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Peripheral Blood Mononuclear Cells (PBMCs) Mitochondrial Respiration after Heart Transplantation (HTx) Potential links with Cellular shift, Mild Diastolic Dysfunction Diagnosed using Echocardiography and/or Acute Rejection

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Summary

The mitochondria are the main source of ATP and oxygen free radicals. A chronic cardiac or respiratory pathology is accompanied by an alteration of the mitochondrial tissue respiration, linked to the severity of the disease. This diagnosis requires an invasive biopsy, but mitochondrial analysis on circulating Peripheral Blood Mononuclear Cells (PBMCs) could be an alternative, due to the reflection of organ damage on PBMCs. In heart failure, this analysis is little explored and there is no data after heart transplantation. The objectives of this work were therefore to review the current knowledge in this field and to analyse the mitochondrial function of PBMCs from heart transplant patients. I wrote two reviews of the literature. Briefly, the decrease in PBMC oxidative capacity is related to the severity of cardiovascular injury, and associated with an increase in ROS production, thus confirming the possibility of using mitochondrial markers on PBMCs to study cardiac injury. In pulmonary pathologies, the mitochondrial function of PBMCs varies according to the pathology observed.

In an original prospective study carried out in heart transplant patients, circulating superoxide anion is increased compared to the control group, accompanied by an alteration of mitochondrial respiration linked to complex II, and a stimulation of complex IV. It appears that the alteration of complex II correlates with parameters, which may correspond to systemic inflammation or even to a minimal impairment of the diastolic function of transplanted hearts. Stimulation of complex IV, on the other hand, could be a compensatory mechanism for maintaining adequate energy production in heart transplant patients. The general inflammatory context objectified by an increased production of ROS and by increased neutrophil/lymphocyte ratios could participate in these results. Two patients were in rejection at the time of the study and had an increase in complex IV activity. It will be interesting, in the future, to confirm this result by a study targeting patients with cellular or humoral rejection.

My work has thus shown, for the first time, an alteration in the mitochondrial function of PBMCs in heart transplant patients compared with matched healthy subjects.

Résumé

La mitochondrie est la source principale d'ATP, et de radicaux libres de l'oxygène (RLO). Une pathologie chronique cardiaque ou respiratoire s'accompagne d'une altération de la respiration mitochondriale tissulaire, liée à la gravité de la maladie. Ce diagnostic nécessite une biopsie, geste invasif, mais une analyse mitochondriale sur des cellules circulantes mononuclées (PBMC) pourrait être une alternative, grâce au reflet de l'atteinte d'organes sur les PBMC. Dans l'insuffisance cardiaque, cette analyse est peu explorée, *et ne l'est pas pour la transplantation cardiaque.* Ce travail avait donc pour **objectifs** de faire le point sur les connaissances actuelles dans ce domaine et d'analyser la fonction mitochondriale des PBMC de patients transplantés cardiaques. J'ai écrit **deux revues de la littérature.** Brièvement, la diminution de la capacité oxydative des PBMC est en lien avec la gravité de l'atteinte cardiovasculaire, et associée à une augmentation de la production de RLO, ***confirmant ainsi la possibilité d'utiliser des marqueurs mitochondriaux sur des PBMCs pour étudier l'atteinte cardiaque***. Lors de pathologies pulmonaires. la fonction mitochondriale des PBMC varie en fonction de la pathologie observée.

Au cours d'un travail prospectif original réalisé chez des **patients transplantés cardiaques**, l'anion superoxyde circulant est augmenté par rapport au groupe contrôle, accompagné d'une altération de la respiration mitochondriale liée au complexe II, et d'une stimulation du complexe IV. Il s'avère que l'altération du complexe II est corrélée avec des paramètres pouvant correspondre à une inflammation systémique voire à une atteinte minime de la fonction diastolique des cœurs transplantés. La stimulation du complexe IV au contraire pourrait être un mécanisme compensateur permettant de maintenir une production énergétique adéquate chez les patients transplantés cardiaques. Le contexte général inflammatoire objectivé par une production augmentée de RLO et par des ratios neutrophiles/lymphocytes augmentés pourraient participer à ces résultats. Deux patients étaient en rejet lors de l'étude et avaient une augmentation de l'activité du complexe IV. Il sera intéressant, par la suite, de confirmer ce résultat par une étude ciblant les patients avec un rejet cellulaire ou humoral.

Mon travail a donc montré, pour la première fois, une altération de la fonction mitochondriale des PBMCs chez les patients transplantés cardiaques par rapport à des sujets sains appariés.

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Abbreviations

ACR	Acute cellular rejection
AMR	antibody-mediated rejection
ARB	angiotensin-receptor blockers
BB	Beta-blockers
CAV	Cardiac Allograft Vasculopathy
CMV	Cytomegalovirus
CVA	Cerebrovascular accident
EMBs	Endomyocardial biopsies
ETC	Electron transport chain
ETS	Electron Transport System
EPR	Electronic paramagnetic resonance
GDTM	guideline-directed medical therapy
GEP	Gene expression profiling
GFR	Glomerular filtration rate
HF	Heart failure
HTx	Heart transplant
HUS	Hôpitaux Universitaires de Strasbourg
ISHLT	International Society for Heart and Lung transplantation
IVC	Inferior vena cava
LVAD	Left Ventricular Assist Device
MIM	Mitochondria Inner Membrane
MRC	Mitochondrial Respiratory chain
Oxidative Phosphorylation	OXPHOS
PBMCs	Peripheral Blood mononuclear cells
PGD	Primary Graft Dysfunction
(rt-PCR)	Real time polymerase chain reaction
SDH	Succinate Dehydrogenase
SVC	Superior vena cava
SV-CHD	Single Ventricle Congenital Heart Disease

Publications Related to the Thesis

1. Peripheral Blood Mononuclear Cells and Platelets Mitochondrial Dysfunction, Oxidative Stress, and Circulating mtDNA in Cardiovascular Diseases.
   Alfatni A, Riou M, Charles AL, Meyer A, Barnig C, Andres E, Lejay A, Talha S, Geny B. J Clin Med. 2020 Jan 22;9(2):311. Doi: 10.3390/jcm9020311.

2. New Insights into the Implication of Mitochondrial Dysfunction in Tissue, Peripheral Blood Mononuclear Cells, and Platelets during Lung Diseases.
   Riou M, **Alfatni A**, Charles AL, Andrès E, Pistea C, Charloux A, Geny B. J Clin Med. 2020 Apr 26;9(5):1253. Doi: 10.3390/jcm9051253.PMID: 32357474 Review.

3. Peripheral Blood Mononuclear Cells (PBMCs) Mitochondrial Respiration and superoxide anioin after Heart Transplantation (HTx).
   Alfatni Abrar, Charles Anne Laure, Sauer François, Riou Marianne , Goupilleau Fabienne ,Talha Samy, Meyer Alain, Andres Emmanuel, Kindo Michel, Mazzucotelli Jean-Philippe, Epailly Eric, Geny Bernard. En révision.

Oral Communications

Poster Session

1) Mitochondrial Respiration in Peripheral Blood Mononuclear Cells in Heart Transplanted Patients. **Alfatni Abrar**, Charles Anne laure, Epailly Eric, Goupilleau F, Georg I Sauer F, Geny Bernard, *CRBS days Strasbourg, 9-10 juin 2022*.

Congress SFC Presentation

2) Peripheral Blood Mononuclear Cells (PBMCs) Mitochondrial Respiration after Heart Transplantation(HTx). **Alfatni Abrar**, Charles Anne laure, Sauer François, Talha Samy, Meyer Alain, Kindo Michel, Mazzucotelli Jean-Philippe, Epailly Eric, Geny Bernard.
   Journées Francophones de l'Insuffisance Cardiaque, des Cardiomyopathies, de l'Assistance et de la Transplantation cardiaque (JFIC-CAT), Strasbourg, 22-23 Septembre 2022.

General Introduction and Objectives

Cardiovascular diseases and especially heart failure are the main cause of mortality worldwide with prevalence gradually increasing as life expectancy increases. Therefore, heart transplantation remains the treatment of choice and the most applicable ways to revoke terminal organ failures allowing major increases in life quality and duration. Despite its effectiveness, the transplant is however vulnerable to a significant morbidity and any therapeutic enhancement would be welcome.

Studies shows that the pathophysiology of cardiovascular disease include mitochondrial dysfunction resulting in insufficient cellular energy production and enhanced reactive oxygen spices release[1]. Interestingly, PBMCs are likely to participate in this systemic disease. Recent research in sepsis suggests that bioenergetic profile of circulating PBMCs may represent mitochondrial activity in organs and is associated with illness severity, immunological changes, and prognosis [2]. Generally, PBMCs rely on mitochondrial respiration to match the metabolic need and it is easily accessible. A tiny volume of blood is drawn to get access to peripheral blood mononuclear cells (PBMCs), which allow mitochondrial function investigation. Lymphocytes, monocytes, and dendritic cells make up PBMCs, which are primarily involved in immunity and inflammation [2].

Also, dysfunction of the core production of the ATP (which is the oxidative phosphorylation OXPHOS) system has a crucial consequence in the progression of a disease particularly in the heart that has high-energy demands. To date there is no data concerning PBMCs mitochondrial respiration in heart-transplanted patients (Htx) funding our approach .

Problematic, objectives, and Hypothesis of the study:

My preliminary research, based on literature reviews, demonstrated a link between cardiovascular disease and PBMC mitochondrial dysfunction associated with increased ROS formation, but the data are relatively limited.

For the first time, our study will investigate if there is a change in mitochondrial function at the level of peripheral blood mononuclear cells in heart transplant patients and, mechanisms behind such eventual changes. We hypothesized that subclinical cardiac diastolic dysfunction and/or rejection, modulated by the immunosuppressives therapies might be involved in mitochondrial modulation.

The objectives:

1. This study aim to determine mitochondrial function in immune cells of peripheral blood, as well as the formation of reactive oxygen species in the venous blood of heart transplant patients.

2. The secondary objective is to investigate potential relationship between PBMC mitochondrial respiration and clinical, biological, echocardiography, and coronarography characteristics of Htx obtained during usual follow up after heart transplantation.

Hypothesis*:*

"An alteration in the mitochondrial respiration of circulating blood cells, associated with an increase in reactive oxygen species production, depends mainly on patient's cardiac and/or systemic characteristics".

This study will therefore provide a novel insight on mitochondrial respiration on PBMCs (OXPHOS in eukaryotic cells) and a better pathophysiological knowledge of mitochondrial function in heart transplantation population.

In the manuscript, we will first present some reminders (Chapter 1 and 2) that are mine but also the teamwork, and then, will focus on results arising from both my reviews and original research work.

Chapter I
Mitochondria and ROS

1. Mitochondria

Mitochondria organelle rich in biochemical components. Its major role is the production of energy. The mitochondria are membranous cytoplasmic organelles defined as a double membrane structure that has it is own DNA distinct from nuclear DNA and has several copies of mtDNA [3]. It is originated in eukaryotic organism, characterized by varying in its functions and features according to the cell type and metabolism.

mtDNA encodes 13 proteins involved in one of the most important functions of mitochondria: mitochondrial respiration for energy production, also called oxidative phosphorylation or respiratory chain [4]. The other proteins involved in this function (about 850) are encoded in the nucleus and transported to the mitochondria.

The mitochondria thus have a role of energy producer. These organelles are the (powerhouse of the cells) which means they are the primary source of metabolic energy inside the cells and anchorage the oxidative phosphorylation system that produce the majority of the ATP essential for cellular process [5,6]. In addition to producing cellular energy, mitochondria are engaged in a variety of other functions, including signaling, cellular differentiation and activation, intracellular calcium regulation, programmed cell death, cell cycle and cell growth regulation[2,7].

In addition, these organelles constitute one of the main sites of free radical production which seems to play a key role in several intracellular signaling pathways.

1.1. Structure

Mitochondria is composed of 4 components which made up of 2 membranes (Figure 1):

1) Outer mitochondrial membrane:

It divides the intermembranous region from the extracellular part. It is a rigid membrane. The outer membrane facilitates the flow of metabolites, cations, and information between mitochondria and cytosol[8].

2) Intermembrane space:

It is the space between the 2 membranes, and it has a major role in energy production and cell death[9].Recently, it was discovered that the intermembranous space plays a critical role in the coordination of mitochondrial activities with other cellular processes. The exchange of proteins, lipids, or metal ions between the matrix and the cytosol, the regulated initiation of apoptotic cascades, signaling pathways that regulate respiration and metabolic functions, the prevention of reactive oxygen species produced by the respiratory chain, and the control of mitochondrial morphogenesis are all examples of these activities [10].

3) Inner mitochondrial membrane:

Has multiple folds (cristae) to increase the surface area. The inner mitochondrial membrane is thus the primary location of ATP creation (oxidative phosphorylation), and its shape reflects this crucial role[6]. First, by folding into cristae, its surface area is significantly enlarged. Furthermore, the inner mitochondrial membrane includes an abnormally high percentage (more than 70%) of proteins involved in oxidative phosphorylation and the transfer of metabolites (e.g., pyruvate and fatty acids) between the cytosol and mitochondria. Rather, the inner membrane is impermeable to most ions and small molecules, which is necessary for the proton gradient that causes oxidative phosphorylation to be maintained[6].

These proteins carriers are:

- Respiratory chain complexes (CR) complexes. There are five of them, one of which is ATP synthase. Responsible for proton gradient maintenance and ATP production

-ANT (adenine nucleotide translocase) transports ATP from the matrix to the intermembranous space. It can exchange ADP with the matrix for ATP with the intermembranous space depending on its conformation.

- Uncoupling Proteins UCP

Allow protons to leak through the mitochondrial inner membrane into the matrix. This causes a slight uncoupling, which results in a loss of energy in the form of heat.

This uncoupling could reduce ROS production by increasing ubiquinone oxidation (a component of CR). As a result, UCP protect against oxidative stress[11].

4) The organelle's interior (or matrix):

Internal compartment of the mitochondria. The mitochondrial genetic system, as well as the enzymes responsible for the central reactions of oxidative metabolism, are housed in the matrix. There is also mitochondrial DNA encoding proteins that make up some of the subunits of complexes I, III, IV, V of the CR, mitochondrial ribosomes, tRNAs, and enzymes necessary for DNA expression[6].

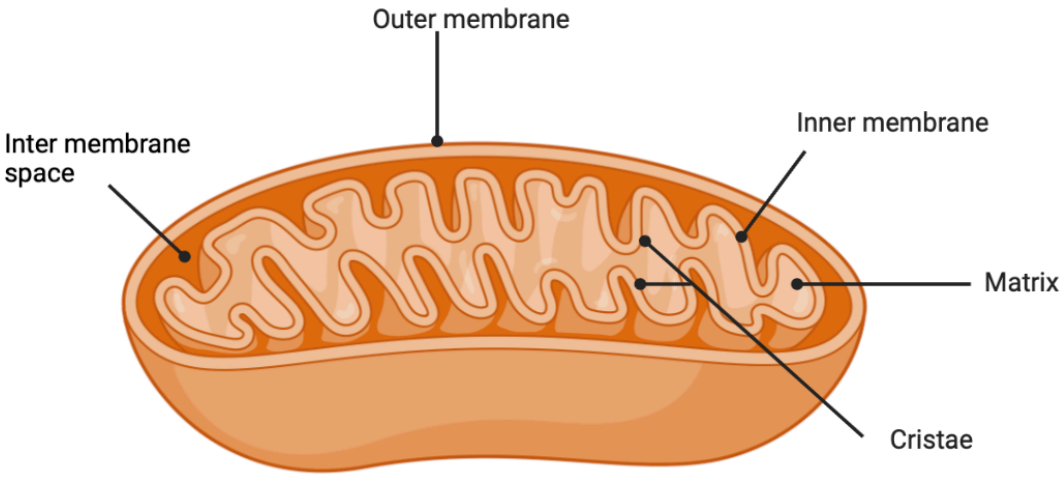

Figure 1.mitochondrial structures. Created with Biorender .com

1.2.Function

1.2.1. Electron Transport Chain

Mitochondrial respiration is an energy-generating mechanism , it takes place in ETC (Electron transport chain) in which five protein complexes(I-V) contained in the mitochondrial inner membrane (MIM) and electron transfer carriers ubiquinone and cytochrome c work together to make ATP [12]. Four of them play a role in the mechanism of oxidative phosphorylation (OXPHOS) and compose the Mitochondrial respiratory chain (MRC)[13,14]. These complexes are Complex I = NADH dehydrogenase, complex II = succinate dehydrogenase, complex III = ubiquinol: cytochrome c oxidoreductase, complex IV = cytochrome c oxidase (COX), and complex V = ATP synthase (Figure 2).
Electrons passing through ETC create energy, and these electrons eventually convert molecular oxygen to water[15].

