA methylation involves the addition of a methyl group to the 5-position of cytosine by DNA methyl-transferases, at areas known as CpG islands. Methyl groups control gene expression by binding to promoter sites of the gene. This changes the affinity of methylation-sensitive binding proteins, and is associated with transcriptional gene silencing [2]. Whilst required for normal development, changes in DNA methylation have been linked to CVD conditions such as atherosclerosis. For example, the athero-protective oestrogen receptor genes ESR1 and ESR2, usually expressed in SMCs, are hyper-methylated in atherosclerosis [3, 4].

Unlike the platelet transcriptome and proteome, the investigation of epigenetic processes is an almost completely unexplored area in platelet biology, as analysis of these mechanisms requires DNA [2]. Although anucleate, platelets have functionally active mitochondria, with mitochondrial DNA (mtDNA) that can also be methylated, moderating the control of mitochondrial gene expression. Interestingly, Zhong and colleagues recently reported that *de novo* DNA synthesis in mitochondria, and its subsequent oxidation, plays a key role in triggering the innate immune response. Mitochondria can regulate how immune cells respond to infection and tissue damage, producing pro- or anti-inflammatory signals by regulating Krebs cycle metabolites or the production of reactive oxygen species (ROS). More and more examples are being found of mitochondrial functions being repurposed in unexpected ways to contribute to many biological processes, including inflammatory signalling [5].

Understanding epigenetic regulation of mitochondrial genes in platelets is proving crucial to understanding their implication in CVD development. Novel research by Baccarelli and Byun 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 [6]. These results suggest that DNA methylation in platelet mitochondria could be a potential contributor to CVD development through the regulation of platelet function.

Histone, proteins that structure DNA into units known as nucleosomes, can be modified at their amino-acid tails. Histone modifications refer to the post-translational alterations of the N-termini of these tails that subsequently modify histone-DNA interactions [7]. 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

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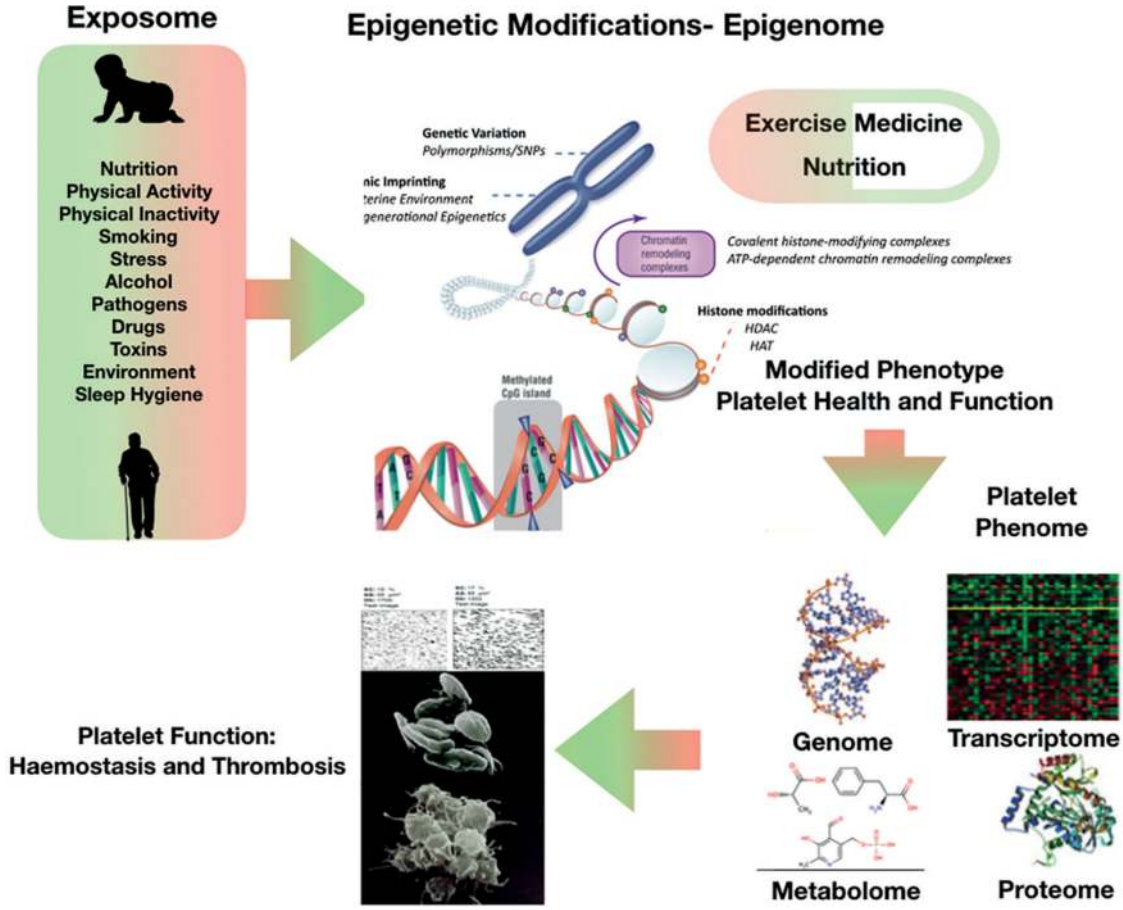

