ASSESSMENT OF PLATELET RESPIRATION AS EMERGING BIOMARKER OF DISEASE

Short title: Platelet Respiration As Emerging Biomarker

Authors:

ALEXANDRA T. PETRUȘ[1,#], DIANA L. LIGHEZAN[2,#], MARIA D. DĂNILĂ[3], OANA M. DUICU[3,*], ADRIAN STURZA[3,*], DANINA M. MUNTEAN[3,,$], IOANA IONIȚĂ[2]

[1]Department of Anatomy, Physiology and Pathophysiology, Faculty of Pharmacy, "Victor Babeș" University of Medicine and Pharmacy, Timișoara, Romania

[2]Department of Internal Medicine I – Hematology, Faculty of Medicine, "Victor Babeș" University of Medicine and Pharmacy, Timișoara, Romania

[3]Department of Functional Sciences – Pathophysiology, Faculty of Medicine, "Victor Babeș" University of Medicine and Pharmacy, Timișoara, Romania

[*]Centre for Translational Research and Systems Medicine, "Victor Babeș" University of Medicine and Pharmacy, Timișoara, Romania

[#]These authors contributed equally to this work.

[$]**Corresponding Author:**

Prof. Danina M. Muntean, MD, PhD

Department of Functional Sciences – Pathophysiology, "Victor Babeș" University of Medicine and Pharmacy of Timişoara,

2, Eftimie Murgu Sq., 300041 Timișoara, Romania

E-mail: daninamuntean@umft.ro

Abstract

Mitochondrial dysfunction is currently acknowledged as a central pathomechanism of most common diseases of the 21st century. Recently, the assessment of the bioenergetic profile of human peripheral blood cells has emerged as a novel research field with potential applications in the development of disease biomarkers. In particular, platelets have been successfully used for the *ex vivo* analysis of mitochondrial respiratory function in several acute and chronic pathologies. An increasing number of studies support the idea that evaluation of the bioenergetic function in circulating platelets may represent the peripheral signature of mitochondrial dysfunction in metabolically active tissues (brain, heart, liver, skeletal muscle). Accordingly, impairment of mitochondrial respiration in peripheral platelets might have potential clinical applicability as a diagnostic and prognostic tool as well as a biomarker in treatment monitoring.

The aim of this minireview is to summarize current information in the field of platelet mitochondrial dysfunction in both acute and chronic diseases.

Key words: blood platelets, mitochondrial respiration, high-resolution respirometry, extracellular flux analysis, acute and chronic diseases

Introduction

Chronic non-communicable pathologies, mainly cardiovascular and neurodegenerative diseases, cancer, and type 2 diabetes are nowadays the leading causes of mortality, being collectively responsible for almost 70% of deaths worldwide (WHO, 2017). Importantly, all lifestyle-related conditions (unhealthy diet, tobacco use, lack of physical activity and alcohol abuse), classic risk factors (obesity, dys/hyperlipidemia, and high blood glucose) and comorbidities (eg, depression) associated with these pathologies negatively impact on cellular metabolism, including mitochondrial function (Arduino *et al.* 2013, Medina-Gomez 2012, Nicolson 2014, Ouyang *et al.* 2013).

More recently, mitochondria have emerged as acute contributors to both pathogenesis and ultimate outcome of acute severe conditions, such as sepsis, trauma and stroke (Arulkumaran *et al.* 2016, Busija *et al.* 2016, Jang *et al.* 2017).

Tissue biopsy, although the most relevant method to confirm various pathological changes in diseased organs, implies high costs and is not feasible in all clinical conditions, especially those related to older individuals who cannot cooperate during the procedure or in the presence of hemostasis disorders (Tyrrell *et al.* 2016). On the other hand, circulating human blood cells such as peripheral blood mononuclear cells (PBMC) and platelets are increasingly used in translational research for the assessment of organ-specific mitochondrial dys/function relevant for the clinical outcome (Chacko *et al.* 2013, Hsiao and Hoppel 2018, Sjovall *et al.* 2013). These abundant, readily available samples, obtained through minimally invasive procedures are ideal tools that allow dynamic monitoring of mitochondrial status, view the possibility to perform serial measurements. Indeed, the impairment of mitochondrial respiration

has emerged in the past decade as a disease biomarker and a quantification tool for the evaluation of disease prognosis and/or therapeutic response.

The occurrence of an energy crisis has been widely acknowledged as a central event in the pathophysiological sequence of common degenerative and metabolic pathologies that are nowadays viewed as "primarily systemic bioenergetic diseases" (Wallace 2013). In this respect, high-throughput assays have been developed and constantly improved in order to allow the assessment of the bioenergetic profile of blood cells as a mirror of the energetic capacity of body tissues (Chacko *et al.* 2014, Tyrrell *et al.* 2016). In particular, there is increasing evidence that measurement of platelet mitochondrial function can serve as a good proxy for tissue-specific defects in cellular respiration (Zharikov and Shiva 2013).