All these complexes are made up of multiple protein subunits. Indeed, Complex II is encoded by nuclear DNA. The other complexes are encoded in both nuclear and mitochondrial DNA[16].

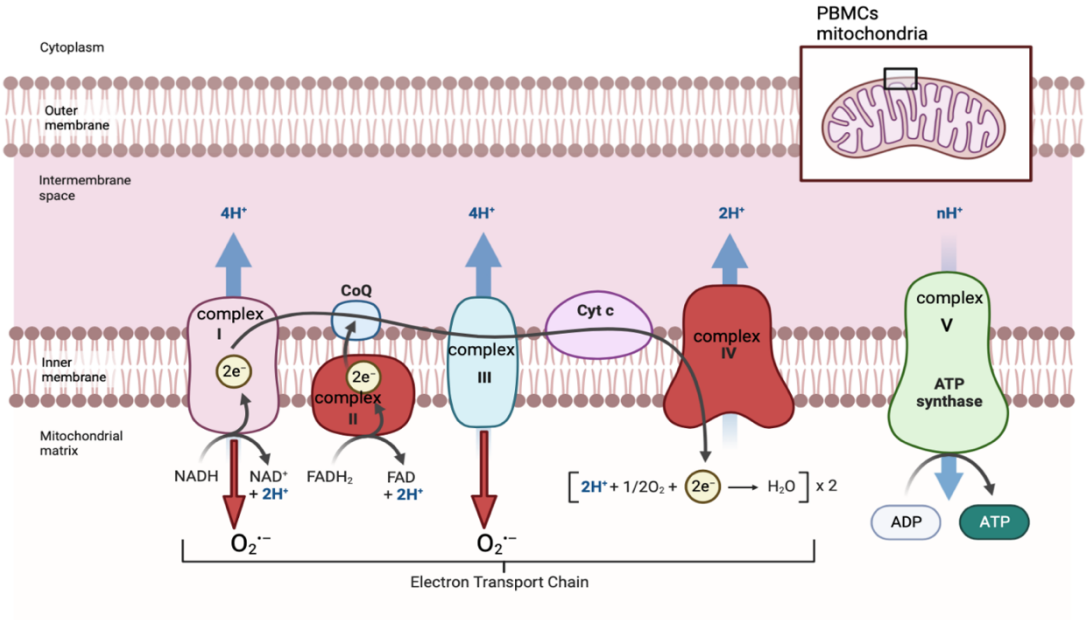

Figure 2.Simplified scheme of basic component of electron Transport chain (Oxidative phosphorylation), sites of superoxide anion generation (red arrows), and proton translocation (Blue arrows). The respiratory chain complexes I, III and IV transfer protons over the inner mitochondrial membrane, driven by redox reactions. Thus, they launch a proton gradient which is then used by the ATP synthase for the phosphorylation of ADP. Via NADH (Complex I) and succinate (complex II), electrons enter in the respiratory chain. Quinone (CoQ) and cytochrome c(cytc) shuttle the electrons between the complexes. The final electron acceptor is oxygen. Created with Biorender.com

1.2.2. The Oxidative Phosphorylation

According to the chemiosmotic theory by Peter Mitchell in 1961, by translocating protons from the matrix into the intermembrane space, the free energy of oxidation is released as an electrochemical potential across the inner membrane at complexes I, III, and IV to drive mitochondrial ATP production [13,17,18]. The high-energy electrons from NADH and FADH2 are transported to molecular oxygen via a succession of carriers in the membrane. The energy generated by these electron transfer processes is transformed to potential energy, which is then stored in a proton gradient across the membrane and utilized to drive ATP production[6].

OXPHOS appears to function as a cellular checkpoint. Normal cellular functions are represented by appropriate OXPHOS performance, while changed, non-adequate OXPHOS performance is a danger signal for the host cell[19].

1.2.2.a. Complex I :

Complex I, also called NADH-ubiquinone oxidoreductasewhich consists of about 40 polypeptide chains, is considered the largest complex among other complexes in the electron transport chain. It is an L-shaped structure located across the inner mitochondrial membrane and the matrix [20].

Furthermore, this structure's three-dimensional form is present at a very poor resolution and consists of multiple subunits (about 45 subunits) whose functions have not been clear yet, which contributes to the conclusion of the complexity of complex I. The main function of this part is to convert NADH to NAD+ by the transference of a pair of electrons onto complex I[21].

1.2.2.b. Complex II :

Complex II is called Succinate Dehydrogenase (SDH), it receives electrons from succinate (an intermediate in the citric acid (Krebs) cycle) and serves as a second entry point to the ETC ,and it composed of four subunits making it the smallest complex in the OXPHOS system .

When succinate is oxidized to fumarate (as a part of Krebs cycle), 2 electrons are accepted by FAD within complex II. FAD passes them to Fe-S clusters and then to coenzyme Q. However, it is the sole complex that does not pump protons across the inner mitochondrial membrane and has all its subunits encoded by nuclear DNA, therefore less ATP is produced with this pathway.

1.2.2.c. Complex III :

Complex III is called *ubiquinol-cytochrome c oxidoreductase*
Cytochrome c reductase is the third protein complex of the electron transport chain. Has 11 subunits including 2 cytochromes b, cytochrome c1, and an iron/sulfur center.

Complex III receives 2 electrons from complex I and II and shunts these electrons across the intermembrane space to cytochrome c which transfer electrons to complex IV[22]. This operation permits to transfer 4 protons from matrix to the intermembrane space.

1.2.2.d. Complex IV :

Complex IV is called Cytochrome C oxidase (COX). Complex IV belongs to the super-family of heme-copper oxidases. This enzyme has 13 subunits , three of them (COX1, COX2, COX3) are controlled by mitochondrial DNA , and ten are controlled by nuclear DNA [23].

It is the last electron acceptor responsible for converting O2 to H2O [12,24].

$$\mathbf{O2 + 4H^{+} + 4\ e^{-} \rightarrow 2\ H2O}$$

Indeed, the electrons given up by cytochrome c enter the protein complex to be transferred to the active site where the binding of oxygen and its reduction to water takes place [25].This protein complex of respiratory chain function considered as the major regulatory location for oxidative phosphorylation (Mitochondrial respiration)"OXPHOS" since this is the location where over 90% of oxygen is consumed [13,26],80% used for ATP production and 20% used for proton leakage. Proton leakage is the passive return of protons into the matrix without passing through complex IV. Thus decrease the efficiency of oxidative phosphorylation.

1.2.2.e. Complex V:

The fifth complex , ATPsynthase, uses the electrochemical gradient force generated across the inner mitochondrial membrane for the phosphorylation of adenosin-diphosphate (ADP) to adenosin-triphosphate (ATP)[27].

Composed of 2 sub-complexes:

- F0 inserted in the internal membrane of the mitochondria
  - F0 carries protons from the intermembrane space to the matrix.
  - Has 5 subunits.

- F1 is in the matrix.
  - F1 converts ADP into ATP or the reverse through the proton gradient.
  - Has 5 subunits.

Table 1. Summary of all complexes with its function

Complex	Function
Complex I **(NADH-ubiquinone oxidoreductase)**	• Oxidize NADH (The key role of CI is to transfer 2 electrons from matrix NADH to ubiquinone in intermembrane space to induce pumping of 4 protons) • Generate superoxide anion • play an important role in causing apoptosis in programmed cell death [27]
Complex II **(Succinate dehydrogenase)**	• Oxidize $FADH_2$ • Generate superoxide anion
Complex III **(Cytochrome c reductase)**	• Receives electron from complex I and II • transferer electrons across the intermembrane space to cytochrome c. which carries the electrons to complex IV Generate superoxide anion
Complex IV **(Cytochrome c oxidase)**	• Receives 4 electrons from complex III 8 protons removed from the matrix (4 used to form 2 water molecules, and the other 4 pumped into the IMS)
Complex V **(ATP synthetase)**	• Utilize the proton gradient formed across the inner mitochondrial membrane to generateATP.For every 4 H+ ions, 1 ATP is formed [27].

1.3.Mitochondrial DNA

Mitochondria has its own genome. A small part of the mitochondrial proteins is encoded in this genome, the rest is encoded in the nuclear genome.

Thus there are regulatory factors encoded in the nuclear genome allowing the expression of either nuclear or mitochondrial genes.

Human mitochondrial DNA is a tiny double-stranded circular molecule of about 16 kilobases [28]. Mitochondria have a variable number of copies of mtDNA. A mutation can be present on all copies or on only a part of them. Encodes 13 mitochondrial respiratory chain proteins, 22 transfer RNAs and 2 ribosomal RNAs[29].

1.4.Mitochondrial Respiration Methods

Generally, significant increases in energy demand or a decrease in energy supply, frequently result in cellular dysfunction and death. Because mitochondria are the primary source of energy in the cell, their dysfunction is frequently pathogenic. As a result, quantitative measurements of cellular and mitochondrial energy utilization and production are critical for understanding disease development and progression [30].

According to Nolfi-Donegan et al, oxidative phosphorylation can be studied by non-invasive and invasive techniques (Figure 3) [11]:

1.4.1. Non- and minimally invasive technologies:

Near-infrared spectroscopy (NIRS) , magnetic resonance spectroscopy (MRS), and positron emission tomography (PET) .

1.4.1.a. Near-infrared spectroscopy

At different wavelengths, a near-infrared light is absorbed by the oxygenated and deoxygenated heme groups of hemoglobin and myoglobin. Then, tissue oxygen consumption is calculated, and permits to have an idea of the mitochondrial respiration. While NIRS is relatively inexpensive.One significant disadvantage of this method is that non-invasive near infrared light cannot reach solid organs such as the heart and therefore is best used for exercising large muscles closer to the skin [11].

1.4.1.b. Magnetic resonance spectroscopy

Is a non-invasive, ionizing-radiation-free method for measuring the presence of metabolites in the heart and other solid organs by measuring the resonance of MR-visible isotopes[11].

1.4.1.c. PET imaging

To measure the accumulation or consumption of metabolic intermediates, injected positron-emitting radionuclide tracers are detected.

While PET and MRS provide a thorough assessment of energetics, both techniques necessitate expensive equipment and extensive training for data acquisition and analysis. Furthermore, PET faces the additional challenge of radiopharmaceutical production and patient radiation exposure. Furthermore, these technologies are highly specialized and unsuitable for high-throughput clinical applications, limiting their widespread application[11].

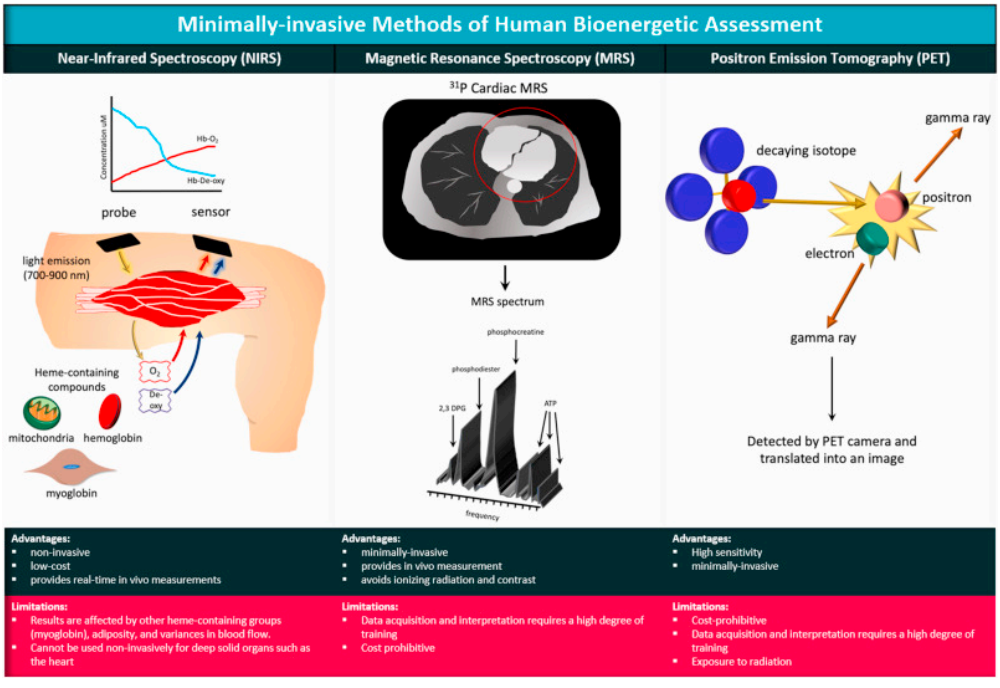

Figure 3.The bioenergetics measurement with minimally invasive methods[11].

1.4.2. Invasive Techniques

They requires a sample such as biopsy or blood . Electron transport complex activity can be densitometrically evaluated in frozen tissue using histochemistry [26]. But, the gold standard for the direct assessment of mitochondrial ETC function in humans is the measurement of mitochondrial function by the Oroboros O2k system which provide a better sensitivity and precision using polarographic measurement of oxygen consumption by isolated mitochondria or cells with a clark type electrode [31,32].

A number of laboratories have recently advanced the concept of taking respirometric measurements in circulating blood cells, such as platelets and peripheral blood mononuclear cells, to assess systemic bioenergetic function. Blood cells have fully functional mitochondria, are abundant, self-renewing, and require little invasiveness to obtain [11]. There are two ways to measure the mitochondrial respiration under this technique (Figures 4,5):

1.4.2.a. Extracellular flux analyser using (Seahorse XF):

The key features of using Seahorse Extracellular Flux Analyzer (EFA) is to measure cellular bioenergetics function in intact and permeabilized cells because it produces efficient, comprehensive, and highly reproducible results. The basic principle is to measure the extracellular acidification rate (ECAR; an indicator of glycolysis) and the oxygen consumption rate (OCR; an indicator of oxidative phosphorylation (OXPHOS) from relatively small numbers of cells in real-time and without radioactivity [33,34]. The Seahorse XF is an assay platform that uses 24 or 96 microplates. The microplate-based assay has the advantage of being able to analyze multiple samples in a single plate with high resolution, providing sensitive high-throughput capability using fluorescent sensor [35].

In this high throughput multi-well format, the Seahorse extracellular flux (XF) analyzer can measure oxygen consumption in an intact monolayer of cells. The Seahorse analyzer measures extracellular pH while also estimating glycolytic rate. Despite significant differences between the two systems, such as the larger sample size required for the Oroboros and the requirement for a monolayer for the Seahorse versus a sample in suspension for the Oroboros, both systems can be used with a series of pharmacologic mitochondrial modulators to provide an ETC function profile [11].

Figure 4. Seahorse Instrument[36]

1.4.2.b. High resolution respirometry using (Oxygraph-2k; Oroboros Instruments).

A high-resolution oxygraph is a device that measures cellular oxygen consumption in biological samples (intact and permeabilized cells, tissues or isolated mitochondria) with very high resolution and sensitivity in a closed-chamber system [31,32]. The high-resolution oxygraph system has two separate 2-mL chambers and measures oxygen concentration (µmol/L) and calculates oxygen consumption within each chamber using polarographic oxygen sensors. The rate of oxygen consumption is determined using software and represented as pmol/sec/cell count [31,35]. The basic principle of the system is to measure the concentration and consumption of oxygen by injecting substrates directly to isolated mitochondria or cells that are suspended in solution within the chamber.