Figure 1. Modifiable risk factors covalently alter the static genome by processes involving epigenetic writers, erasers and readers, which fine-tune gene expression in an age and lifestyle dependent manner, a process known as *epigenetic drift*. The term epigenetics refers to stable patterns of gene expression and they are not dependent on dynamic changes in coding DNA. These gene expression states are encoded in the epigenome—a collection of chemical marks on DNA or on histones that are established during embryogenesis and are modified by age and environment over a person's lifetime. Studies have uncovered stochastic DNA methylation drift that reflects imperfect maintenance of epigenetic marks. We hypothesise that *drift* creates epigenetic mosaicism in ageing haemopoietic stem cells in the bone marrow. This in turn impacts on HSC differentiation, maturation and platelet production. This may highlight platelets as a functional diagnostic index of cardiovascular competence. The fact that the initiation and progression of CVD is characterised by a lag time between onset and clinical manifestation provides a window of opportunity for the implementation of intervention strategies to reduce the CVD burden. In addition, such studies will better inform primary, secondary and tertiary preventive strategies—promoting an ageing well paradigm and optimising a person's disease free years.

deacetyltransferases (HDACs). This mechanism alters chromatin structure (heterochromatin versus euchromatin) to influence gene expression [3].

RNA-based epigenetic processes involve non-coding RNA (ncRNA) and are factors in the chromatin-based regulation of gene expression [8]. 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) is a subset of ncRNA that negatively regulates gene transcription by degrading

or repressing target mRNA [9]. miRNA can control the expression of important epigenetic regulators such as histone deacetylases and DNA methyl-transferases and similarly, DNA methylation and histone modification can control the expression of some miRNA, thereby forming a feedback loop [10]. 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.

Individuals age differently and lifestyle factors such as exercise or smoking have been shown to delay or accelerate the ageing process, respectively [11]. 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 [12]. *Epigenetic drift* is the term given to epigenetic modifications as they occur as a direct consequence of age [13]. This was previously observed when DNA methylation marks in identical twins differed increasingly as a function of age [1]. 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. Disparity in these epigenetic marks between twins may be as a result of lifestyle influences such as diet, physical activity levels, stress and smoking.

Epigenetic drift affects the majority of the genome over time leading to biological ageing (**Figure 2**). Ageing is a natural process associated with the de-regulation of histone tags, senescence-associated lncRNA, a gradual de-regulation of DNA methylation, in a potential linear fashion depicted by age-predictive linear models [14]. However, an individual exposed to either environmental or genetic risk factors may show signs of premature ageing as a result of either lifestyle or environmental risk factors. De-regulation of DNA methylation can increase the susceptibility to chronic diseases like CVD. Furthermore, it has been hypothesised that a healthy lifestyle may reserve a more intact epigenome, promoting longevity [15]. In a recent compelling study by Horvath and colleagues, a novel, sensitive and highly robust DNAm age estimator (based on 391 CpGs) was developed for human fibroblasts, keratinocytes, buccal cells, endothelial cells, lymphoblastoid cells, skin, blood and saliva samples [16]. This seminal research builds upon and overcomes the limitations of the two ground-breaking studies on the epigenetic clock (biological/molecular age) and its relationship with chronological age. These studies, one a blood-based age estimator [14] and the other a pan-tissue estimator [17], facilitated age estimates (DNAmAge) that are widely used in epidemiological studies. The novel 'skin and blood clock' overcomes the technical and sensitivity limitations of the previous DNAmAge biomarker panels.