The aim of this mini review was to summarize current information in the field of platelet mitochondrial dysfunction in both acute and chronic pathologies.

SEARCH STRATEGY

The PubMed data base was searched for English-language literature following MeSH (Medical Subject Headings): "peripheral platelets", "circulating blood platelets", "mitochondrial dysfunction", "mitochondrial respiration", "bioenergetics", "profile", "biomarker", "mechanism", "interaction, "correlation"", "human", "acute", "chronic", and "disease". All articles addressing the topic were studied in detail and additional relevant literature was extracted from the references of the cited papers. The "related articles" function on PubMed was also used to further identify relevant information. No publication date restrictions were applied.

Platelet Function Regulation and Mitochondria

Platelets are short-lived (5-7 days), anucleated blood cells primarily involved in the regulation of hemostasis and thrombosis (Koupenova *et al.* 2018) that also play important roles in inflammation, immunity, and cancer (Li 2016, Rondina *et al.* 2013, Semple *et al.* 2011).

Despite having a relatively low number of functional mitochondria, platelets are highly energy consuming, metabolically active cells. Accordingly, the main role of platelet mitochondria is to provide the ATP required for their activation within the process of thrombus formation (Rendu and Brohard-Bohn 2001, Zharikov and Shiva 2013). Although platelets in basal state are powered by both oxidative phosphorylation and glycolysis, the former has been reported to be the prevalent source of energy when platelets are activated (Aibibula *et al.* 2018, Chacko *et al.* 2013). Platelets have a higher oxygen consumption rates as compared to leucocytes since higher levels of ATP are required for the normal functioning of ion channels that maintain the intracellular ionic balance (particularly of calcium ions) that is essential for preventing platelet activation in basal conditions (Kramer *et al.* 2014). Another important platelet trait is the fact that mitochondrial complex III and IV proteins are low (Kramer *et al.* 2014) which means that even a slight degree of mitochondrial damage could have a very severe impact on platelet function. Also, this feature serves to validate the important role of platelet mitochondrial respiration monitoring as a very useful, minimally invasive diagnostic and prognostic tool in widespread pathologies proven to affect mitochondrial complexes III and IV, such as diabetes (Raza *et al.* 2011), Alzheimer's disease (Parker *et al.* 1990b) or Parkinson's disease (Benecke *et al.* 1993, Haas *et al.* 1995).

In platelets, as in other cells, oxidative phosphorylation and glycolysis are inter-linked in such a way that a decrease/inhibition of one process leads to the compensatory increase of the

other (Akkerman *et al.* 1979). It has been reported more than 2 decades ago that a significant increase in lactate production (a hallmark of basal glycolysis) secondary to the impairment of oxidative phosphorylation occurred in platelets isolated from elderly people (as compared to young individuals) suggesting that changes in peripheral blood cells reflect the ones reported in post-mitotic tissues with ageing (D'Aurelio *et al.* 2001). Also, since it is now clear that there is an age-related decline in platelet counts for both male and female patients (Mahlknecht and Kaiser 2010) it is tempting to assume that platelets with disrupted mitochondrial function, as reflected by the impaired oxidative phosphorylation, have a higher clearance than the healthy ones. Indeed, a direct link between excessive reactive oxygen species (ROS) generated by inadequate mitochondrial respiration and platelet apoptosis has been described, suggesting that human platelet senescence is at least in part mediated by mitochondrial dysfunction (Wang *et al.* 2017, Wang *et al.* 2015b).

There has been a debate about the roles of the two processes, oxidative phosphorylation or glycolysis, in platelet aggregation. While early studies supported the major role of the latter as the main source of energy (Chaudhry *et al.* 1973, Misselwitz *et al.* 1987), more recent ones concluded that ATP provided by mitochondrial oxidative phosphorylation plays the critical part (Barile *et al.* 2012, Yamagishi *et al.* 2001). Recent bioenergetic studies in intact platelets demonstrate that, upon thrombin stimulation, oxidative phosphorylation is rapidly engaged and the process is supported by both L-glutamine and fatty acids oxidation (Ravi *et al.* 2015). Moreover, in order to evaluate whether the inhibition of mitochondrial respiration can disrupt platelet function, several groups assessed the effects of various chemicals on the electron transport chain complexes. Thus, Barile *et al.* 2012 tested different heterocyclic compounds belonging to the tetrazole, thiazole and 1,2,3-triazole classes. These compounds were able to

interfere with blood clotting, most probably via the inhibition of cytochrome oxidase. Tomasiak *et al.* (2004) also proved that impairment of mitochondrial complex III (cytochrome oxydase) by nitric oxide (NO) or mitochondrial complex IV reduced mitochondrial energy production which in turn inhibited platelet aggregation and secretion, thus demonstrating that platelet-activated coagulation depends on adequate mitochondrial respiratory function.