While the respirometric techniques explained previously can be applied to any cell type, skeletal muscle biopsies or cells cultured from skin grafts have traditionally been used as a source of viable tissue/mitochondria in human studies. This project successfully applied Oroboros to circulating cells to demonstrate the feasibility of this methodology. These studies show that alterations in blood cell bioenergetics in a variety of natural biological conditions or pathologies correlate with physical or clinical parameters of these conditions, and that bioenergetics of circulating cells reflect bioenergetics in solid tissues such as the heart as it has been proven before in the other studies [11].

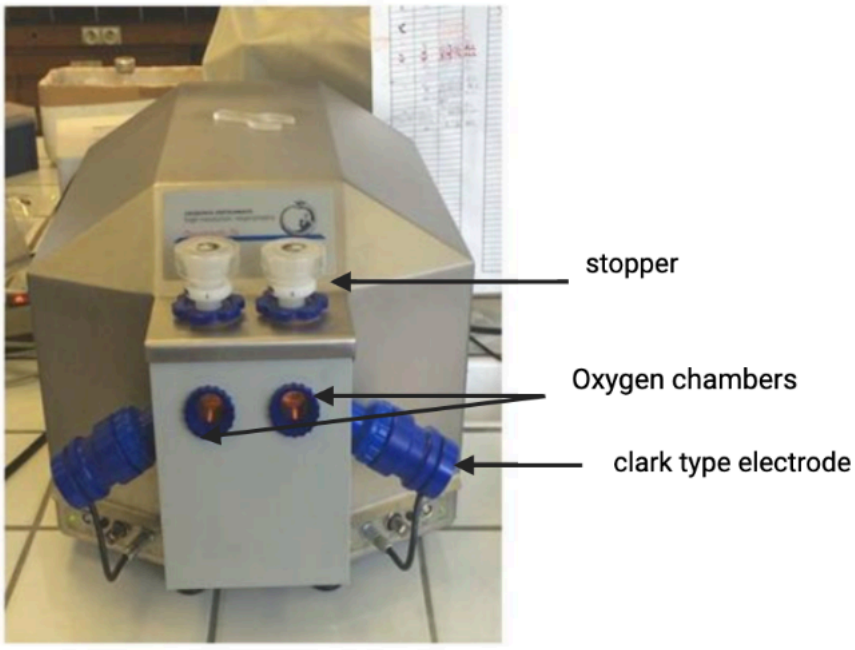

Figure 5.Oroboros Instrument

J'ai utilisé l'oroboros dans mon projet.

L'analyse de la fonction mitochondriale se fait dans des chambres d'oxygraphie avec une température controlée à 37°C, grâce à un effet peltier et sous agitation continue. L'appareil est un oxymètre à haute résolution (Oroboros Instruments, Innsbruck, Autriche)

présentant des électrodes de Clark dans chaque cuve d'analyse. Un logiciel spécifique (Oroborosdatlab) permet la mesure de la concentration d'oxygène au cours du temps et l'analyse de la vitesse de consommation d'oxygène dans chacune des chambres, reflétant ainsi l'activité de la chaîne respiratoire.

Les électrodes de Clark sont d'abord calibrées. Un protocole de « titration de substrats, découpleurs, et inhibiteurs » (SUIT) est utilisé pour activer les différents complexes de la chaîne respiratoire.

5×10^6 PBMC ont été introduits dans la chambre de l'Oxygraph-2k contenant 2,1 mL d'une solution tampon de Miro5+ Créatine.

La concentration en oxygène a été exprimée en µmol. L^{-1} et la consommation d'oxygène par les mitochondries en pmol $O_2.s^{-1}.10^6$ cellules (Figure 6).

La consommation de dioxygène a été analysée à l'aide du logiciel DatLab 8.4.3 (Oxygraph-2k; Oroboros Instruments, Innsbruck, Autriche). Un étalonnage en présence d'oxygène (air ambiant) était nécessaire avant chaque mesure.

Les membranes cellulaires ont été perméabilisées avec de la saponine (125 µg/mL), et le complexe I a été activé avec du glutamate (5 mM), et du malate (2 mM). Cette étape est destinée à soutenir le flux d'électrons à travers le complexe I (CI) du système de transport d'électrons (STE).

Ensuite, différents substrats et inhibiteurs ont été introduits dans la chambre de l'oxygraphe pour étudier les différents complexes de la chaîne respiratoire mitochondriale. Les différents réactifs ont été ajoutés dans l'ordre suivant :

Oxygen concentration and electron respiratory chain activity

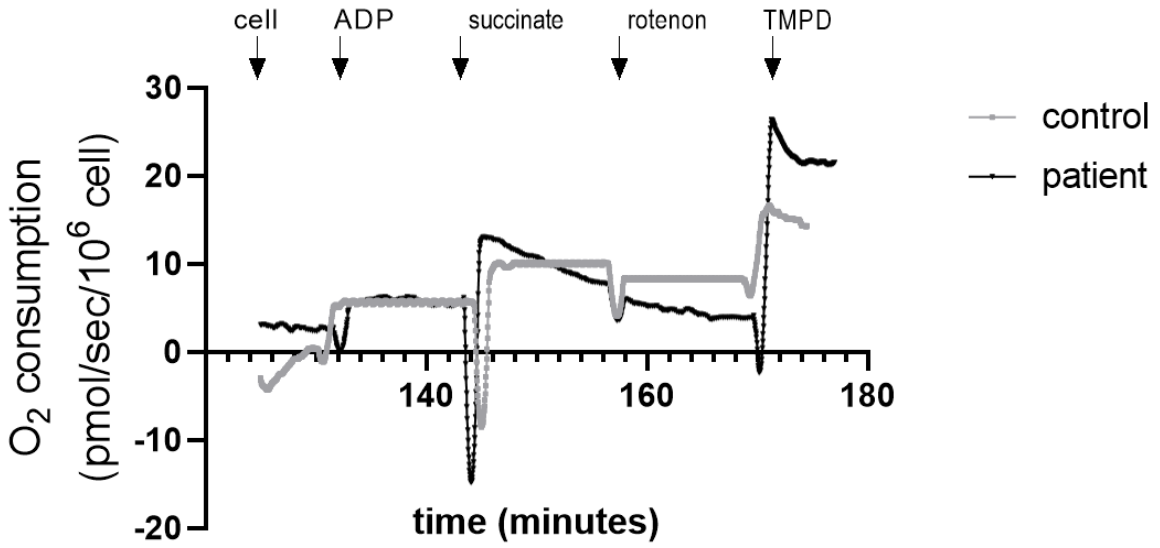

Figure 6. This curve illustrating an example of mitochondrial respiration of HTx patients compared to control.Cell: (represent CI leak, or basal respiration)Cell membrane is permeabilized with Saponin, complex I is activated using Glutamate & Malate, then PBMCS are introduced. In this stage, oxygen consumption of complex I at the leakage state is obtained which correspond to mitochondrial respiration compensating for proton leakage without activation of ATP synthase.

- L'ADP (2 mM) a induit l'activation de l'ATP synthase (OXPHOS par CI). A cette étape, on parle de phosphorylation oxydative soutenue par les complexes I, III, IV, et V.
- le succinate (25 mM) a été ensuite introduit pour étudier l'OXPHOS par CI&II. Cet état permet de voir l'activité de tous les complexes I, II, III, IV, et V.
- La roténone (0,5 µM), qui inhibe le complexe I, permet d'analyser l'OXPHOSCII, c'est-à-dire l'activité des complexes II, III, IV et V.
- L'ascorbate/ TMPD (0,5 mM/0,5 mM) ont enfin été ajoutés pour activer le complexe IV= Cytochrome C oxydase.

Le résultat a été exprimé en pmol/s/10^6 cellules.

2. Oxidative stress

Oxidative stress refers to elevated intracellular levels of reactive oxygen species (ROS) that cause destruction to lipids, proteins and DNA. Oxidative stress has been associated to a numerous of pathologies. However, elevated ROS are also signaling molecules [37].

Reactive oxygen species (ROS) are well-known for their ability to function both destructively and constructively[38]. Indeed, ROS are involved in a variety of cellular redox-regulating actions that help to maintain cellular homeostasis. However, it has been observed that excessive production creates oxidative stress, which is a harmful process that is involved in cell structure destruction and causes a variety of diseases [38].

2.1. Types of ROS

Reactive oxygen species defined as highly reactive unstable molecules that can undertake a number of reduction events, potentially causing injury and harm the normal cells [38].A free radical is any molecular species that can exist independently and has an unpaired electron. The presence of an unpaired electron results in certain properties that most radicals share. Many radicals are extremely reactive and unstable. They can either donate electrons from other molecules, and thus act as oxidants and often it starts a cascade of ROS production or otherwise accept electrons , in this case they are reductants [39].

The Oxygen O_2 can capture 1 or 2 electrons to give $O_2^{-\cdot}$and then H_2O_2 , thus at the origin of ROS. In the ROS family there are: superoxide anion ($O_2^{-\cdot}$), hydroxyl radical ($HO^{\cdot}$), and hydrogen peroxide (H_2O_2) but which is not radical [40].

Superoxide anion ($O_2^{-\cdot}$) produced by the reduction of O_2 which captures an electron. Its dismutation leads to the formation of oxygen and H_2O_2.

H_2O_2 is not a free radical because it has no unpaired electrons, but it is extremely toxic. Being uncharged it easily passes the membranes and can move far in the body.

Then according to the Fenton reaction:

$H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+}$

2.2. Producers (Sources)

Several sources in our body including autooxidation of small molecules, enzymes such as xanthine oxidase, and NADPH oxidase, peroxisomes and finally mitochondria.

ROS can be generated by exogenous or endogenous sources [41,42]. Pollution, alcohol, diet, ultraviolet light, tobacco smoke, heavy metals, industrial solvents, pesticides, and radiation are all examples of exogenous factors.[43,44]. Endogenous pro-oxidant compounds, are derived from cellular systems of organisms with high oxygen consumption, such as plasma membranes, cytosols, peroxisomes, and mitochondria [45]

Peroxisomes are also involved in the formation of $O_2^{-\cdot}$, $HO^{\cdot}$, $NO^{\cdot}$ and especially H2O2, whose production is partly regulated by β-oxidation of fatty acids [46]. Furthermore, cytosolic enzyme systems, such as the NADPH oxidase (NOX) family, produce RONS through the NADPH-dependent one-electron reduction of oxygen to superoxide [47–50] .The Xanthine Oxidase (XO) enzyme is also an important source of RONS [51].

Most of the intracellular ROS are derived from mitochondria. Under physiological settings, 0.2-2 % of the electrons in the ETC do not follow the regular transfer order and instead leak out and combine with oxygen to form superoxide or hydrogen peroxide. Each site of mitochondrial ROS generation is differentially sensitive to factors such as substrate supply, rate of ATP production and Δp depending on how these conditions affect the reduction of particular site of ROS production.

2.2.1. Complex I

Two sites of $O_2^{\bullet -}$ generation have been identified at Complex I:

1) The FMN cofactor which accepts electrons from NADH and
2) the Q binding site at which two electrons are transferred the terminal Fe–S to Q.

Forward electron transport involves electrons from NADH completely reducing the flavin mononucleotide center (FMN), which then reacts with oxygen to produce $O_2^{\bullet -}$.Conditions that promote NADH accumulation, such as low ATP demand resulting in

decreased respiration or ETC damage, result in a greater reduction of the FMN and increased $O_2^{\bullet -}$production at this site [11].

Among others, the Complex I inhibitor rotenone, which binds to the Q binding site, promotes $O_2^{\bullet -}$production by increasing electron accumulation and decreasing FMN site reduction.

According to popular belief, complex I (NADH-ubiquinone oxidoreductase) is the primary source of ROS in mitochondria. However, complex I ROS production is conditional; as a result, complex I becomes a major ROS source under pathological conditions rather than a dominant source under resting and healthy conditions[40].

2.2.2. Complex II

While Complex II is not generally regarded as a major source of ROS in comparison to Complexes I and III, several reports describe the production of $O_2^{\bullet -}$by the complex II. While the exact site of ROS is still being debated, Brand and colleagues have shown that the flavin site, where FAD binds the active site of the enzyme, is responsible for the production of ROS [11]. Also, growing evidence has determined that complex II mutations lead to oxidative stress, genomic instability and tumorigenesis [52].

2.2.3. Complex III

It is well known that complex III generate superoxide anion into either the matrix or intermembrane space [22].In complex III, Antimycin is the only effective ROS inducer among the bc1 complex inhibitors (antimycin A, myxothiazol, and stigmatellin).
Notably, as with ROS production in complex I, conformational changes detected in the bcl_1 complex after antimycin A binding may be a prerequisite for dramatic molecular rearrangements in the complex, resulting in significant ROS production.[40]

2.3.Antioxidant Defences

Every cell undergoes chemical reactions, including molecule oxidation and reduction. These reactions can result in the generation of free radicals. Organic substrates such as lipids, proteins, and DNA are attacked by free radicals. Free radicals cause damage to these molecules through oxidation, disrupting their normal function and potentially contributing to a variety of diseases. The anti-oxidation system, as a means of defence in our body, which includes both enzymatic and non-enzymatic antioxidants, protects against Ros production and oxidative stress. If this system is defected, tissue damage will increases [53].

2.3.1. Non-enzymatic systems:

Non-enzymatic antioxidants system deal with disturbing free radical chain reactions[54]. Few examples of non-enzymatic antioxidants include transition elements, vitamins C and E, and reduced glutathione [55].

2.3.2. Enzymatic systems[56]

Enzymatic antioxidants is the first line of defence against ROS. work by reducing and eliminating free radicals. In a multistep process involving cofactors such as copper, zinc, manganese, and iron, antioxidant enzymes convert dangerous oxidative products to hydrogen peroxide (H_2O_2) and then to water [54]

Glutathione peroxidase, catalase, and superoxide dismutase are the most effective enzymatic antioxidants [55]

2.4.Cell damages

Damage induced by ROS including Lipid peroxidation, protein oxidation, and DNA mutations.

These damages can lead to the death of the cell, by apoptosis[57].

a) Lipid peroxidation

Lipid peroxidation is a process generated naturally in small amounts in the body, mainly by the effect of several reactive oxygen species. These reactive oxygen species attack the fatty acid membrane very easly[58]. The effect and consequence of lipid peroxidation is modification of the membrane fluidity and permeability[59].

b) Protein oxidation

Protein oxidation is structural modification of a protein caused by either direct reactions with reactive oxygen species (ROS) or indirect reactions with secondary oxidative stress by-products leading to a loss of function[60]

c) DNA mutations

Cellular damage of DNA occurs at high level of ROS which leads to different disease pathology. More specifically , mitochondrial DNA due to the origin of the production of ROS especially mitochondrial [61].

2.5.Reactive Oxygen species production Methods

Currently, no standard methodologies exist to directly measure mitochondrial ROS production in humans.

La mesure de l'anion superoxyde dans le sang veineux total a été réalisée par résonance paramagnétique électronique (RPE), E-scan, Bruker-Biospin, Rheinstetten, Allemagne) à 37 °C [62]. En bref, 1 mL de sang veineux a été conservé sur la glace afin de réaliser l'analyse 1 heure après le prélèvement. 25 µL de sang ont été mélangés avec la sonde de spin CMH (1-hydroxy-3-méthoxycarbonyl-2,2,5,5-tétraméthylpyrrolidine HCl, 200 µM). Le mélange a ensuite été introduit dans un tube capillaire RPE en verre (Noxygen Science Transfer & Diagnostics, Elzach, Allemagne), puis placé dans la cavité du spectromètre e-scan (Bruker, Rheinstetten, Allemagne) pour l'acquisition des données. La détection de la production de ERO a été réalisée avec les paramètres RPE suivants: champ central g = 3477,452 ; largeur de balayage 60 G ; puissance micro-ondes 21,85 mW ; amplitude de modulation 2,4 G ; constante de temps 40,96 ms ; temps de conversion 10,24 ms ; nombre de points de courbe de retard 6.