DNA methylation undergoes extensive changes during differentiation of self-renewing stem cells [18, 19]. Indeed, DNA methylation is involved in the production of MKs and subsequent transcription. 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

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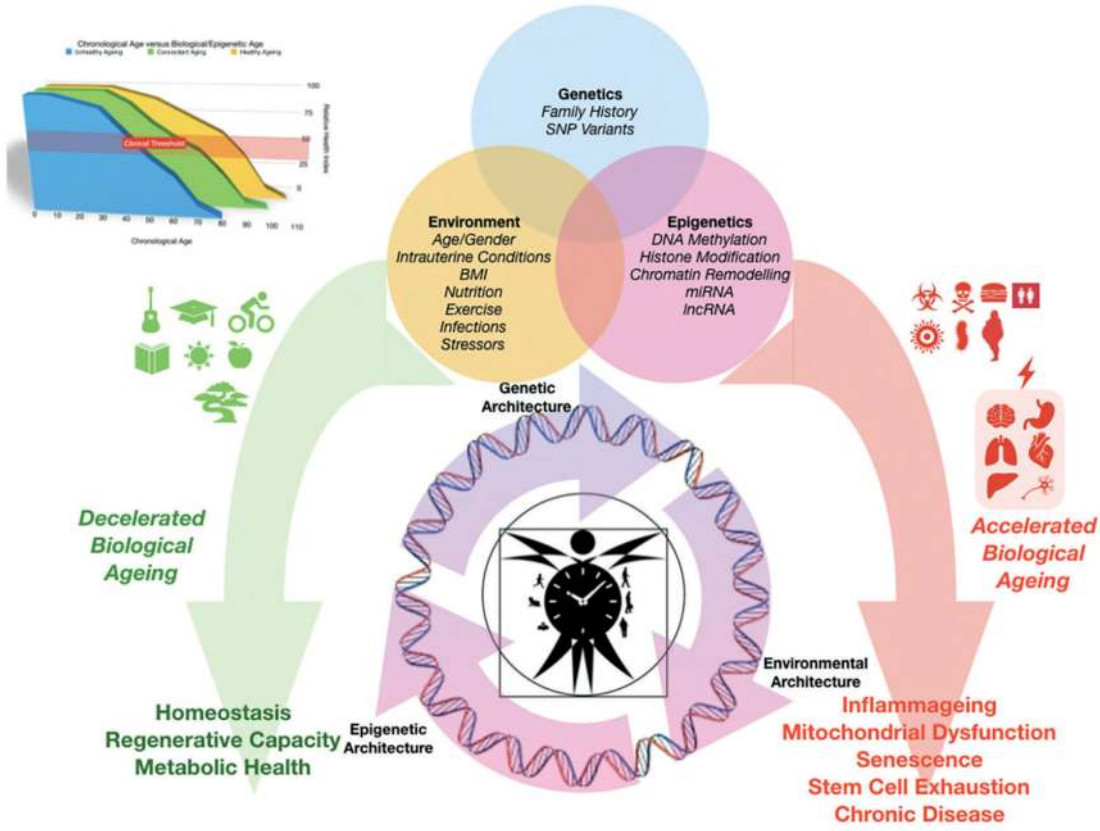

Figure 2. Schematic representation of ageing well versus accelerated ageing. Poor lifestyle choices such as inactivity and diet are rapidly becoming a global pandemic, accelerating many chronic illnesses such as cardiovascular disease, metabolic disorder, diabetes, Alzheimer's disease and cancer. Understanding their pathophysiology is important for the development of future therapeutic interventions, stratification of clinical trials and to challenge our current perception of engaging with cost effective measures such as lifestyle management. The aetiology of age-related chronic illnesses involves a complex interplay between many biological processes and is modulated by non-modifiable and modifiable risk factors.

determining PLT or changes in histone acetylation with aging have been suggested to play an important role in platelet function [20].

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 silencing of various genes [21, 22], hence now termed 'fine-tuners' of cellular phenotypes. They are thought to be involved in the regulation of ~60% of human genes [23, 24]. miRNA can be classed as intronic, exonic and intergenic miRNA, according to the location of their encoding genes [25]. Intronic miRNAs account for approximately 70% of all transcribed miRNA [26, 27]. Intergenic miRNAs are found between two protein-coding genes and employ their own promoters and regulatory molecules. As miRNAs target mRNA by imperfect binding, each miRNA has multiple targets, enabling miRNAs to regulate over half of the human genome [28]. 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 and 220 h has been reported) and are very stable [29]. 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 [30]. 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 hyper-methylated). Post-transcriptional mechanisms define modifications in miRNA processing and stability [31].