However, mitochondria also regulate the activation of platelets during thrombogenesis via non-ATP-mediated mechanisms, such as the mild generation of signalling molecules including mitochondrial ROS or the increase in mitochondrial calcium (Pignatelli *et al.* 1998). Importantly, these pathways that can equally trigger platelet apoptosis when released in high amounts (Lebois and Josefsson 2016, Lopez *et al.* 2007) - Fig. 1. Eukaryotic cells display a high efficiency of oxidative phosphorylation but this process inevitably leads to mitochondrial ROS release, thus rendering mitochondria as the main source of cellular ROS (Muntean *et al.* 2016). Complexes I and III of the electron transport chain also play a significant role in platelet ROS production as they generate the superoxide ion, which is then converted by superoxide dismutase into hydrogen peroxide (H_2O_2) (Pietraforte *et al.* 2014). These ROS are now recognized as second messengers in collagen-stimulated platelet activation (Zharikov and Shiva 2013). Indeed, exogenous treatment with H_2O_2 induces platelet activation while intracellular H_2O_2 scavenging inhibits calcium mobilization and platelet aggregation (Pignatelli *et al.* 1998). ROS production is regulated mainly by the redox state of the electron transport chain and therefore by the proton motive force/mitochondrial membrane potential ($\Delta\Psi$) (Lambert and Brand 2004). In this regard, several studies demonstrated a link between hyperpolarization of the mitochondrial membrane, ROS production and platelet activation (Matarrese *et al.* 2009, Yamagishi *et al.* 2001). For example, platelets from patients with diabetes expressed a decreased rate of oxygen consumption

together with hallmark signs of increased ROS production (Avila *et al.* 2012). Matarrese *et al.* (2009) demonstrated that an activator of the complement system can also induce membrane hyperpolarization, oxidative stress and platelet activation. Moreover, the group of Brownlee reported that hyperglycaemia induces membrane hyperpolarization in healthy platelets, a process that increased ROS generation and elicited platelet activation (Yamagishi *et al.* 2001).

Apart from superoxide and H_2O_2, mitochondria are able to produce •NO (Rusak *et al.* 2006). A rapid reaction between superoxide and NO leads to the formation of peroxynitrite ($ONOO^-$), a powerful oxidizing and nitrating compound. In collagen-stimulated platelets, $ONOO^-$ was reported to decrease ATP concentration and mitochondrial respiration via the inhibition of complexes I, II and IV of the respiratory chain (Rusak *et al.* 2006) - Fig. 1 (left dotted line). Moreover, the inhibitory effect observed on platelet secretion (but not on aggregation) may be due, at least in part, to the decrease of mitochondrial ATP production (Rusak *et al.* 2006).

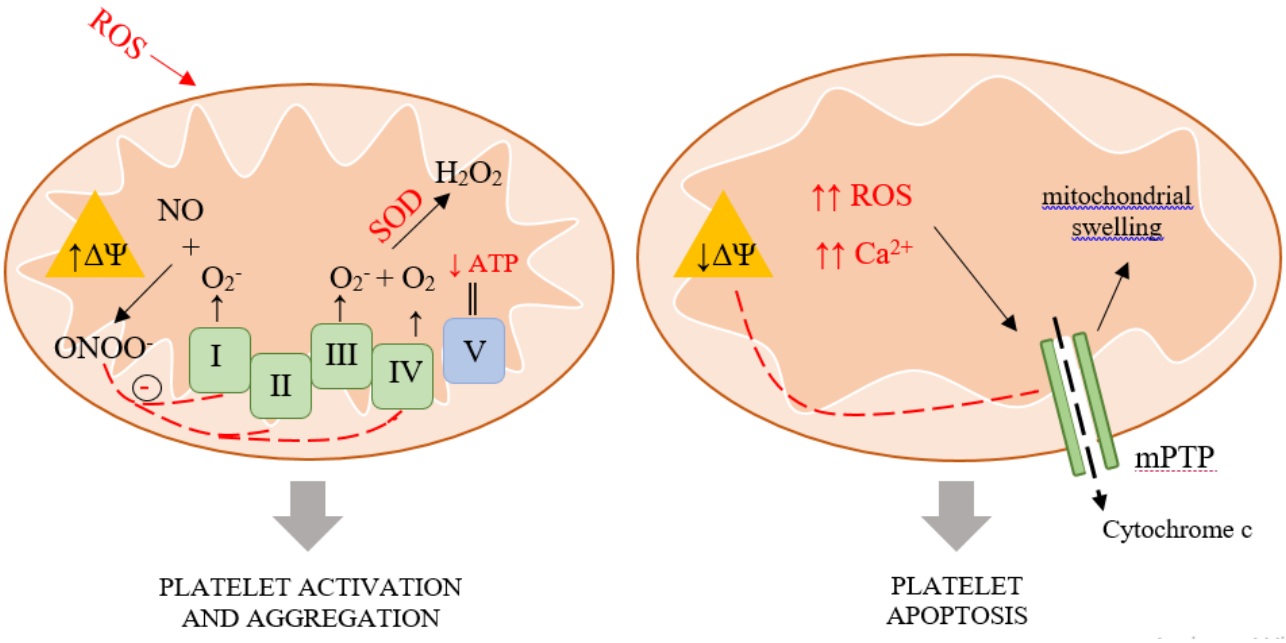

Figure 1. Mitochondrial regulation of platelet function (explications in text).