Le signal RPE est proportionnel au nombre d'électrons non appariés. Le résultat a été exprimé en µmol/min.

3. PBMCS and mitochondrial respiration

3.1. What are PBMCs?

Human peripheral blood mononuclear cells (PBMCs) are defined as any blood cell with a round nucleus (e.g., lymphocytes, monocytes, natural killer cells (NK cells), or dendritic cells) isolated from peripheral blood. Density gradient centrifugation separates the cell fraction corresponding to red blood cells and granulocytes (neutrophils, basophils, and eosinophils) from whole blood. A gradient medium with a density of 1.077 g/ml separates whole blood into two fractions: PBMCs, which remain in the low density (upper fraction), and red blood cells and PMNs, which have a higher density and are found in the lower fraction(Figure 7)[63].

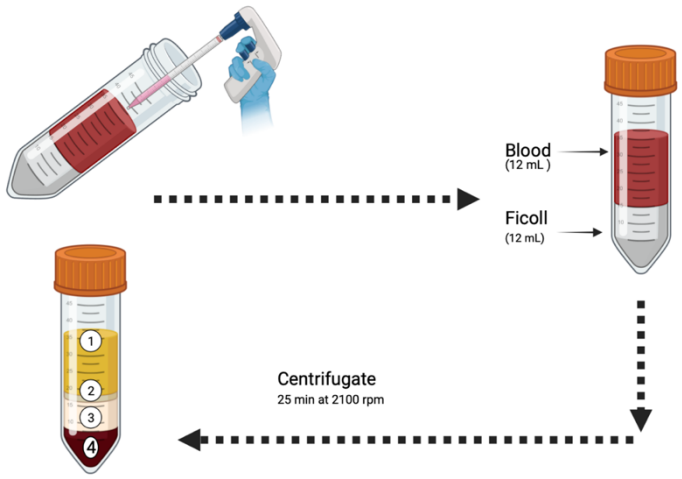

Figure 7.Representation of blood deposite on the Ficoll and the appearance of the tube after centrifugation.1) Plasma, 2) Cell ring: PBMCs (2/3 Lymphocyte, 1/3 monocytes and some circulating dendritic cells), 3) Ficoll, 4) Globular cap: red blood cells (RBCs) composed of granulocyte (neutrophils, eosinophils, and basophils). Created with BioRender.com

These cells are therefore increasingly used as biomarkers. They are easy to extract and reflect the pathology at the systemic level. Their use in heart transplant patients remains to be done.

3.2. Méthods de Extraction des cellules mononucléaires du sang périphérique (PBMC):

L'extraction des cellules a été réalisées extemporanément. La méthodologie d'extraction des PBMCs est similaire dans toutes les procédures réalisées au laboratoire.

Cette technique a été importée du laboratoire d'allergologie (HUS, Strasbourg).

Trente mL de sang a été utilisé pour extraire les cellules mononucléaires du sang périphérique (PBMC). Brièvement, le sang a été placé sur un gradient de densité ficoll (Eurobio, Lymphocytes separation medium, Courtabeauf France, France) et centrifugé (980g, 25 min, 18 °C, sans frein) pour obtenir une séparation des différents éléments du sang. En bas, on retrouve les globules rouges, au milieu les PBMC sous forme d'un anneau trouble, et enfin au-dessus le plasma.

Ce dernier est récupéré et congelé à -80°C pour de futures analyses.

L'anneau cellulaire contenant les PBMCs (lymphocytes et monocytes) est ensuite lavé avec une solution saline de DPBS (Dulbecco's Phosphate Buffer Saline 0067M, Hyclone, Utah, USA), et centrifugé à 569g, 10 minutes, 18°C. Le culot cellulaire est ensuite traité à la versalyse, si une présence de globule rouge est notée. Ce produit contient une amine cyclique qui, au contact d'une enzyme présente dans les hématies (anhydrase carbonique), va se transformer en un composé hautement lytique pour ces cellules. (Beckman coulter, Villepinte, France) [64].

A la suite de ce traitement un nouveau lavage au DPBS est réalisé suivi d'une centrifugation (569g, 10 minutes, 18°C).

Le culot de cellules est alors repris dans du DPBS et les cellules sont comptées par cytométrie de flux (Muse Cell Analyser, Merck Millipore, Darmstadt, Allemagne).

The whole methodology used in this project is summarized in this (Figure 8):

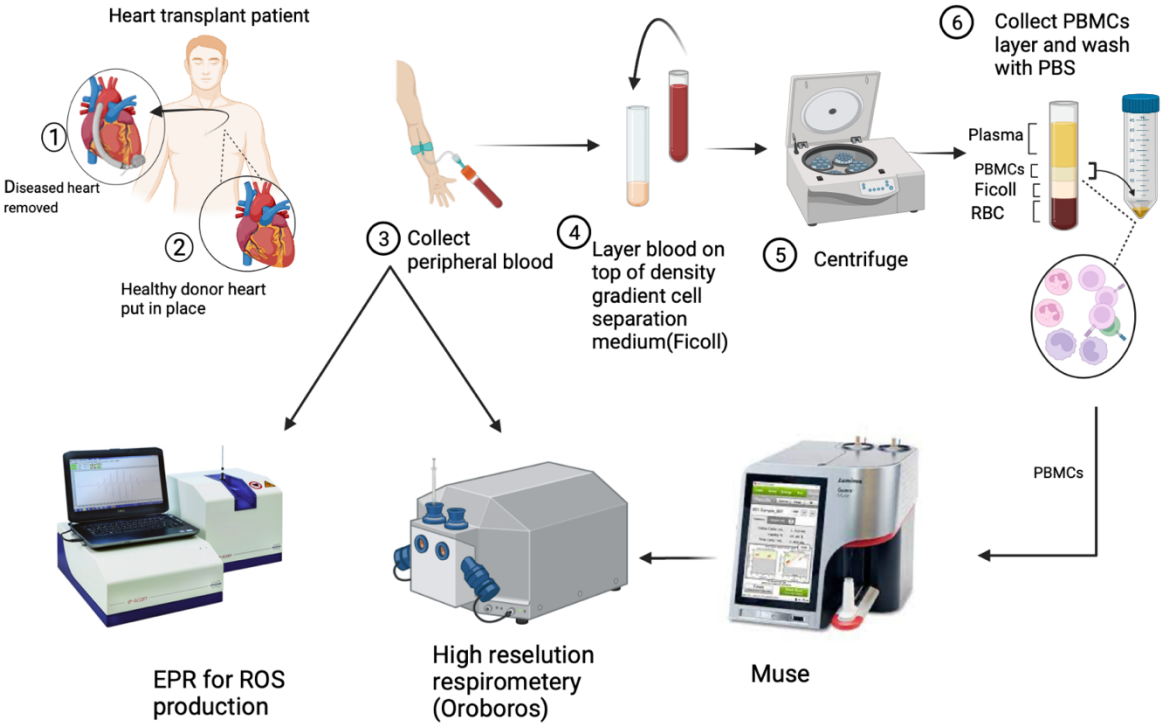

Figure 8.Methodology Applied in this Study

Chapter II

Heart Transplantation

1. Definition and Epidemiology

Heart transplantation, defined as the surgical replacement of a diseased heart by a healthy one, it is a treatment of choice for end-stage heart failure (HF). The first human to human heart transplantation was performed fifty five years ago by Christiaan Barnard, on the 3rd of December, 1967 [65,66].

Nowadays, HF impact nearly 5.7 million adults in the united states and almost half of those individuals die within five years of diagnosis [67]. Thus, heart transplant is considered as a gold standard treatment for severe refractory heart failure [68–70]. However, the number of heart transplant performed around the world is very limited [71]. Even more, since the beginning of Corona pandemic COVID-19, there is a considerable decline in the number of heart transplant and more generally on organ transplant in Europe and USA [72,73] (Figures 9,10,11,12,13) .

While patient is on waiting list for a donor's heart, once can survive on inotropes (medications that improve the force of the cardiac contractions) or mechanical support (LVAD) [70].

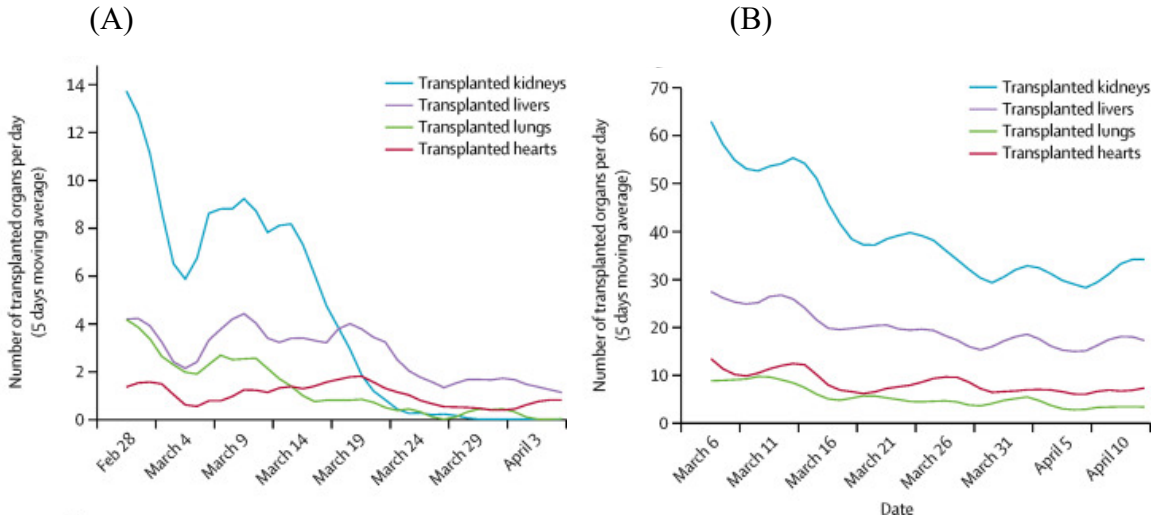

Figure 9. Total number of transplants during the beginning of Covid pandemic, with separate trend lines for Kidney, liver, heart, and lung in A)France; and B) USA[72].

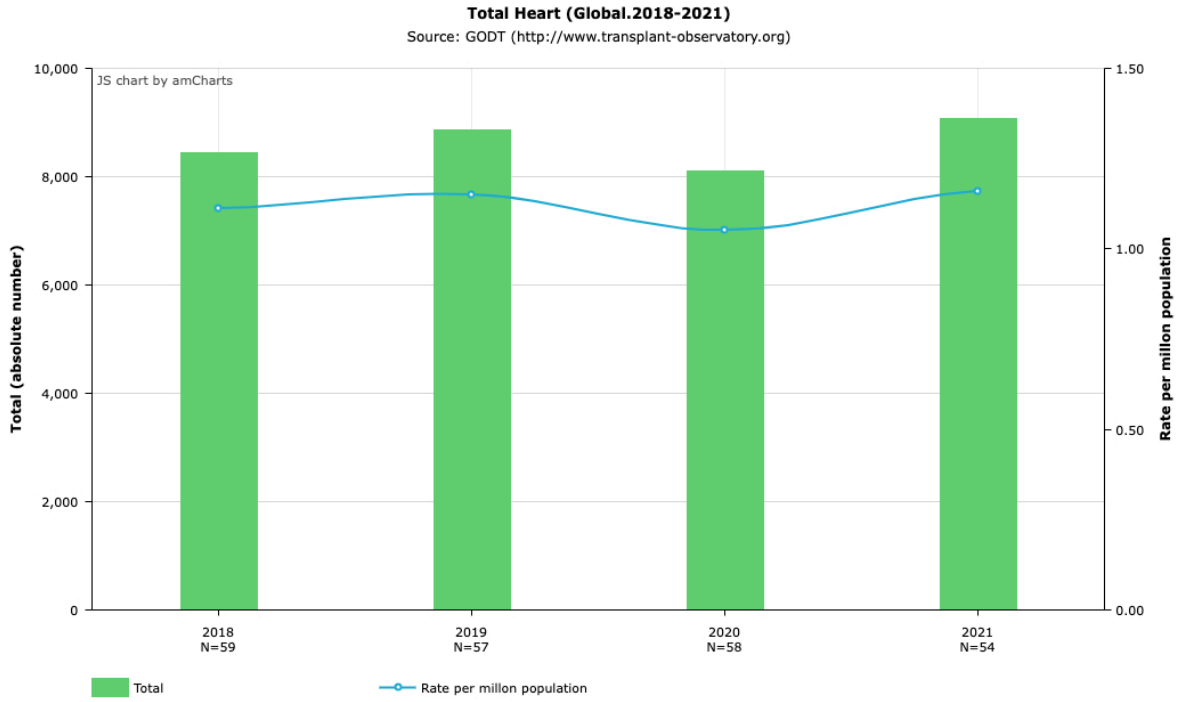

Figure 10. Total (Absolute number) of heart transplants globally before and during Covid Pandemic, N (number of countries included).). In 2018(N=59 countries) Total number of heart transplant =8.450, rate per million population (percentage) =1.11%. In 2019 (N=57 countries) Total number of heart transplants =8.857, rate per million population =1.15%. In 2020 (N=58 countries) Total number of transplants= 8,103, rate per million population =1.05%. In 2021 (N=54 countries) Total number of transplants =9.064, rate per million population =1.16%. Data obtained from (Global Observatory on Donation and Transplantation GODT (transplant-observatory.org/data-charts-and-tables/chart).

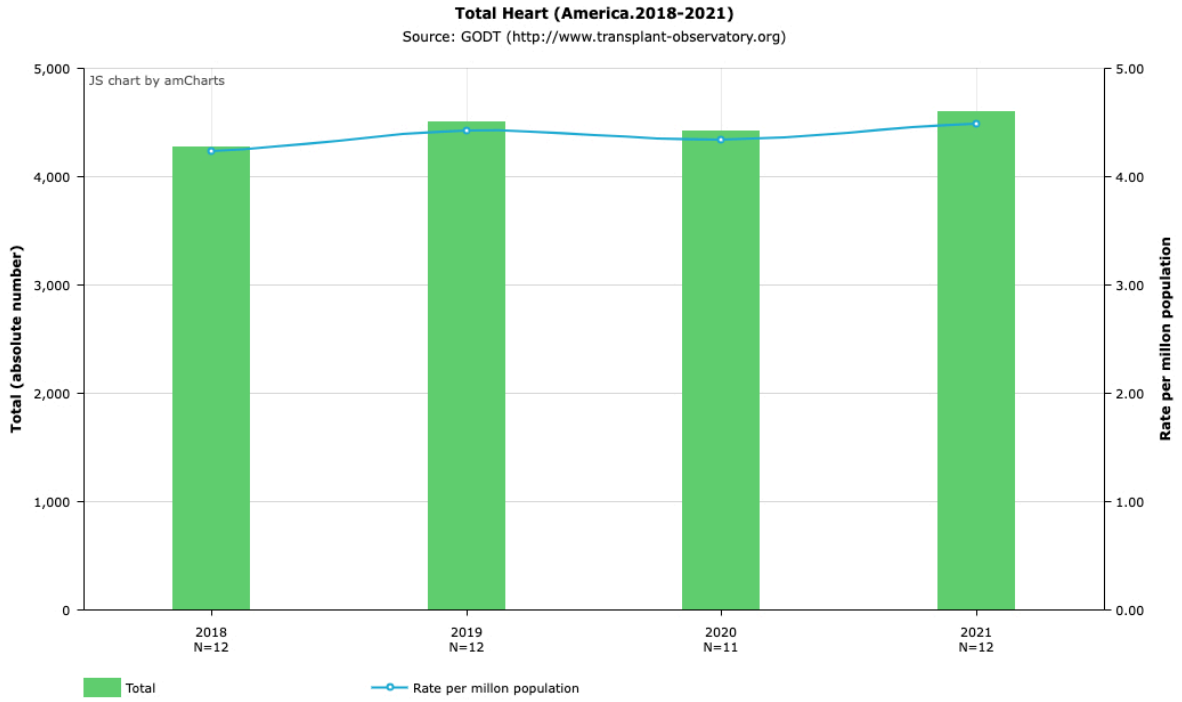

Figure 11. Total (Absolute number) of heart transplant in USA before and during Covid Pandemic, N (number of countries included).). In 2018(N=12 countries) Total number of heart transplant =4.273, rate per million population (percentage)= 4.23 %. In 2019 (N=12 countries) Total number of heart transplants =4.506, rate per million population = 4.42%. In 2020 (N=11 countries) Total number of transplants= 4.419, rate per million population = 4.34 %. In 2021 (N=12 countries) Total number of transplants =4.599, rate per million population =4.48%. Data obtained from (Global Observatory on Donation and Transplantation GODTtransplant-observatory.org/data-charts-and-tables/chart).