2. Platelet Epigenetics

2.1. 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. Approximately 32% of all human genes are present in platelets at the mRNA level [23]. It is well accepted that platelets contain the necessary splicing machinery, rough ER and polyribosomes that allow the synthesis of proteins required for their functioning [32]. 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 h, 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 post-transcriptional RNA mechanisms. 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 [33]. 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 [34], who showed by locked nucleic acid (LNA) microarray profiling, that platelets harboured an impressive number (219) of miRNA. 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 [34, 35] as pre-miRNAs have been identified at low levels (21 transcripts) in platelets [23]. Star or passenger strand miRNA have also been identified [36]. 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 [34].

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 [37]. 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 et al. [38], 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 [38]. 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, gender and race [39, 40]. Other reports on agonist-induced platelet activation by thrombin (and ADP) show differential expression of platelet miRNA compared to resting platelets [41].

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 [23, 35, 42]. Transcriptomic approaches show that miRNA make up the majority (80%) of all small RNAs in platelets. 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.

A number of highly expressed miRNA have been characterised in human platelets, some of which are involved in myeloid cell differentiation, megakaryocytopoiesis and thrombopoiesis. miR-223 has been identified as the most highly expressed platelet miRNA [38, 43, 44] and has roles in thrombopoiesis and megakaryocyte differentiation [24]. miR-223 regulates ADP $P2Y_{12}$, a target for existing anti-platelet 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 [45]. miR-126 plays central roles in vascular inflammation and is thought to be the second most highly expressed miRNA in platelets [46]. 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 [47].

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 [48]. The majority of platelet miRNA are supposedly formed in the megakaryocyte and packaged into platelets upon formation. For example, miR-146b positively regulates megakaryopoieses by targeting and down regulating the megakaryopoieses inhibitor PDGFRA [49, 50]. 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 [51]. 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 [34, 52, 53].

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 (PMV) and/or microparticles (PMP), fulfilling novel processes of gene regulation in target cells [54]. Initial studies by Laffont and Gidlöf demonstrated the functionality of platelet miRNA [24, 55]. Functional complexes of miR-223 and Argonaute 2 protein (Ago2) packaged in MVs from activated platelets were found to modulate the expression of targeted endothelial cell endogenous mRNA transcripts FBXW7 and EFNA1. 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 [56].

Gidlof et al. suggested that platelet miRNA could modulate vascular endothelial inflammatory responses [55]. 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., showed that platelet-released miR-223 through platelet MPs can encourage lung cancer cell invasion by targeting the tumour suppressor EPB41L3 [57].

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 down-regulation 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 [58]. 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 [13].

2.2. Platelets, lifestyle and miRNA in the aetiology of CVD

Efforts in coping with CVD require further understanding of its aetiology in order to develop effective management strategies. Epidemiological studies in adults have acknowledged a set of characteristic risk factors that predict the probability of a person developing clinical manifestations of disease [59, 60]. CVD risk factors are 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 [61] 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 [62]. Physical inactivity and obesity are primary potent risk factors, both of which can severely impact platelet physiology.

Platelets have central roles in CVD [63] contributing to both early stages of endothelial dysfunction and advanced stages of the plaque rupture [64]. Platelets participate in early stage disease initiation through multiple mechanisms enabling adhesion to 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

[65]. 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 [66]. CD40L is thought to be at the heart of the atherosclerotic process, with 90% of circulating CD40L residing in platelets. CD40L 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*. 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 [66]. 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., 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 [67].

Platelet-derived microparticles released upon activation may further amplify the progression of atherosclerosis through processes of adhesion, coagulation, inflammation and lipid metabolism [68]. 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 and T-cells [66]. 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 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 [64]. Given the critical roles of platelets in the pathogenesis of atherosclerosis and the development of acute thrombotic events, anti-platelet 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 and phosphodiesterase inhibitors (block degradation of cyclic nucleotides) [69, 70].