Therefore, accumulating evidence suggests that platelet mitochondria are not limited to providing ATP from oxidative phosphorylation but that they play crucial roles in triggering platelet activation through many interlinked mitochondrial processes: *i)* the increase in ROS production, caused by a dysfunctional electron transport chain; *ii)* the collapse of the mitochondrial membrane potential ($\Delta\Psi m$) caused by the impaired proton shift towards the mitochondrial intermembrane space and *iii)* the opening of a "megachannel", the mitochondrial permeability transition pore (mPTP) (Hottz *et al.* 2013, Leytin *et al.* 2009, Lopez *et al.* 2007) by increased to ROS and ROS-induced calcium mobilization via phospholipase C activation (Pietraforte *et al.* 2014). Thus, a vicious circle is created, in which the decreased $\Delta\Psi m$ opens the mPTP which will further reduce the mitochondrial membrane potential (Wang *et al.* 2017) - Fig 1 - right. Several studies have demonstrated that mPTP opening is associated with an increase of the inner mitochondrial membrane permeability to different compounds (ions, protons, and small metabolites) that cause mitochondrial swelling and the consecutive collapse of the mitochondrial membrane potential which finally leads to ATP exhaustion and cell death (Bernardi *et al.* 2015, Ong *et al.* 2015). Apart from this, mPTP also seems to be an important determinant of platelet PS (phosphatidylserine) exposure to the plasma milieu, an event that holds an important place in normal homeostasis since it is involved in thrombin production regulation (Lentz 2003). Studies have demonstrated that in stimulated platelets, exposure of PS is closely linked to mPTP formation (Jobe *et al.* 2008, Remenyi *et al.* 2005), release of cytochrome c and subsequent apoptosis (Pietraforte *et al.* 2014). Elevated levels of cytoplasmic Ca^{2+} are a necessary but insufficient condition for an initial PS exposure signal; conversely, inhibition of mitochondrial Ca^{2+} entry abolishes elevation of mitochondrial Ca^{2+} levels along with mPTP formation and PS exposure but does not affect platelet granule release or aggregation (Choo *et al.* 2012).

Besides their physiological role in platelet plug formation within the process of primary hemostasis (Cimmino and Golino 2013), it has been shown that platelets can recruit leukocytes at the site of inflammation and/or vascular injury, with the subsequent release of several inflammatory mediators and angiogenic factors (Smyth *et al.* 2009). Moreover, since platelets express toll-like receptors on their surface that are able to recognize pathogen-associated molecular patterns, they also play a significant role as mediators of innate immune response against invading microorganisms (Aslam *et al.* 2006). Platelet activation also lead to the release of respiratory-competent mitochondria. Boudreau *et al.* (2014) demonstrated that these extracellular platelet mitochondria are key mediators in inflammatory conditions. When found in high amounts in platelet concentrates, extracellular mitochondria induce transfusion-related acute reactions (e.g. fever, skin manifestations, etc.). Also, due to the presence of mitochondrial *N*-formylated peptides, leukocyte recruitment and neutrophil rolling along the vascular wall are promoted (Boudreau *et al.* 2014, Schiffmann *et al.* 1975). Owing to their ancestral bacterial origin, extracellular mitochondria serve as a substrate for the bactericidal sPLA2-IIA (secreted phospholipase A2-IIA), leading to mitochondrial integrity disruption and the release of ATP and mitochondrial DNA (mtDNA). Moreover, the interaction between mitochondria and sPLA2-IIA induces neutrophil activation and the formation of neutrophil extracellular traps (Boudreau *et al.* 2014) which in turn promotes platelet activation and aggregation (Fuchs *et al.* 2010, Gros *et al.* 2014); this might explain the long- time observed clinical link between sterile inflammation states (e.g. cancer) and thrombosis. It is important to mention that, since platelets lack nuclei. they also represent the ideal tool for future mtDNA studies aimed at anti-inflammatory therapeutic development (Wang *et al.* 2017).

As previously reported in the literature, not only thrombocytes but also leukocytes have been started to be used as biomarker of mitochondrial dysfunction in various pathologies such as neurodegenerative diseases, diabetes, cancer and cardiovascular pathologies (Briet *et al.* 2003, Clayton and Vinograd 1969, Cordero *et al.* 2010, Japiassu *et al.* 2011, Widlansky *et al.* 2010). Specifically, monocytes are considered a good sensor of metabolic stressors, such as hyperlipidemia or hyperglycemia. Also, lymphocytes bioenergetics can be used an index of disease processes that are associated with inflammation, as recently reviewed by Kramer *et al.* (Kramer *et al.* 2014).