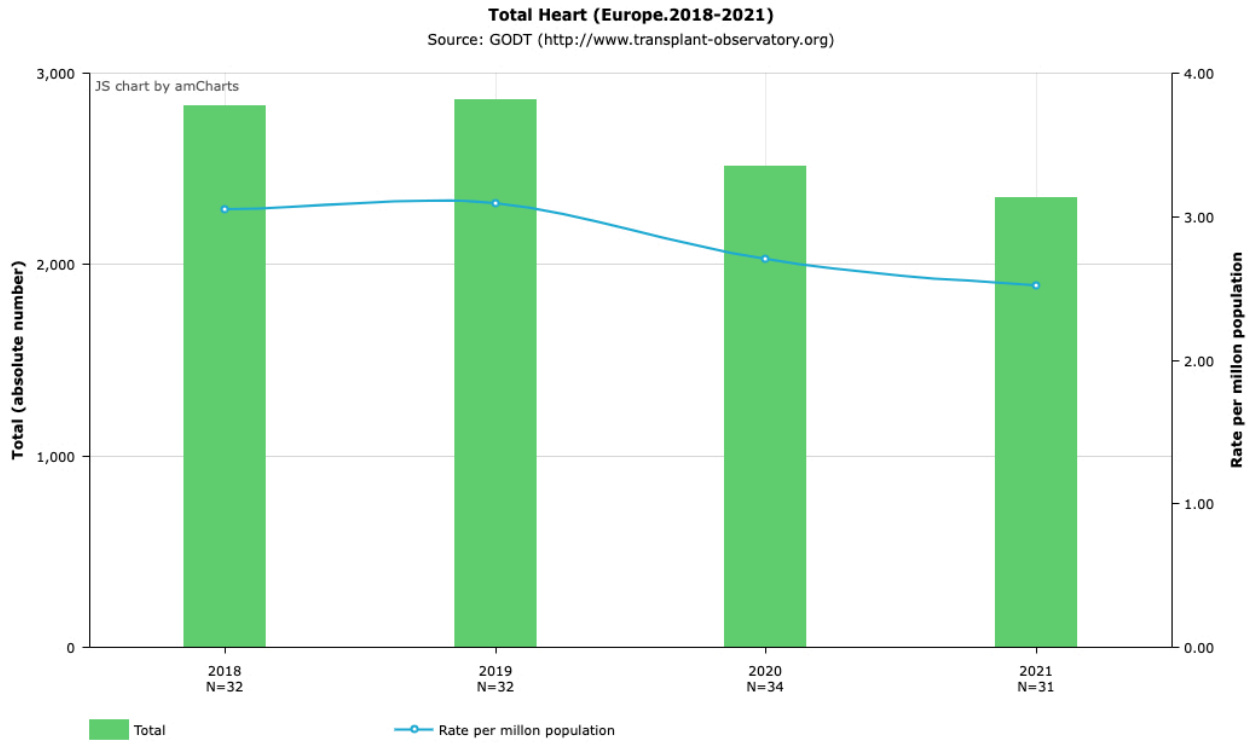

Figure 12. Total (Absolute number) of Heart transplants in Europe before and during Covid Pandemic. N (number of countries included).). In 2018(N=32 countries) Total number of heart transplant =2.827, rate per million population (percentage) =3.05 %. In 2019 (N=32 countries) Total number of heart transplants =2.862, rate per million population = 3.09 %. In 2020 (N=34 countries) Total number of transplants= 2.515, rate per million population = 2.7 %. In 2021 (N=31countries) Total number of transplants =2.350, rate per million population =2.52%. Data obtained from (Global Observatory on Donation and Transplantation GODT transplant-observatory.org/data-charts-and-tables/chart).

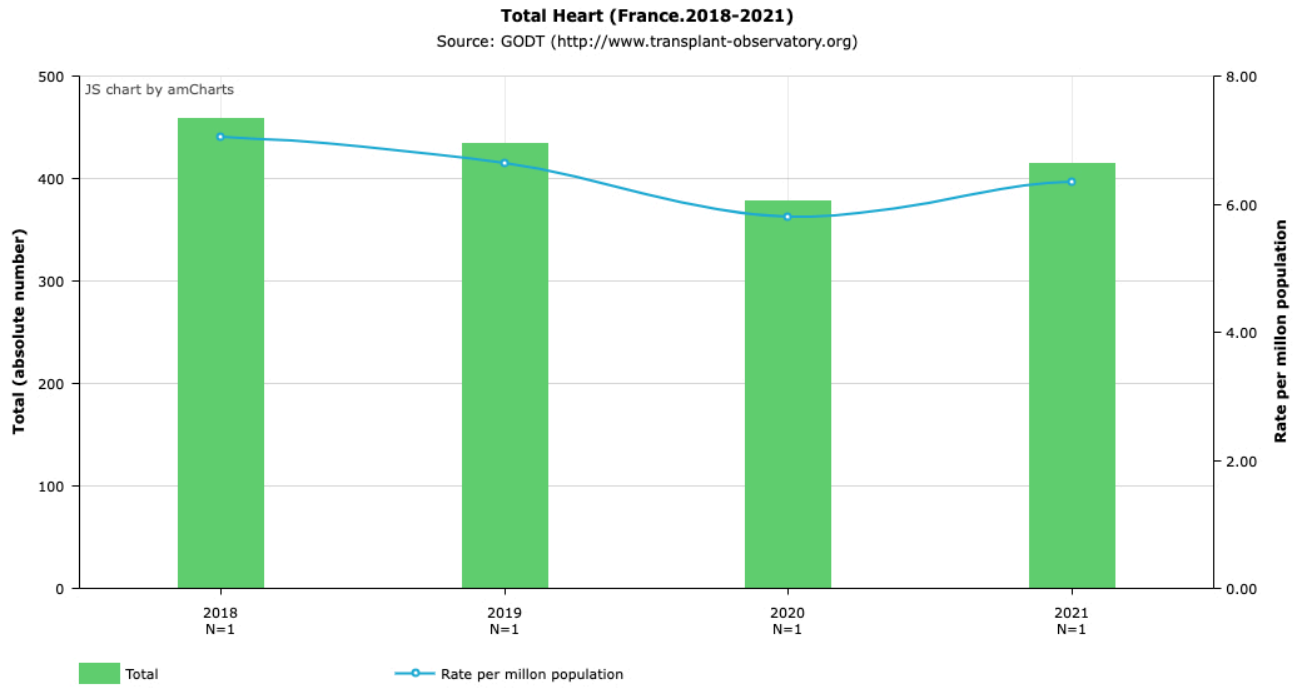

Figure 13.Total (Absolute number) of heart transplant in France before and during Covid Pandemic. N (number of countries included).). In 2018(N=1 county) Total number of heart transplant =459, rate per million population (percentage) =7.04 %. In 2019 (N=1 country) Total number of heart transplants =434, rate per million population = 6.63 %. In 2020 (N=1 country) Total number of transplants= 378, rate per million population = 5.79 %. In 2021 (N=1country) Total number of transplants =415, rate per million population =6.35%. Data obtained from (Global Observatory on Donation and Transplantation GODT.transplant-observatory.org/data-charts-and-tables/chart).

As a postoperative problems, heart transplantation surgeries generated poor outcomes until the introduction of cyclosporine as an immunosuppressant medication in 1980 [68]. Indeed, the heart tend to be a tolerance resistance organ, meaning that it has a high rejection rate, and a recipient heart transplant should be under immunosuppressant drugs in order to achieve a long term survival [74,75]. However, regardless of this improvement in management, Khachatoorian et al ,2021 mentioned that "The 5-year post transplant survival rate is about 72.5%, with the median long term survival of 13 years among those surviving the first year post-transplantation" [67] (Figures 14,15,16,17).

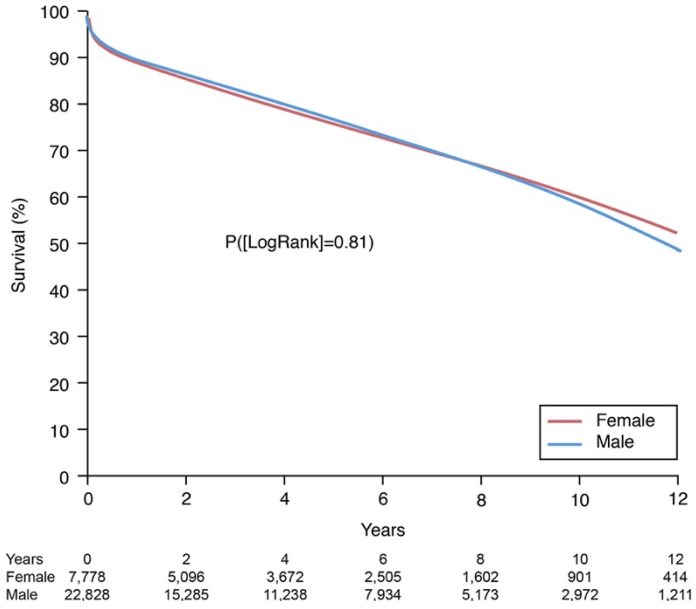

Figure 14.Survival of heart transplant recipient stratified by sex in USA[76]

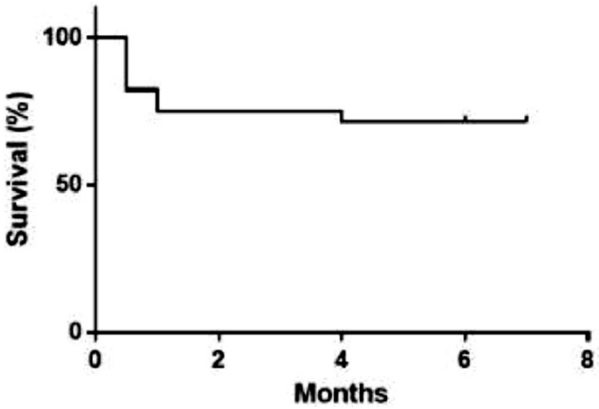

Figure 15. Six-month Survival rate of heart transplant recipinict infectd by COVID-19 in USA [77].

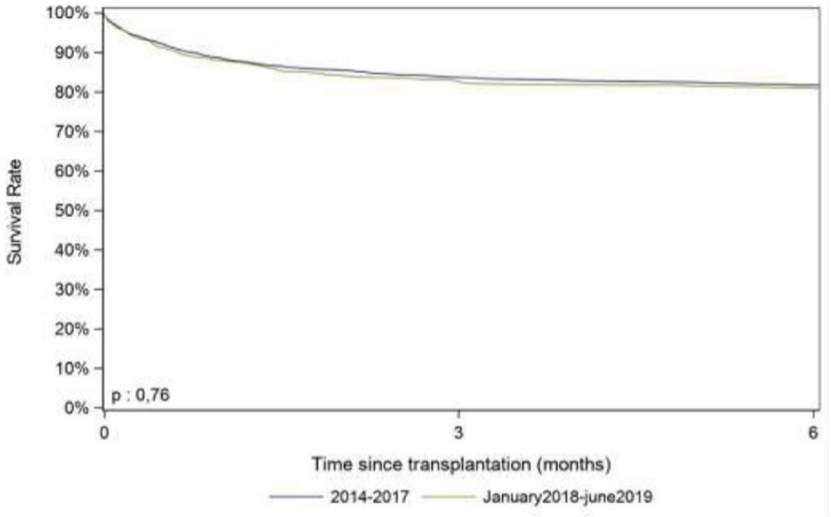

Figure 16. Six-month survival rate after heart transplant in France[78]

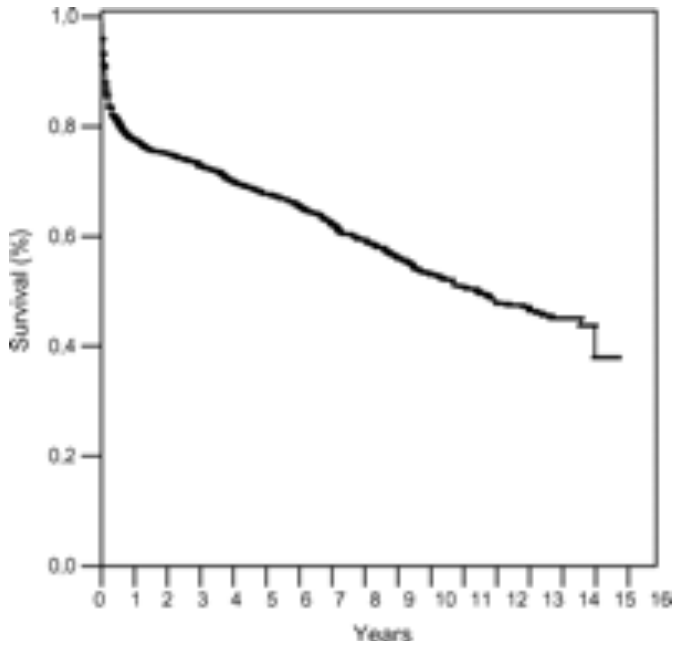

Figure 17. Long term survival of adult heart transplantation in Europe[79]

More recent data on the life expectancy after heart transplantation (2021, figures not shown)
-One year survival rate in Europe 80%
-One year survival rate in USA 90 % [80].

2. Patients' Selection

In fact, not all heart transplant patients can undergo the operation; there are some criteria that must be met in order to receive heart transplantation [81].

2.1 Indications and Contraindications:

At present, during Covid-19 epidemic era, the indications for adult heart transplantation has been updated according to the Registry of the International Society for Heart and Lung transplantation [82]. ISHLT implement an imperative strategy to allow continuing transplant activities for life-saving demand. However, in general, the most common indications and relative contraindications for recipient selection are listed below (Table 2).

Table 2. Lists of the Standard Criteria for Cardiac Transplantation

Indications [70,83]	**Contraindications** [3,15]
*Advanced Cardiogenic shock requiring highest dose inotropic treatment *Advanced Cardiogenic shock requiring percutaneous mechanical circulatory support * Maximal oxygen uptake (VO2 max) < 10 ml /kg/min. *Refractory pulmonary edema insusceptible to diuretics and demanding positive pulmonary pressure and ventilation.	*Cirrhosis *Irreversible pulmonary parenchymal disease *Recent pulmonary embolism *Advanced pulmonary artery hypertension (PASP>60mmHg) *Severe irreversible multisystem disease *Severe kidney disease (GFR<30ml/min/1.73m²)

*End-stage ventricular arrhythmia or angina despite maximum medical and surgical treatment. * End stage heart patients on guideline-directed medical therapy (GDTM) such as beta-blockers (BB) and angiotensin-receptor blockers (ARB) with poor quality of life and who still have limiting symptoms on strain. *Deteriorating renal function ascribed to the cardio renal syndrome. *Hypotension or renal failure avoiding the use of GDTM. * Gradual deterioration of right ventricular function or increasing pulmonary artery pressure resulting from left heart failure. *Other not corrected symptoms such as anaemia, low sodium level in the blood, weight loss, liver dysfunction ascribed to heart failure.	*Acute or chronic infection (should be treated before transplantation) *Recent cerebrovascular accident (CVA) *Symptomatic peripheral vascular disease. *Diabetes with organ impairment. *Active malignancy (Oncologist decision for transplantation). *Severe obesity (BMI>35 kg/m^2) *Wight loss (cachexia) *Psychosocial aspects involving active smoker or drug use, lack of social support, mental disorders and psychiatric conditions.