Given the impact of miRNA gene regulation, it is unsurprising that the dysregulation of miRNA is implicated in CVD. miRNAs 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 [71] 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 up-regulated in patients compared to controls (miR-340* and miR-624*), although whether or not they are the cause or consequence is currently unknown [72]. Besides their roles as mediators and biomarkers of CVD, platelet miRNA act as novel surrogate measures of the responsiveness to anti-platelet therapies used in CVD [73]. miR-223 levels are significantly down regulated in low responders to anti-platelet therapy [45, 74]. Furthermore, expression of platelet miR-26a has been linked with clopidogrel resistance during coronary stenting [75]. 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 [76]. Other research investigating the effects of anti-platelet 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 vivo*, aspirin intake resulted in platelet inhibition and lower circulating platelet-derived miR-126 levels than were seen in untreated subjects [77]. Greater understanding of the meaning of platelet miRNA in CVD patients could aid in the diagnosis and treatment of these diseases.

2.3. Effect of obesity on platelet function

Obesity is a multifactorial condition involving a plethora of interrelated processes such as alterations in lipid metabolism, insulin resistance, inflammation, endothelial dysfunction, adipokine imbalance and oxidative stress. These metabolic aberrations have been postulated to be involved in platelet hyper-aggregability. Indeed, platelet activation markers are described as elevated in obesity, contributing to the inflammatory and prothrombotic state [78]. Subjects with overweight and obesity display increased platelet activation markers urinary-11-dehydro-TXB2 [79], MPV [80] and PLT [81]. 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 [82, 83]. 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+} [84]. 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 [85, 86].

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 [87]. 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 [88]. Activated platelets also produce ROS [89], amplifying their own aggregatory potential by increasing

αIIbβ3 and CD40L expression [90, 91] 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. describes a decrease of nitric oxide synthase (NOS) activity and cGMP levels with simultaneous platelet hyper-aggregability in obese subjects compared to healthy controls with impaired antioxidant responses as potential contributors [92]. Anfossi et al. showed that platelet sensitivity to anti-aggregatory effects of PGI_2 and NO is reduced in obesity [84]. Importantly, weight loss in obese subjects marks a reduction in platelet activation markers and can potentially reverse the platelet responsiveness to NO and prostacyclin [93, 94]. 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 [95] and P-Selectin expression in overweight CAD patients [96].

Although an association between obesity and platelet activation is evident, the molecular mechanisms responsible have only begun to surface [97]. Platelet RNA is reflective of pathological disease states where inflammatory transcript profiles (e.g., INFG, IL1R1, IL6 and TLR2) correlate significantly with increasing BMI [98], 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 [99], 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 [36].

2.4. Role of physical activity on platelet function

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 [100]. Differences in these known factors have been demonstrated to explain a large proportion of the inverse relationship between physical activity and CVD risk [101, 102]. 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 [103].

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 [104]. 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. Exercise effects on platelet function in both diseased and healthy

populations have 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 and lack of consistency between research groups [105]. Different platelet adhesion experimental protocols have provided no definitive consensus on the platelet response to acute exercise in healthy adult subjects [106–110]. 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 increased platelet aggregation after acute exercise [111]. In general, it appears that acute vigorous exercise induces a hyper-reactive haemostatic state [112] and a transient increase in agonist-induced platelet adhesion and aggregation *in vitro* and *ex vivo*. However, there is no definitive consensus regarding the short-term effects of exercise on platelet function.

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 [113–116]. 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 [106, 117]. The actual relationship between CRF and platelet function has been referred to in a recent breakthrough study by Heber et al., who investigated platelet function and CRF in 62 young women [118]. 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 expressions (non-agonist induced). Interestingly, basal platelet function in the MF and HF were almost equal, indicating a definite influence of CRF 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 5×/week 30 min/day), reduced shear stress-induced platelet activation and ox-LDL-potentiated platelet function [109, 111]. Importantly, after 12 weeks of de-conditioning, the beneficial effects of exercise on platelets were non-existent and platelet function returned to its pre-training state.

De Meirelles et al. reported that chronic physical activity had favourable effects on platelet activation in hypertensive patients at rest [119]. Twelve weeks of regular exercise (75–85% VO_2 max 5×/week for 45–60 min) reduced platelet aggregation in response to collagen. Santilli et al. investigated the effects of regular high intensity (60–75%) aerobic exercise for 2 months in low and intermediate CVD risk sedentary subjects [120]. Exercise training was associated with reductions in TxA_2, plasma P-Selectin and platelet-derived CD40L, despite no reduction in CRP (representing systemic inflammation). Evidently, physical activity and exercise affects nearly all facets of platelet function [121]. Studies on the effects of acute exercise appear to

heighten platelet reactivity. Regular exercise can improve this response, seems to have an antithrombotic effect on platelets and could represent a portion of the protective effects of exercise on CVD risk factors. 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 [122].