Methods Used To Assess Respiratory Mitochondrial Function In Platelets

A large number of studies have already shown that mitochondrial dysfunction is present in various frequent pathologies, such as diabetes (Duicu *et al.* 2016), cancer (Sturza *et al.* 2018), neurodegenerative pathologies (Nicolson 2014) and cardiovascular diseases, the most significant cause of morbidity and mortality globally in the last 15 years (World Health Organization, 2018). As previously stated, in such pathological settings the easily accessible platelets demonstrate the potential for being used as markers for disease monitoring and recovery after therapeutic interventions.

The investigation of maximal enzymatic activity of the respiratory chain complexes is a widespread spectrophotometric method used to assess mitochondrial function (Sjovall *et al.* 2013). However, the evaluation of isolated complex activity does not offer an accurate account on the global mitochondrial function because respiratory chain complexes are interdependent and work as a whole, regulating up-stream other enzymes and supercomplexes (Lenaz and Genova 2009). Therefore, in order to correctly analyse mitochondrial function, a technique that operates as close as possible to a physiological environment (such as platelet-rich plasma) and without or

at least with a minimal cell disturbance should be used. Of note, assessment of platelet respiration can be traced back to the late 60s (Kitchens and Newcomb,1968).

Nowadays high resolution respirometry using the Oxygraph-2K equipment (Oroboros Instr.), is widely used for the assesment of peripheral blood cells respiration, including platelets (Sjovall *et al.* 2010). The development of this equipment allows nowadays the study of either intact or permeabilized cells for which exogenous substrates, inhibitors or uncouplers can be added, in order to analyse mitochondrial function (Pesta and Gnaiger 2012, Sumbalová Z 2018).

There is an increasing interest of the research community in performing mitochondrial respiratory studies in blood cells yet standardization of the assays is still needed. This is one of the objectives of the COST Action CA15203 MITOEAGLE (MitoEAGLE 2018). The group of Eskil Elmer was among the first to attempt standardization of the assay. Currently, two consecutive centrifugations at room temperature are required to obtain platelet-rich plasma for respirometry studies (Sjovall *et al.* 2013). These researchers also evaluated the impact of whole blood storage at either room temperature or at 4 °C and found that respiration remained stable after 24 hours but after 48 hours platelet mitochondria showed reduced respiratory capacity. A decline of both mitochondrial respiration and platelet function with the storage was also demonstrated by other studies, platelet storage time being associated with an increase in proton leak, ROS production and platelet apoptosis (Perales Villarroel et al. 2013, Ravi et al. 2015). Further studies are required to address the effects of time of sampling, anticoagulants used, transportation, separation procedures etc on platelet activation and how they will impact on respirometry studies.

Despite the fact that evidence points to the major role of oxidative phosphorylation in platelets, glycolysis remains also an important source of energy. The study of both processes is

possibly by means of the extracellular flux analyzer (Agilent Seahorse Bioscience) that requires small numbers of intact cells to evaluate the oxygen consumption rate related to mitochondrial respiration as well as pH modifications ascribed to changes in the glycolytic process (Avila *et al.* 2012, Chacko *et al.* 2013, Kramer *et al.* 2014).

Impairment of Platelet Mitochondrial Function in Particular Diseases

The process of collecting an appropriate amount of a viable tissue samples in systemic or organ-specific pathologies requires a high degree of invasiveness, thus the development of relevant cellular models to study mitochondrial function in humans is hindered. From this point of view, blood platelets are abundant and are easily obtainable through a simple blood draw, therefore they represent an attractive source for mitochondria studies in man (Zharikov and Shiva 2013). The study of platelet mitochondrial function in various disease states provides an effective way to investigate the underlying pathology. There is an abundance of literature demonstrating the association between platelet mitochondrial dysfunction and ageing/ageing-related diseases; therefore platelet respirometry is viewed as a viable marker of systemic mitochondrial dysfunction (Hauptmann 2006, Sjovall *et al.* 2013, Sjovall *et al.* 2010). Besides, due to the lack of nuclei, metabolic alterations can be investigated without the transcriptional regulation interference.

Table 1 reviews several studies aimed at elucidating platelet mitochondrial function in acute and chronic pathologies together with the reported change in mitochondrial function.

Table 1. Mechanisms of platelet mitochondrial dysfunction in various pathologies

Pathologies	Special conditions	Mitochondrial functional changes	Reference
ACUTE CONDITIONS			
Sepsis		Platelet mitochondrial uncoupling Increased respiratory capacity	(Sjovall *et al.* 2010)
		Lower mitochondrial nicotinamide adenine dinucleotide dehydrogenase (NADH) Decreased CI,III and IV activity	(Protti *et al.* 2015)
		Non-survivors presented a higher basal and maximal respiratory rate as compared to survivors, rates associated with organ failure and initial lactate level	(Puskarich MA 2016)
Cardiac arrest	Cardiac arrest induced in a porcine model via an asphyxia-associated ventricular fibrillation	Platelet mitochondrial bioenergetics are correlated with cerebral bioenergetic function Increase of CII-driven convergent respiration	(Ferguson MA 2016)