3. Surgical Techniques used for Heart Transplantation:

There are two types of heart transplant procedures, the most common of which is orthotopic heart transplant. This is considered as the most utilized procedure and performed by the elimination of the recipient's heart and insertion of the donor's heart in the chest area of the recipient [70]. This type of procedures can be achieved in three different ways ; biatrial , bicaval, and total techniques [84]. Biatrial is the first developed technique of orthotopic also called the standard surgical technique [84]. This involved a biatrial anastomosis, meaning that the donor's left and right atrial cuffs are attached to the remnant left and right atria of the recipient respectively [85]. The main advantage of performing this procedure was to reduce the allograft ischemic time [86].

On the other hand, bicaval, is the more recently and widely using technique since it mends the heart anatomy, physiology and postoperative outcome [84].This technique was elucidated in detail by Toscano et al, showing all steps of the procedure step by step in a videotape [86]. Basically, it sustains the donor right atrium intact and perform left atrial anastomosis, two arterial anastomosis (aorta and pulmonary artery), two caval anastomosis (IVC and SVC) (Figure 18) [86]. The major advantages of this are minimizing the incidence of late atrial dilation, tricuspid regurgitation, onset of supraventricular arrythmias, and temporary pacemaker [86].

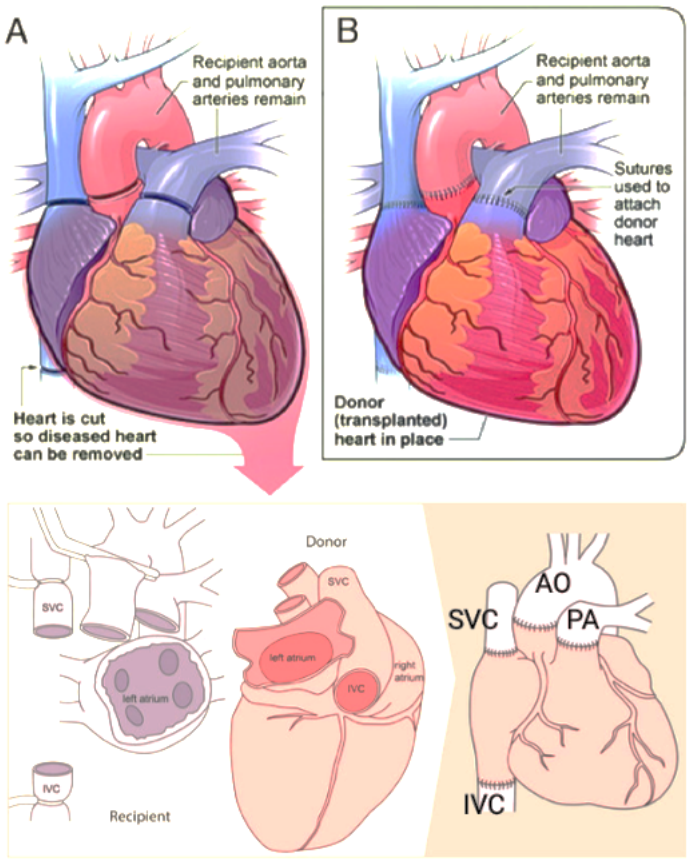

Figure 18. Illustration of Bicaval Heart Transplantation technique. Ao: Aorta, PA: Pulmonary Artery, SVA: Superior vena cava, IVC: Inferior vena cava. Adapted from[86,87]

Lastly, is the total orthotopic technique, where the complete excision of the recipient left and right atria take place [84].

The less common type of heart transplantation is the heterotopic transplant which is rarely used nowadays. It maintains the recipient's heart in its position and connect the graft to the native heart in a parallel manner. The main indication of this surgical procedure involved an advanced pulmonary hypertension and a donor-recipient size mismatch [88,89]. The fundamental advantage of heterotopic technique was to permit the recipient's heart to support the donor's heart when severe rejection and/or donor right ventricular failure are comprised [83].

4. Complications after Heart Transplantation:

Generally, complications following transplantation are divided into early and delayed complications [70]. Early complication occurs soon within hours or days after transplantation. These include:

4.1. Primary Graft Dysfunction (PGD):

Primary graft failure is described as the primary cause of early mortality [90]. It is defined as cardiac allograft impairment occurring within 24 hours of transplantation which is not ascribed to another health issues such as hyper acute rejection, pulmonary hypertension or any other surgical problem [70,90]. PGD is classified into two different types with a greatest probability of death associated with severe PGD (Figure 19) [90]:

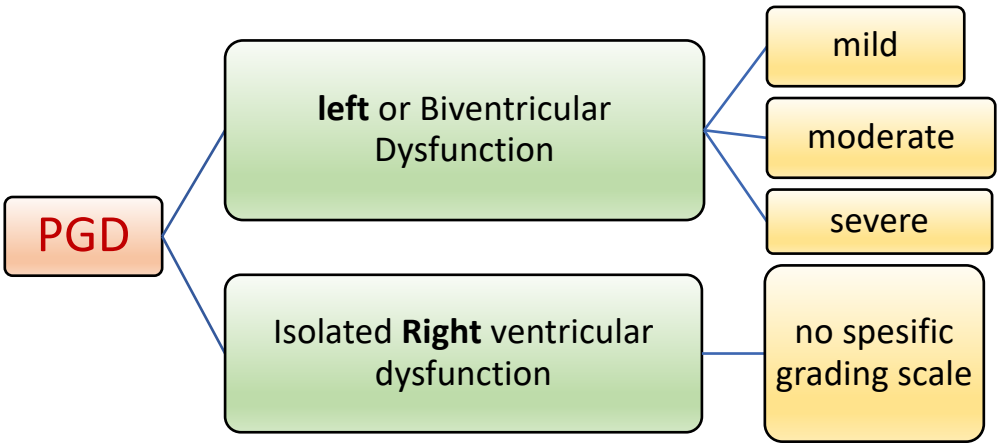

Figure 19.Classification of Primary Graft Dysfunction[90]

4.1.1. Potential Aetiology of PGD:

PGD occurs when identifiable cause of allograft malfunction is established such as hyper acute rejection, graft malfunction due to pulmonary hypertension, or recognized intraoperative complication. Regarding the echocardiography, criteria consider a left ventricular ejection fraction of 40% to be diagnostic for PGD (in the absence of secondary causes). High filling pressures, i.e. a right atrial pressure (RAP) > 15 mmHg and pulmonary capillary wedge pressure (PCWP) > 20 mmHg, suggest PGD if they occur in the setting of a low cardiac index (CI) (2.0 L/min/m2) lasting at least 1 hour [91]. There are some risk factors predisposing to PGD which are donor-related, recipient-related, and procedural-related factors. Thus, when explaining the pathophysiology of PGD, it is critical to consider the precise time points at which the heart is injured, such as brainstem death, cold ischaemia, warm ischaemia, and ischaemia-reperfusion damage[92]. Acute ischaemia-reperfusion damage with myocardial shock has been proposed as the primary cause of PGD. In addition , The donor's brain death is

linked to a set of events that result in reduced cardiac contractility which ultimately leads to PGD [93].

4.2. Rejection:

Rejection happens when there is interface between the patient's immune system and the donor's organ (allograft), which stimulate the immune response against the foreign antigen in the body [70,94]. Rejection can appears immediately intraoperatively and/or many years after transplantation [94]. Thus the timing of occurrence helps in determining prognosis and diagnosis and aid on classification [94,95].

According to the immune response, cardiac rejection can be divided into hyperacute, acute, and chronic rejection [95]. Hyperacute happens directly within minutes or hours after transplantation but it is in very rare incidence [95]. On the other hand, acute and chronic rejections are frequent following heart transplantation [95].
Acute rejection is classified into: a) acute cellular rejection (ACR) , and b) antibody-mediated rejection (AMR), that is humoral rejection [67,70,94]. These two types usually occur during the first postoperative months While chronic rejection is recognized as cardiac allograft vasculopathy (CAV) [95]. Acute cellular rejection (ACR), which is usually asymptomatic, remains a risk for patients following heart transplantation (Htx), particularly in the first year . When rejection is mild or severe, immunosuppressive therapy is frequently intensified, and this is referred to as treatment needing ACR (TRACR) [96].

And according to severity, it is classified into three types: a) mild rejection with no allograft impairment b) moderate rejection, and c) sever rejection accompanying with hemodynamic changes [70].

It has been noted that acute cellular rejection as an early complication can lead to chronic allograft vasculopathy as a delayed complication usually in a couple of years after operation [70]. Acute rejection can be detected and diagnosed by tissue endomyocardial biopsy (EMBs) which is considered a gold standard [97,98]. However, there are some limitation on EMBs as these studies are not affordable to anyone, considered as a highly invasive test, and further they are capable of sampling error [67]. Also it has been noted some risks with this kind of

procedure; including arrythmias, tricuspid regurgitation, cardiac tamponade, ventricular septal perforation, and right ventricular free wall peroration [99].

In addition, ACR manifests as a mononuclear inflammatory response invading cardiac tissue, with lymphocytic cells predominating [94].

In the domain of imaging modality, tissue doppler ultrasonography with the analysis of the posterior wall can also identify rejection as a non-invasive method [100].

4.2.1. Rejection score:

The classification of the presence of acute cellular rejection was graded according to the international Society of Heart and Lung Transplantation (ISHLT) classification proposed in 1990 (Table 3) [94,101].

Table 3. Old Grading Classification of ISHLT

Rejection grade ISHLT	Classification
1A	Focal, mild acute rejection
1B	Diffuse, mild acute rejection
2	Focal, moderate acute rejection
3A	Multifocal , moderate rejection
3B	diffuse, borderline severe acute rejection
4	severe acute rejection

Then, in 2005, another updated classification was proposed as follows:
Acute Allograft Rejection grades:

Table 4.ISHLT Acute Cellular Rejection (ACR) Grading [94]:

ISHLT Grades	Classification
0	No rejection
1R	Mild , Interstitial and/or perivascular infiltrate with up to one focus of myocyte damage. (grade 1A, 1B and 2 in 1990 system)
2R	Moderate , Two or more foci of infiltrates with associated myocyte damage. (grade 3A in 1990 system).
3R	Severe , Diffuse infiltrate with multifocal myocyte damage, with or without edema, haemorrhage, or vasculitis. (grade 3B and 4 in 1990 system).

Table 5. Acute Humoral /Antibody Rejection Grading (AMR)[94]:

Grades	classification
0	Negative histologic and immunopathologic findings
1	Presence of positive histologic and immunopathologic findings
2	Presence of positive histologic and immunopathologic findings
3	Presence of severe histologic plus immunopathologic findings

Table 6. ISHLT of Cardiac Allograft Vasculopathy (CAV) for the assessment of Coronary angiography[102]

ISHLT -CAV Grades	Classification
ISHLT-CAV_0	implies that there is no visible angiographic lesion
ISHLT-CAV_1	Mild, denotes angiographic left main <50%, or primary vessel with a maximum lesion of <70%, or any branch stenosis <70% (including diffuse constriction) without allograft malfunction
ISHLT-CAV_2	Moderate, shows angiographic left main stenosis <50%, a single primary artery ≥ 70%, or isolated branch stenosis ≥ 70% in branches of 2 systems, without allograft malfunction
ISHLT-CAV_3	Severe, implies angiographic left main stenosis ≥ 50%, or two or more primary arteries with ≥ 70% stenosis, or isolated branch stenosis ≥ 70% in all 3 systems, or ISHLT-CAV_1 or ISHLT-CAV_2 with allograft dysfunction

4.2.2. Biological Markers (Biomarkers) for Heart Transplant Rejection:

Biomarkers are defined as a biological molecule found in the blood stream ,body fluids, or tissues which works as hallmarks or indicators of normal or pathological medical conditions that can be measured objectively [103,104].

From the biological and physiological aspect, the evolution of science and technology creates a giant advancement in research. The focus is to discover non-invasive biomarkers targeting heart-transplanted patients for investigating allograft rejection. Liquid biopsy is the current trend clinical application that is applicable in this field [98].

In genomics studies, donor-derived cell-free DNA (dd-cfDNA) levels has been used to predict the rejection even before it appears in EMBs [67]. Moreover, in transcriptomics studies, PBMCs is considered as a new intact source of diagnostic and prognostic biomarkers in several diseases [103]. Numerous studies used PBMCs as a biomarker to detect the alteration of mRNAs/microRNAs in different disorders [103]. Regarding heart disease, for

instance, gene expression profiling (GEP) (Allomap™ test) of circulating PBMCs have been used to diagnose transplant rejection [67]. This is a non-invasive blood test in transplantation medicine focused on quantifying mRNA in PBMCs using real time polymerase chain reaction (rt-PCR) and categorizing different genes that are able to rule out acute rejection in heart transplant [67].

mRNA from PBMCS could allow the detection of acute cellular rejection at an earlier stage using Allomap [99,105]. This kind of test along with echocardiography has been incorporated for detecting moderate /severe ACR in stable heart transplanted patient [67]. Other applications of PBMCS as biomarkers used for a diagnostic and prognostic purpose are presented in (Figure 20) [103].

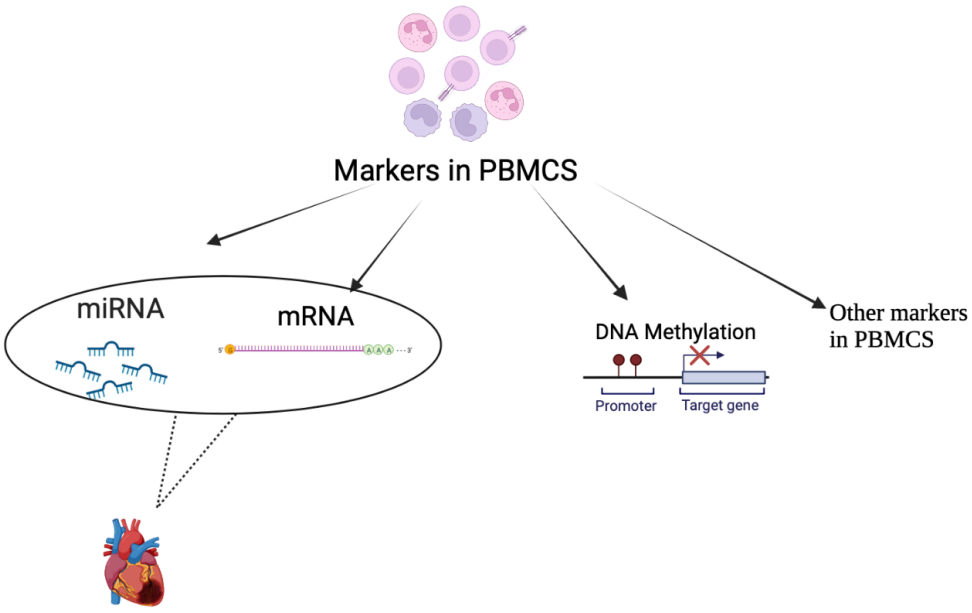

Figure 20. PBMCs as a Source of Biomarker

In addition, when the rejection of allograft takes place, microRNAs are released into blood circulation and considered as biomarker for monitoring graft rejection [67]. Also, it has been illustrated that after heart transplantation, there are some dynamic changes in the level of cardiomyocyte -enriched microRNA, which increase can predict early graft damage [67].

Besides, in the study of metabolomic biomarkers, some were found to be raised during heart transplant rejection; these include Neopterin and B2, and thromboxane A2 metabolomic [67]. Lately, one of the spectrometry analysis identified four metabolite biomarkers that have increased sensitivity and specificity levels for early acute rejection in rats model [67]. These involves D-tagatose, choline, C16 sphinganine, and D-glutamine [67]. Also, there is another metabolite biomarker that has been found to be diminished during heart transplant rejection; this biomarker is the sarcoplasmic reticulum Ca2+ -ATPase [67].