2.5. Role of physical inactivity and sedentary lifestyle on platelet function

In contrast to physical activity, physical inactivity/sedentary behaviour is a universal leading cause of death and independent CVD risk factor [123, 124]. Sedentary behaviour refers to any waking activity characterised by an energy expenditure ≤1.5 metabolic equivalents in a sitting or reclining posture [125]. 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 [126], some mechanisms involved in the pathogenesis of physical inactivity are similar to that of obesity such as insulin resistance [127], hypertension and increased inflammation [128]. 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 [129] and arterial remodelling [130, 131].

2.5.1. 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, 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 [34]. 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 [132–134]. Work by Baggish et al. showed altered expression of circulating miRNA (c-miRNA) in response to acute and chronic exercise in athletes [135]. 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 up-regulated 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. Moreover, evidence of these physical activity-specific microRNA signatures [136–138] has 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. recently described indications for epigenetic variation (by miRNA expression) as a link between physical activity and sedentary lifestyle [139]. An epigenetic adaptation to habitual exercise has been described [140, 141]. Similarly, an epigenetic adaptation to physical inactivity may exist.

2.6. Physical activity/inactivity and platelet epigenetic drift

It has been well recognised that regular exercise may reduce risk of major vascular thrombotic events and protect against CVD [123]. Differences in known factors explain a large percentage of the inverse relationship between physical activity and CVD risk [101, 102]. Nevertheless, 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. 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 [120, 142]. Therefore, it is imperative that future studies explore 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.

Low cardiorespiratory fitness is associated with physical inactivity [143]. This 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 [144]. 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.

3. Future research avenues

Our group, in collaboration with the European Space Agency (ESA), the Centre National d'Etudes Spatiales (*CNES*) and *MEDES* (Institute for Space Medicine and Physiology, Toulouse, France) have employed ground-based models of microgravity, i.e., 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 [145]. 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 [146]. DI presents a unique opportunity to analyse the specific effects of physical inactivity on platelet physiology/function and related biomarkers.

Recent studies reflect the first comprehensive attempts to evaluate the relationship between platelet function and physical activity, physical inactivity and overweight. While exploratory in nature to date, 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. A relatively low dose of exercise has been shown to be

sufficient to normalise platelet function in low fit females [118]. 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 would develop this knowledge. 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 may also impact platelet function in CVD patients. Exercise interventions in these populations could be beneficial in terms of reducing anti-platelet therapy dosage or combining anti-platelet therapy with exercise [147], i.e., prescriptive exercise medicine as an adjuvant management strategy/therapy.

The investigation of epigenetic processes is almost a completely unexplored area in platelet biology as analyses of these mechanisms require DNA [2]. Platelets have functionally active mitochondria [148]. 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 [6]. Furthermore, miRNA have recently been linked with platelet mitochondrial health in stored platelets [149]. Platelets contain the machinery to process pre-miRNA to mature miRNA [34]. Platelets contain higher levels of pre-miRNA than other blood cells [56], and the maturation of pre-miRNA could contributed to altered miRNA profiles due to physical activity and inactivity. This may represent a more focused and efficient method of monitoring platelet function. Targeting levels of other biogenesis molecules in the miRNA pathway would also be an interesting avenue of platelet miRNA biology. Recently, Elgheznawy et al. showed that Dicer was decreased in patients with TD2M compared to healthy controls, whereas interestingly, Argonaute 2 levels did not differ [150]. Experiments investigating levels of miRNA processing machinery such as Dicer and Argonaute 2 in physically active and sedentary populations would be of major interest.

Long-term lifestyle choices such as physical inactivity may incur epigenetic penalties in megakaryocytes, and in the biological processes of *megakaryocytopoiesis* and *thrombopoiesis*. Thus, platelet miRNA could reflect these epigenetic changes, holding substantial predictive potential of both health and disease. Epigenetic changes in the megakaryocyte genome such as methylation of genes determining platelet biogenesis or changes in histone acetylation with aging have been suggested to play an important role in platelet function [20]. Prescribed exercise could induce epigenetic changes in megakaryocytes to produce a healthier phenotype of platelets with a direct change in platelet reactivity.

4. Conclusion

It is evident that lifestyle factors such as physical activity, physical inactivity and overweight do impact platelet function. 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. Future research in this area, will no doubt contribute to a greater mechanistic understanding of the relationship between epigenetics, cardiovascular health, lifestyle factors and platelet biology.

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Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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