Cardiogenic shock		Lower mitochondrial nicotinamide adenine dinucleotide dehydrogenase (NADH) Decreased CI,II,III and IV activity	(Protti *et al.* 2015)
CHRONIC CONDITIONS			
Ageing		Decreased stability of CI Decreased energy conservation Decreased CIV activity	(D'Aurelio *et al.* 2001)
Asthma		Astmatic patients present increased oxygen utilization and efficients usage of substrates in platelets as previously shown in the airways	(Weiling Xu 2015)
Pulmonary hypertension	-	Increased mitochondrial reserve capacity Increased CII activity	(Nguyen *et al.* 2017)
Dyslipidaemia	Treatment with statins	Decreases ADP stimulated respiration Decreases CI-linked respiration	(Vevera *et al.* 2016)
Cardio-pulmonary bypass		Unchanged platelet mitochondrial function	(Mazzeffi *et al.* 2016)
Sickle Cell Disease		Decreased complex V activity Decreased mitochondrial respiration	(Cardenes *et al.* 2014)

		Membrane hyperpolarization Increased ROS production	
Bipolar affective disorder	Manic patients Depressive patients Patients in remission	Increased CI-linked respiration Decreased CIV activity Increased CI-linked respiration	(Hroudová *et al.* 2016)
Depression	Intact platelets Permeabilized platelets	Decreased physiological respiration, ETS capacity and respiratory rate after complex I inhibition No changes in mitochondrial respiratory rates	(Hroudova *et al.* 2013)
Alzheimer's disease	 Intact platelets	Decreased CIV activity Decreased endogenous basal respiration rates, CIV activity and ETS capacity, increased respiratory rates after CI inhibition	(Parker *et al.* 1990b), (Mancuso *et al.* 2003)

	Permeabilized platelets	Mitochondrial respiration was completely rescued by the addition of CI substrates	(Fisar *et al.* 2016)
Parkinson's disease		Mild CI defect	(Krige *et al.* 1992)
		Decreased CI and CIV activity	(Benecke *et al.* 1993)
		Decreased CI and CII/III activity	(Haas *et al.* 1995)
Huntington's disease		Decreased CI activity	(Parker *et al.* 1990a)
		Decreased mitochondrial CI and CII function, lower maximal phosphorylation capacity	(Johannes K. Ehinger 2016)
Schizophrenia	High positive schizophrenics	Increased CI activity	(Ben-Shachar *et al.* 2007)
	Low positive schizophrenics	No changes in CI activity	
Amyotrophic lateral		Decreased CIV activity	(Ehinger *et al.* 2015)

sclerosis			

Platelet Mitochondrial Function In Diabetes Mellitus

Cardiovascular disease (CVD) remains the world's most common cause of death with more than 17 millions annual global deaths (Roth *et al.* 2017). Moreover, CVD is the most common comorbidity in type 2 diabetes mellitus (DM); overall CVD affects approximately 1/3 of diabetic patients and accounts for approximately 1/2 of all deaths (Einarson *et al.* 2018). Several factors such as endothelial dysfunction, oxidative stress, increased coagulability and chronic inflammation are direct contributors to the development of CVD (Leon and Maddox 2015). As presented above, besides their central role in coagulation, platelets are involved in inflammation, due to their ability to secrete several mediators, with the acceleration of both atherogenesis and progression towards diabetic micro- and macroangiopathy (Siewiera *et al.* 2016, Vieira-de-Abreu *et al.* 2012). Currently, it is widely known that atherosclerosis is the most frequent complication of both type 1 and 2 DM (Ersoy *et al.* 2015, Tomkin and Owens 2015) and platelet dysfunction is contributing to the occurrence of cardiovascular complications (Tomkin and Owens 2015). Nevertheless, there are rather few studies that comprehensively aimed at elucidating the role of platelet mitochondrial dys/function in DM (Tschoepe *et al.* 1991). Brownlee stated already back to (Brownlee 2001) that mitochondrial dysfunction can be at the root of pathomechanisms by which hyperglycaemia causes diabetic complications. Moreover, it has been reported that a high concentration of glucose can induce mitochondrial damage in heart muscle fibres, skeletal muscle, brain and kidney tissue (Chowdhury *et al.* 2011) and therefore, the hypothesis of platelet mitochondrial dysfunction in high glucose conditions is conceivable. Indeed, Wu *et al.* (2015) demonstrated platelet mitochondrial changes such as