In the clinical setting, counting PBMCs is used frequently as a significant indicator for diagnosing disease [106]. It has been noted that the number of circulating monocytes could be changed with excessive stress and persistent exercise which may indicate severe infection in most cases [106]. Recently, growing studies illustrated that PBMCs count is associated with cardiovascular disease, cerebrovascular accident, and cancer prognosis. For example, it has been found that low PMBCs count is inversely correlated with aortic valve stenosis, meaning that with increased severity of stenosis, PBMCS count is degraded [106].

Specifically, studies shows the existence of inverse relationship between inflammatory marker Lymphocyte to monocyte ratio (LMR) and severity of calcific aortic stenosis [107]. In a recent study on Chinese population by Chen et al ,2022 confirmed that Lymphocyte to monocyte ratio (LMR) was significantly reduced in patients with severe post stenotic-aortic dilatation [108] .

However, the Neutrophil/Lymphocyte Ratio (NLR) has been proposed also as a systemic marker of inflammation. There is a statistically significant correlation between increased NLR and severity of calcific aortic stenosis in patients with normal systolic function[109]. Similarly, Platelet-to-lymphocyte ratio (PLR) is consistent with the finding of NLR, is that increased PLR correlates with the severity of calcific AS[110].

Along with these biomarkers, there are some biochemical and inflammatory markers that contribute in the evaluation of allograft rejection for the diagnostic and prognostic purpose [67]. For example, B-type natriuretic peptide, cardiac troponin and circulating inflammatory biomarkers (C-reactive protein) plasma levels can be used for the diagnosis of rejection [97,111]. Studying these biomarkers for prognosis and diagnosis is also interesting [67]. For instance, some studies stated that, in heart transplanted patient, increased BNP level is strongly correlated with rejection and increased risk of complication [67]. Also, higher level of troponin and CRP is associated with allograft dysfunction [67]. On the other hand, several researchers found that allograft rejection makes no changes in BNP, troponin and CRP after one year transplantation [67]. Thus, these markers are not specific.

There is a comparative study performed to analyze the values of six biomarkers for the detection of rejection [97]. Theses biomarkers include C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-a), sialic acid, fibrinogen protein (Fgp) and function (Fgf) [97]. Only CRP showed a significant difference and could be considered as the most useful parameters for diagnosing rejection after heart transplant [97].

Additionally, Zhuo et al. addressed in detail and performed an overview of all the markers of immune function in heart transplant and the methods of assessing cardiac allograft rejection (Figure 21) [99].

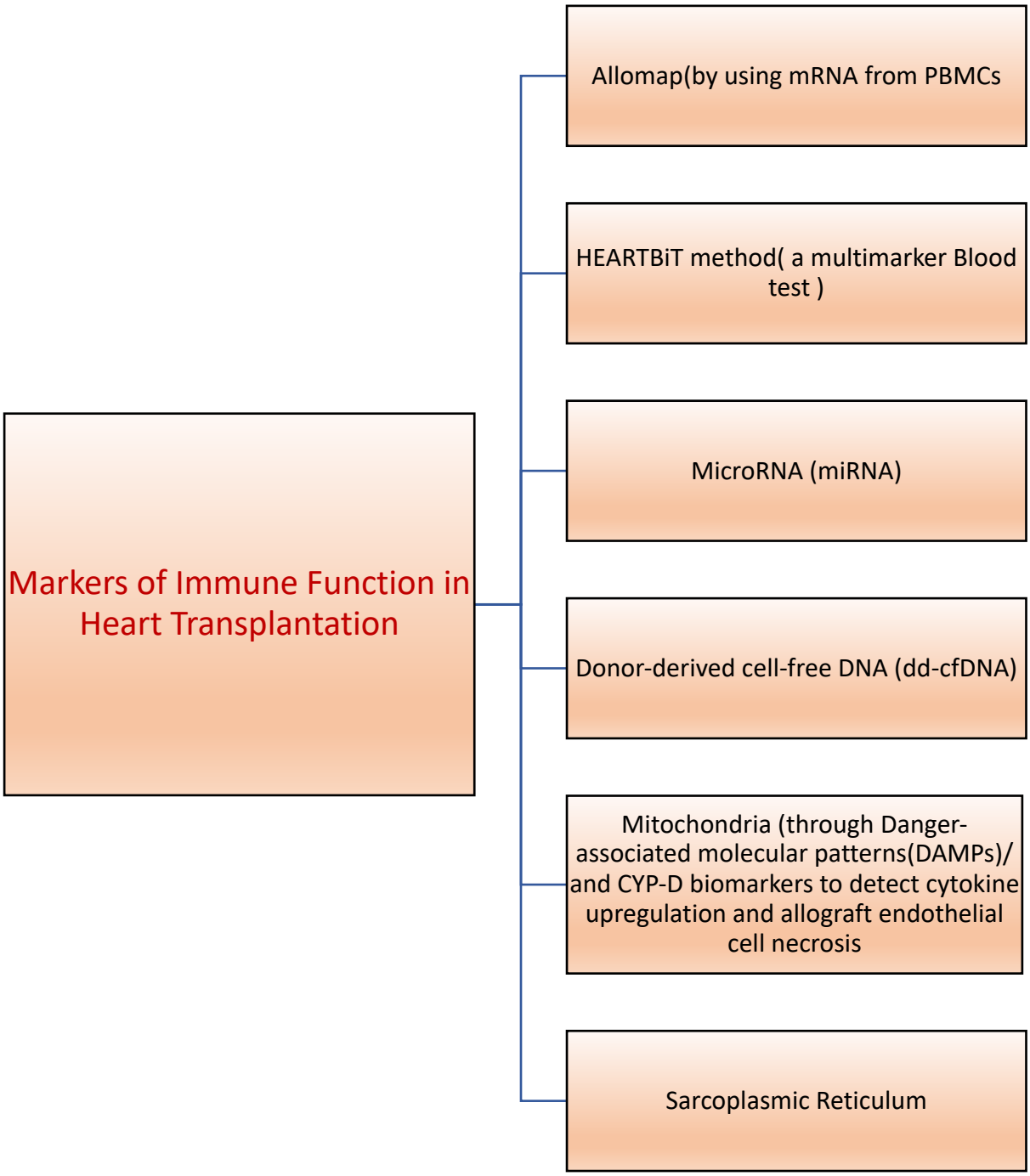

Figure 21.Overview of the non-invasive methods for the surveillance of acute cardiac allograft rejection[99]

4.3. Infection

Patients who are under immunosuppression drugs are more vulnerable to infections [70], which increase the risk of morbidity and mortality during the first year post-transplantation[70,112]. Growing studies have reported the incidence of viral infections with percentage of 9-45% with the most commonly one being the cytomegalovirus (CMV) and bacterial infection with a percentage of 20-30% [112]

Whereas late complications takes place after months or years of transplant [70], these includes:

4.4. Cardiac Allograft Vasculopathy (CAV)

CAV is a distinctive form of coronary atherosclerosis disease affecting heart transplant patients characterized by intimal thickening of coronary vessels [113]. It is a leading cause of graft dysfunction and death following heart transplantation [114]. The prevalence of CAV is about 30% within the first 5 years and 50% at 10 years after heart transplantation [95]. CAV is triggered by both immune and non-immune factors, but the exact pathogenic mechanism is not clarified.

The possibility of PBMCs GEP has been evaluated also to identify patient at risk of CAV [67,114]. For the evaluation of severe CAV, Nuclear osteopontin protein has been found as a novel proinflammatory biomarker in several cardiac disease [67].

4.5. Malignancy

After 1-5 years post transplantation, the possibility of new episode of solid malignancy is about 10% of transplanted patient with a rate of occurrence of 39.1 % at 10 years [70,115]. The use of higher doses of immunosuppressive drugs appears as a primary cause of malignancies after cardiac transplantation [115].

4.6. Immunosuppression Related side effect

a. Human papillomavirus (HPV) related squamous cell cancer
b. Ebstein-Barr virus-related post-transplantation lymphoproliferation (PTLD)
c. renal disease
d. diabetes
e. hyperlibidemia
f. metabolic derangement [70].

5. Usual Follow-up after Cardiac Transplantation

The most common diagnostic tools used to evaluate the severity of heart failure and distinguish patients are eligible for heart transplant are; a) the Heart Failure Survival Score and b) Seattle Heart Failure Model [83]. However, after heart transplantation early diagnosis is critical to look for functioning graft and avoid any complication that leads to graft failure (dysfunction), Thus, optimal and regular follow up is needed for longer survival and notable improvement in the quality of life [116]. These evaluations include:

5.1. Endomyocardial biopsy (EMB):

The effective gold standard of monitoring rejection during the first year is believed to be the invasive endomyocardial biopsy. Therefore, at periodic intervals post procedure, patients perform a follow up biopsy of the right ventricular myocardium via intravenous catheterization. The biopsy findings may indicate early rejection and necessitate extra medication; in certain situations, the biopsy may discover other cardiac disease such as infection[117]. However, the optimal duration of routine follow-up by endomyocardial biopsy (EMB) have been questioned in the present time of heart transplantation (HT), where the development in immunosuppression and donor selection criteria have led to decrease the incidence of acute allograft rejection. Surprisingly, a very recent study shows that EMB was determined to be a low-yield screening method for rejection after 6 months post HTx[118].

5.2.General medical/clinical management:

All heart transplant recipients must have a routine visit and follow up on a regular basis for basic health maintenance. Those patients require general medical monitoring including age-appropriate cancer screening for malignancies of the cervix, breast, colon, and prostate. Additionally, long-term problems, such as renal failure, hypertension, dyslipidemia, diabetes, osteoporosis, and gout. However, transplant recipients must be instructed to notify the transplant center of any new drug prescribed, since there may be unanticipated interactions that must be monitored [119] .

5.3. Biological surveillance:

As discussed earlier, there are significant variety of circulating biomarkers that have been studied for noninvasive rejection diagnosis. The most relevant biomarkers in this discipline include B-type natriuretic peptide, troponin, and inflammatory markers[111]. Also, nowadays, evaluating the gene expression profile of peripheral blood is used as a genomic marker of acute rejection [98] .

5.4. Echocardiography:

According to Badano et al., to evaluate cardiac chamber morphology and function during follow-up studies, a comprehensive echocardiographic study at 6 months after cardiac transplantation is recommended as a baseline, with careful quantitation of cardiac chamber size, RV systolic function, both systolic and diastolic parameters of LV function, and pulmonary artery pressure. Subsequent echocardiographic investigations should be examined in the context of the findings of the 6-month study. An echocardiographic study, that reveal no change from the baseline study, has a high negative predictive value for graft rejection [120].

Following heart transplantation, acute rejection (AR) and cardiac allograft vasculopathy (CAV) must be diagnosed in a timely and regular manner. Because CAV can start and advance without symptoms, and because subclinical AR might favor CAV formation, there are some standard approaches such as regular endomyocardial biopsies (EMBs) and

coronary angiographies (CA) conducted at predetermined time intervals. However, these invasive screening procedures are time-consuming and costly, as well as inconvenient and dangerous for patients.
And sometimes they cannot detect all subclinical AR or coronary stenoses before a clinical episode [121]. Thus, the establishing of another non-invasive strategy for early diagnosing and monitoring is required.

Transthoracic echocardiography (TTE) is an essential imaging modality that has versatile use for the evaluation of heart transplant patients. This non-invasive free of risks, easily performed- as often as necessary allows for the investigation of cardiac structures and overall functions either postoperatively and during the follow up [122,123]. Also, it aids in predicting outcomes and monitoring complications like identifying rejection and diagnosing graft dysfunction [123]. It appeared as one of the most favourable tools to achieve those purposes, particularly after the additional introduction of Doppler tissue-imaging (DTI) and strain imaging for ventricular wall motion and myocardial deformation analysis [121].

More interestingly, with chronic rejection, it is been noted that echocardiography has a more crucial and significant role in recognizing CAV [123]. While with acute rejection, echocardiography is more valuable with confirmation by biopsy [123].

5.4.1. Echocardiographic of cardiac allograft

5.4.1.a. Assessment of Rejection-free Transplanted heart with normal coronary artery using echocardiography

- In a normal situation, the dimensions of the left ventricle (LV) cavity are typically normal, but the right ventricular (RV) cavity size and wall thickness of both ventricles are frequently enlarged when compared to those of normal native hearts [121]. The increased incidence of RV size and shape changes is mostly due to greater pulmonary vascular resistance (PVR) in heart transplant recipients (especially early after HTx)[121].

- Abnormal atrial geometry and function is present when using biatrial surgical techniques which creates large atrial cavity and echo-dense ridge at mid-atrial level (anastomosis between the residual recipient atrial tissue and the donor atria), best visible in the apical 4 chamber view (Figure 22). These morphological changes can produce abnormal LV filling patterns, tricuspid and mitral valve- ring distortion during ventricular systole, and also enable atrial thrombus development.

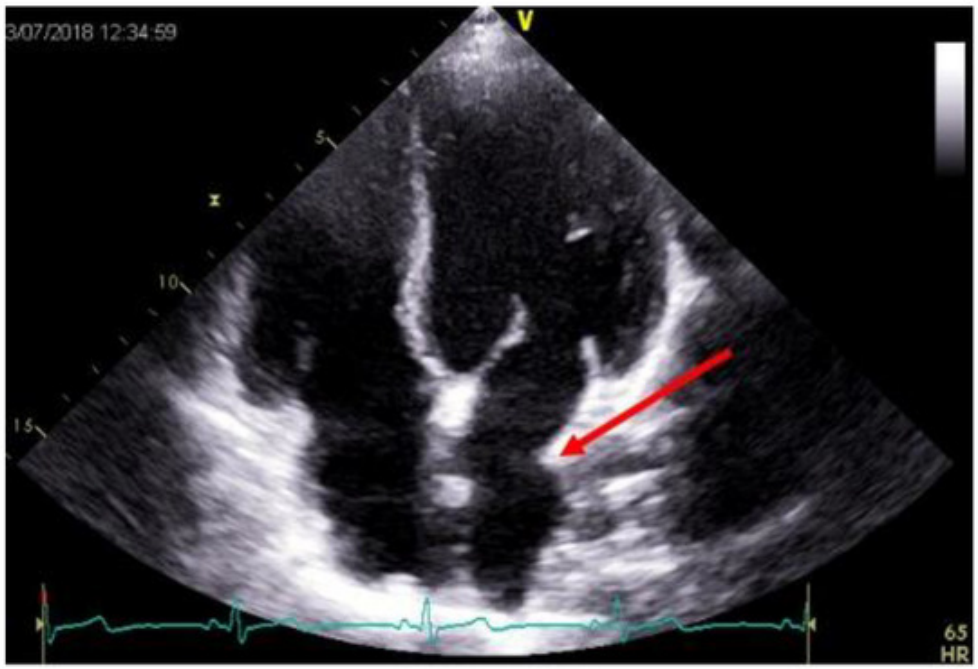

Figure 22.Echocardiography with Apical 4-champer view after heart transplantation (Red arrow indicated the anastomosis between recipient and donor atria[123]

- Tricuspid regurgitation and pericardial effusion are also another feature of transplanted heart.
- Abnormal septal motion detected by time–motion (M-mode) recordings, which can be misleading for recognition of ischemia-induced wall motion alteration.
- LV segmental wall motion abnormalities (WMAs) revealed by two- dimensional (2D) echocardiography can be often detected in both early and long term cardiac transplanted patients without rejection or CAV.
- Within the first year post transplantation, both LV relaxation dysfunction and restrictive physiology are visible; in advanced years , restrictive physiology becomes increasingly more obvious.