swollen mitochondria with damaged inner membrane, an increased ROS production and a decreased ATP content in diabetic rats and patients, concluding that platelets can be used as a model to further analyse the changes of mitochondrial function in DM complications (Wu *et al.* 2015). Similarly, Siewiera *et al.* (2016) showed that untreated DM produces an increase of the mitochondrial mass and also changes in the bioenergetic profile of platelets by hyperpolarization of the mitochondrial membrane. At variance, other studies found no differences in platelet size/volume between type 2 DM and healthy patients (Schaeffer *et al.* 1999). Also, non-proliferative diabetic retinopathy seems to hamper platelet submitochondrial particles membrane fluidity and to increase F0/F1-ATPase hydrolytic activity, both of which being recognized markers of mitochondrial dysfunction (Rodriguez-Carrizalez *et al.* 2014). Regarding the role of mitochondria in DM as sources of ROS Fink *et al.* (2012) suggested that acute or chronic exposure of isolated human platelets to high glucose concentrations does not accelerate the mitochondrial oxidative metabolism and hence, the probability of an enhanced ROS production/altered redox status. However, several studies have shown that, apart from its ability to potentiate collagen-mediated platelet activation, hyperglycemia leads to increased ROS production and that ROS scavengers could prevent platelet hypersensitivity caused by activation (Yamagishi *et al.* 2001, Wu *et al.* 2015, Wang *et al.* 2017). Two antidiabetic drugs, metformin and phenformin (the latter being withdrawn due to an increased incidence of lactic acidosis) were tested on isolated platelets from healthy volunteers; respirometry results revealed that both compounds inhibit mitochondrial respiration via a complex I defect (Piel *et al.* 2015). As a consequence of this inhibition, ATP production was reduced, the enzyme AMP-activated protein kinase (AMPK) was activated, whereas the glucose turnover was accelerated via an increased

glycolysis (Piel *et al.* 2015). In addition, mitochondrial inhibition was considered a direct consequence of lactic acidosis in patients treated with phenformin (Piel *et al.* 2015).

Platelet Mitochondrial Function In Cancer

The development of cancer-specific therapies is the current aim of numerous research studies. In the past decade, as we attained new insights into cancer cells, mitochondria have emerged as key players of cell survival and growth signalling pathways as well as chemotherapy-induced apoptosis. It is now a known fact that mitochondria are effectors of cell death by being involved in the regulation of the intrinsic and extrinsic apoptotic pathway, the autophagic cell death pathway and the necrotic pathway - for a comprehensive review the reader is referred to the paper by Giorgi *et al.* (2008). Nowadays, scientists have proposed several mechanisms by which the Warburg effect observed in cancer cells (i.e., the shift of cancer cells to a high rate of glycolysis even if oxygen is in a high concentration) can take place. The proposed mechanisms include: *i)* mitochondrial dysfunction that leads to a decrease in ATP production; *ii)* adaptation of cancer cells to hypoxia; *iii)* interference of oncogenes with mitochondrial function; *iv)* a high upregulation of enzymes and glycolysis processes by cancer cells; and *v)* mtDNA mutations that can either increase ROS production and tumour proliferation or the adaptation of cancer cells to new environments (reviewed in (Kroemer 2006).

The role of platelets in cancer is partially elucidated. It is known that platelets work in favour of cancer progression being involved in crucial steps of cancer evolution such as angiogenesis (by releasing both pro-angiogenic and anti-angiogenic regulators), cancer invasion (by releasing factors that control vessel permeability), tumour growth (by releasing growth factors), and cancer cell adhesion (by forming platelet-cancer cell conjugates) that facilitate

cancer metastasis (Li 2016). Moreover, cancer itself seems to induce platelet activation via the release of platelet-activating factors like thromboxane A2, ADP and thrombin (Li 2016).

In patients with ovarian cancer, (Wang *et al.* 2015a) observed a significant difference in platelet mitochondria and microtubule system when compared to healthy donors. Thus, cancer patients had 50% more platelets mitochondria as compared to healthy donors and these organelles occupied a larger part of the platelet area. When doxorubicin, a chemotherapeutic agent that has thrombocytopenic side-effects, was tested on platelet function, it was found that the drug dose-dependently impaired platelet adhesion and aggregation, induced mitochondrial-mediated apoptosis via the intrinsic pathway, and increased mitochondrial ROS generation (Wang *et al.* 2015b). Interestingly, these effects were prevented by a mitochondrial selective ROS scavenger , an observation which stresses once more mitochondrial ROS are key players in platelet apoptosis and mitochondrial ROS scavengers are potential therapeutic agents in platelet-associated disorders that also involve mitochondrial oxidative damage, respectively (Wang *et al.* 2015b). Several studies reported on the effects of various compounds to induce platelet apoptosis via the mitochondria pathway. Another chemotherapeutic drug, cisplatin, increased ROS production, induced the mitochondrial translocation of the proapoptotic Bax with subsequent mitochondrial membrane depolarization (Thushara *et al.* 2015). Of note, two of increasingly used supplements, resveratrol and melatonin, were reported to also induce platelet apoptosis via Bax translocation into mitochondria, increase cytochrome c release and caspase-3 activation (Thushara *et al.* 2015).

Platelet Mitochondrial Function In Hematologic Malignancies

Hematologic malignancies (HM) are tumours of hematopoietic and lymphoid tissues that include leukemias, lymphomas, and plasma cell neoplasms (multiple myeloma). Even though the general tendency of HM survival rates has slightly increased over the past decade due to treatment advances (Dunham-Snary *et al.* 2014), these diseases represent the fourth most common type of cancer in both male and female populations (Caimari *et al.* 2010). In this context, a biomarker that can be correlated with the evolution, progression, severity and treatment response, might be advantageous in HM monitoring and therapy.

Mitochondrial dysfunction has been reported to play a critical role in the development and progression of HM, especially in leukemias where various mtDNA alterations (of mitochondrial-encoded cytochrome b, cytochrome c, COX-I and COX-II genes) were described - reviewed by (Sternfeld *et al.* 2009). Schimmer and Skrtic (2012) showed that in leukemic cells a significant increase of mitochondrial mass accompanied by a higher rate of oxygen consumption, as compared to normal cells occur. Moreover, at variance from other neoplasias, in acute myeloid leukemia cancer cells are more dependent on oxidative phosphorylation than on glycolysis (Ferguson *et al*, 2016, Ehinger *et al*. 2016). Interestingly, in both leukemia and lymphoma cells it was demonstrated that a mitochondrial respiratory defect exhibits a survival advantage (Puskarich *et al*. 2016). The mitochondrial uncoupling process (the disruption between substrate oxidation and ADP phosphorylation into ATP) has also been reported in leukemia cell, where it supports a shift to oxidation of fatty acids with increased resistance to the intrinsic apoptosis pathway (Weiling Xu *et al*. 2015).

In a recent pilot study we aimed at assessing the respiratory function of platelets isolated from patients with a number HM (non-Hodgkin lymphoma, acute myeloid leukemia, chronic

lymphocytic leukemia). A significant decrease of all respiratory parameters in the HM group vs. control was found; a decrease of 80% for OXPHOS, 81% for State 2 and 75% for State 4 were recorded *(unpublished data)*. These preliminary results clearly suggest that platelet mitochondrial function is impaired in patients with HM and further studies are mandatory to dynamically assess these parameters and their correlations with the prognostic, severity, survival rate and treatment response.

Platelet Mitochondrial Function In Neurodegenerative Disorders

The literature review shows that mitochondrial dysfunction is involved in neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS), disorders characterized by a gradual and selective loss of related neuronal system (DiMauro and Schon 2008, Lin and Beal 2006, Reddy 2008). As neurons have a high-energy demand, they rely on mitochondria for a proper function, thus being sensitive to every mitochondrial dysfunction. Neuronal damage and/or death can occur as a consequence of mitochondrial dysfunction via apoptosis, excitotoxicity, ETC abnormalities and increased ROS production, that can alter mitochondrial ATP production, calcium homeostasis, mitochondrial membrane potential and the opening of mPTP (Dong *et al.* 2009, Emerit *et al.* 2004, Rao *et al.* 2014, Schapira 1993).

A crucial finding in the assessment of mitochondrial function in patients with neurodegenerative disorders was made by studies that revealed that blood cells can recapitulate the mitochondrial alterations present in the central nervous system (Parker *et al.* 1989, Schapira 1993). Accordingly, it was firstly reported that patients with Parkinson's disease presented a complex I deficiency in both mitochondria from substantia nigra and platelets, respectively

(Parker *et al.* 1989, Schapira 1993). Low complex I, complex II/III and complex I and complex IV activities in platelets from Parkinson's disease patients were described by Benecke *et al.* (Benecke *et al.* 1993) and Haas *et al.* (Haas *et al.* 1995), respectively. Interestingly, Bronstein et al. (Bronstein *et al.* 2015) found no impairments of ETC activity in PD patients. In Alzheimer disease, researchers found a decreased activity of complex IV (Mancuso *et al.* 2003, Parker *et al.* 1989) and later, also in complex III (Valla *et al.* 2006). A more recent study reported besides a decreased activity of complex IV, a low concentration of coenzyme Q10 in blood and an increased activity of complex I in platelets isolate from patients with AD (Fisar *et al.* 2016). In platelets from ALS patients, complex IV activity was also decreased, accompanied by a compensatory increase in cellular mitochondrial content (Ehinger *et al.* 2015). In HD, studies yielded unclear results. Accordingly, Powers *et al.* (2007) found no significant difference in complex I or complex I/III activities in these patients, while a more recent study by Ehinger et al. (2016), revealed a decreased function of complex I, but this result was not uniformly confirmed.

Albeit a straightforward relationship between mitochondrial respiration and the pathogenesis of these disorders is lacking, assessment of platelet respiration has emerged as a putative biomarker in these pathologies as well.

Conclusions

An increasing number of studies are currently focused on mitochondrial dysfunction in disease, as they emerged a key organelles at the crossroad between health and disease and valuable tools for drug development. Due to their content of functional mitochondria, platelets are considered to be an easily accessible and reliable source for the assays aimed at evaluating mitochondrial dys/function in various pathologies. Assessment of mitochondrial platelet

respiration has emerged as a minimally invasive tool able to provide insights about the systemic mitochondrial function. Future studies are required for the standardization of the assays using platelets as a substitute to characterize organ mitochondrial function in various pathologies.

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