5.4.1.b. Assessment of primary graft dysfunction using Echocardiography

Primary graft dysfunction (PGD) is the leading cause of death in HTRs in the first 30 days. PGD usually occurs within 24 hours of transplantation and manifests as cardiogenic shock, such as a systolic blood pressure of less than 90 mmHg for more than an hour and/or a cardiac index of less than 2 L/min/m2 despite adequate right ventricular filling pressures[123]. To make the diagnosis of PGD, all other possibilities must be ruled out, including sepsis and cardiac failure.

In this case, tamponade, bleeding, immunological processes (hyperacute rejection), or chronic severe pulmonary hypertension that is unresponsive to pharmacological treatment. Are possible.

In the clinical setting, echocardiography plays an important role in documenting the presence of systolic dysfunction (with a left ventricular ejection fraction of 45%), loss of contractile reserve, and other cardiac abnormalities [123]**.**

5.4.1.c. Assessment of Acute graft Rejection using Echocardiography

Previous studies have found increases in LV wall thickness with ACR potentially due to myocardial inflammation and edema, although other authors have demonstrated limited accuracy of this measurement [96]**.**

The isovolumic relaxation time (IVRT) and the mitral pressure half time are noninvasive measures of left ventricular diastolic function[124]. Both are used in the follow-up of Htx which are their own controls. A decrease by at least 20 % of their values suggest occurrence of rejection. In 1995, a study by Díez et al. indicate that acute heart rejection decreases the isovolumetric relaxation time in both the left and right ventricles. However, the isovolumetric relaxation time of the right ventricle appears to be a more useful parameter than the isovolumetric relaxation time of the left ventricle, as it allows for the determination of whether an acute heart rejection is treatable or not[125].

Tissue Doppler Imaging (TDI) measures the velocity of myocardial motion using Doppler principles [126]. The utility of tissue doppler in diagnosing ACR has been reported in many studies [96]. More than two decades ago, tissue Doppler echocardiography revealed that wall motion velocities recorded at the left ventricular (LV) posterior wall or the left lateral mitral annulus were significantly linked with the existence of ACR [96]. Ortiz M et al. (2020), confirmed after a broad review of all current evidence and with the recommendation suggested by European and Brazilian society the usefulness of echocardiography after cardiac transplantation. It suggested that TDI seems to have a good accuracy to exclude ACR with good negative predictive value [96].

5.5. Coronarography (or coronary angiography)

A most serious complications of heart transplantation is the development of an aggressive type of vasculopathy in the graft (cardiac allograft vasculopathy, CAV), which results in occlusive coronary artery disease, characterized by intimal hyperplasia coronarography [127]. Coronarography is the standard method for the detection and evaluation of allograft arteriopathy [128].

Recently, karaçaglar et al. 2020, shed the light in a scoring system named Ginsini score which is widely and simply used to determine the severity of coronary artery disease by angiography. It to determine severity score for each coronary stenosis according to degree of luminal narrowing and location. This study concluded that heart transplant patients can be evaluated for CAV using Ginsini score as it provides a valid assessment in clinical practice[129].

6. Immunosuppressive Therapy after Heart Transplant

Management post-transplantation is important, this will aim in maximizing the heart function and minimizing the risk of allograft rejection. For that reason, patients undergoing transplantation must have successful immunosuppressive therapy. However, there are still some patients who experienced some kind of adverse effects to drugs [130].

6.1. Immunosuppressive Therapies

Immunosuppressive drugs and therapies in HTx can be categorized into induction (temporary) and maintenance (lifelong) treatment [95].

i) Induction therapies include:

- Rabbit antithymocyte globulin (rATG) – Thymoglobulin® (Genzyme) or ATG-Fresenius ® (Fresenius)
- Horse antithymocyte globulin (hATG)- ATGAM® (Pfizer)
- IL-2(Interleukin-2) receptor antagonists – basiliximab (Simulect ®, Novartis) or daclizumab (Zenapax ®, Roche).
- Anti-CD3 antibodies – Muromonab-CD3 (Orthoclone OKT3 ®, Janssen-Cilag)
- Anti-CD52 antibodies -Alemtuzumab (Campath ® , Genzyme and Lemtrada ® , Sanofi) [95].

ii) Maintenance immunosuppression after heart transplantation include:

- CSs
- Calcinurin inhibitors-CNI (CSA or TAC)
- Antimetabolite (azathioprine or mycophenolate mofetil (MMF or Cellcept))
- M-TOR inhibitors
- Everolimus
- Sirolimus [95]

Importantly, underdose immunosuppression therapies could result in rejection, and overdose therapies may result in other medical complications such as chronic kidney disease, infections and malignancies [95].

6.2 PBMCs and Immunosuppressive Therapy

Evaluation of immune system response and /or damage is an essential component in the investigation of toxicity of the drugs [131]. Since the PBMCs are a crucial component in immune system and are the major cells in the human body to fight infection, evaluation of these blood cells might give responses in term of immunity (Figure 23) [131].

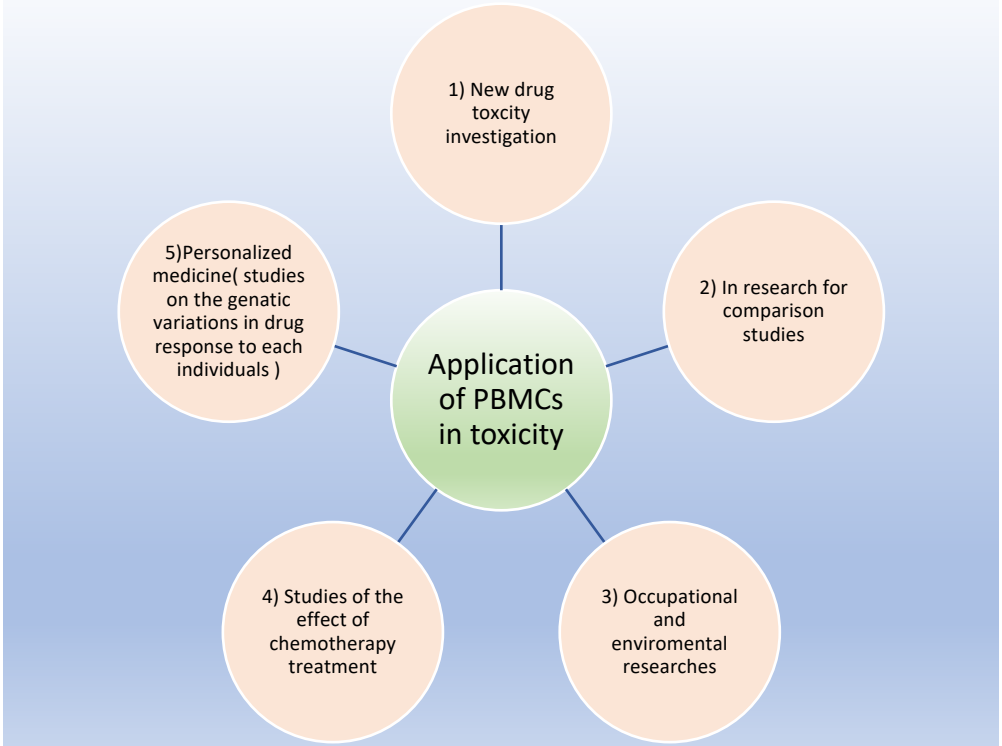

Figure 23.Application of PBMCs in Toxicity

Despite the clinical utility of immunosuppressive drugs for heart transplanted patients in the prevention of allograft rejection, these medication could have an effect on mitochondria [132]. There are some researches that have been done to examine the response of PBMCs to immunosuppressive drugs [130]. Regarding Calcinurin inhibitors, and monitoring tacrolimus specifically, it is been noted that determining its concentrations inside PBMCs could be a favorable way[133]. Quantification of TAC in PBMCs this is accomplished by using a liquid chromatography tandem mass spectrometry method [133].

In heart transplant patients, data regarding PBMCs concentrations of TAC are lacking but there are some data reported for renal and liver transplant patients [133].

For example, Mijiti et al. performed such study on cirrhosis patients undergoing liver transplantation and watch the effectiveness of some immunosuppressive treatment on those patients [130]. Prednisolone, methylprednisolone, cyclosporine, and tacrolimus were evaluated

and in vitro blastogenesis of PBMCS and the drug concentration IC50s value were calculated [130]. The study found some variations in the results; one patient revealing high PBMCs sensitivity to tacrolimus and exhibiting no allograft rejection until 5 weeks post transplantation [130]. In contrast, another patient demonstrating relatively decreased PBMCs sensitivity to tacrolimus showing rejection 1 week after transplantation [130]. This research conclude that the PBMCs from liver transplanted patients are susceptive to immunosuppressive treatment of prednisolone and calcineurin inhibitors[130].

Another example of calcineurin inhibitors drug investigation performed on lymphocyte was reported by Flack et al. on kidney transplant patients [133,134]. This study examined the effect of cyclosporine in intra-lymphocyte and whole blood [133]. It found that the concentration of cyclosporine decreases in intra-lymphocyte a few days before acute rejection occurrence, whereas continue in therapeutic ranges on the whole blood[133]. Thus, it concludes that the concentration of cyclosporine in intra-lymphocyte could be valuable for predicting acute rejection [133].

Lemaitre et al. examined for the first time the concentration of TAC in PBMCs on heart transplant patients [133].

A very recent study assessed the effect of the most common maintenance immunosuppressive treatment such as Calcinurin inhibitors, MMF, rapamycine on mitochondrial function of PBMCs specially on T-cell [132]. The study showed that only MMF showed decrease mitochondrial respiration and increased ROS production and produced significant level of apoptotic cells death that were correlated with increase in cytochrome c that were demonstrated by Western blot [132].

Chapter III

Results

1. Result I : Review of PBMCs and Cardiovascular Disease

Le Résumé

Les maladies cardiovasculaires (MCV) sont des troubles dévastateurs et la principale cause de mortalité dans le monde. La physiopathologie des maladies cardiovasculaires est complexe et multifactorielle et, ces dernières années, le dysfonctionnement mitochondrial et la production excessive d'espèces réactives de l'oxygène (ERO) ont suscité une attention croissante. En effet, les maladies cardiovasculaires peuvent être considérées comme une altération systémique, et il semble utile de comprendre l'implication éventuelle des cellules sanguines circulantes que sont les cellules mononucléaires du sang périphérique (PBMC) et/ou les plaquettes, et en particulier leur fonction mitochondriale, la production de ERO et la libération d'ADN mitochondrial (ADNmt) chez les patients souffrant de déficiences cardiaques. Il est intéressant de noter que les rapports démontrent de façon constante une réduction de la capacité oxydative de la chaîne respiratoire mitochondriale liée au degré de sévérité des MCV et à une production accrue de ERO par les PBMC. De plus, le niveau d'ADNmt circulant était généralement modifié chez ces patients. Ces données constituent des étapes cruciales dans la compréhension des maladies cardiaques et d'autres études sont nécessaires pour évaluer l'adjonction possible de la dysfonction mitochondriale des PBMC et des plaquettes, du stress oxydatif et de l'ADNmt circulant comme biomarqueurs du diagnostic et du pronostic des MCV. Cette nouvelle approche pourrait également permettre de nouveaux développements thérapeutiques intéressants.

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Review

Peripheral Blood Mononuclear Cells and Platelets Mitochondrial Dysfunction, Oxidative Stress, and Circulating mtDNA in Cardiovascular Diseases

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Abstract: Cardiovascular diseases (CVDs) are devastating disorders and the leading cause of mortality worldwide. The pathophysiology of cardiovascular diseases is complex and multifactorial and, in the past years, mitochondrial dysfunction and excessive production of reactive oxygen species (ROS) have gained growing attention. Indeed, CVDs can be considered as a systemic alteration, and understanding the eventual implication of circulating blood cells peripheral blood mononuclear cells (PBMCs) and or platelets, and particularly their mitochondrial function, ROS production, and mitochondrial DNA (mtDNA) releases in patients with cardiac impairments, appears worthwhile. Interestingly, reports consistently demonstrate a reduced mitochondrial respiratory chain oxidative capacity related to the degree of CVD severity and to an increased ROS production by PBMCs. Further, circulating mtDNA level was generally modified in such patients. These data are critical steps in term of cardiac disease comprehension and further studies are warranted to challenge the possible adjunct of PBMCs' and platelets' mitochondrial dysfunction, oxidative stress, and circulating mtDNA as biomarkers of CVD diagnosis and prognosis. This new approach might also allow further interesting therapeutic developments.

Keywords: cardiovascular diseases; mitochondrial dysfunction; circulating cells; PBMCS; platelets; oxidative stress; reactive oxygen species (ROS); mitochondrial DNA (mtDNA); biomarkers; herat failure

1. Introduction

Cardiovascular diseases (CVDs) rank as one of the first diseases leading to death worldwide [1,2]. The 2019 report of the American Heart Association shows that between 2013 and 2016, CVDs, including hypertension, heart failure (HF), coronary heart disease, and stroke, were present in

about 48% of patients older than 20 years in the United States [3–5]. Significant progress has been made concerning CVD diagnosis and therapies, particularly considering neuro-hormonal modulation, such as natriuretic peptide (NP)-guided therapy [2,6–9], but it seems that a plateau has begun to be reached, suggesting new approaches. In this view, since CVDs are generally systemic diseases, an attempt based on circulating cells might be proposed to better understand CVD pathophysiology and to discover new biomarkers. Indeed, growing evidence suggests that the assessment of mitochondrial respiratory function of circulating peripheral blood mononuclear cells (PBMCs) and platelets might be viewed as a marker to detect the mitochondrial dysfunction in different tissues, including the heart [10–14].

The myocardium possesses one of the highest number of mitochondria in the body, allowing heart pumping activity through ATP production. Mitochondria are known contributors to the pathogenesis and outcome of several cardiovascular diseases. Indeed, regardless of cardiac disease etiology, most evidence demonstrates that mitochondrial dysfunction is widely observed in the pathological heart.

Mitochondrial dysfunction might be inferred from tissues' or cells' oxygen consumption (reflecting mitochondrial oxidative capacity) and the mitochondrial membrane potential (reflecting the ability of the electron transport system to maintain the gradient of proton driving ATP production). Thus, the failure of mitochondria to produce ATP results in an energy deficit, impairing cells, and finally, organ function [14–19].

Mitochondria have also been identified as significant sources of reactive oxygen species (ROS) [20]. Research reveals that oxidative stress, due to increased ROS and/or reduced antioxidant capacity, plays a considerable role in the development of HF and determines patient prognosis. Increased ROS accumulation and inflammation play a key role in the cardiac and vascular functional and structural damage underlying all major causes of CVDs [21]. However, the fundamental mechanism of ROS production in HF deserves to be further investigated [22]. Thus, it would be interesting to further monitor ROS levels and mitochondrial function in circulating cells in order to improve both diagnosis and follow-up of patients with CVDs.

Indeed, if tissue biopsies are relevant to the investigation of pathological changes and study of mitochondrial function in diseased organs, they are invasive and not always feasible. Alternatively, peripheral blood mononuclear cells (PBMCs) and/or platelets represent an easily available population of cells allowing mitochondrial function studies. Analysis of the energetic profile (mitochondrial function) of circulating blood cells in experimental animals and humans appears as a new research field with potential applications in the development of disease biomarkers in several settings, including respiratory and CVDs [10–14,19,23,24].

This review presents data exploring the PBMCs' and platelets' mitochondrial function, together with their ROS production and mitochondrial DNA release in order to assess whether such key parameters are modified and might be considered as biological markers of CVDs with diagnosis, prognosis, and even prognosis interests.

2. Is Mitochondrial Function Accessible in all Circulating Cells in the Blood?

2.1. Classification of Circulating Cells

There are many circulating cells in the blood, ranging from different population subtypes of white cells involved in immunity and inflammation, to platelets modulating blood aggregation and to red cells mainly transporting O_2. Several techniques might be used to separate blood cells in the blood, but gradient centrifugation is generally performed (Figure 1). Theoretically, the oxidative capacity of all circulating cells—through mitochondrial respiratory chain complexes activities assessment—might be explored, but there is a noticeable exception. Unlike in birds, for instance, human red blood cells do not present with mitochondria [25].

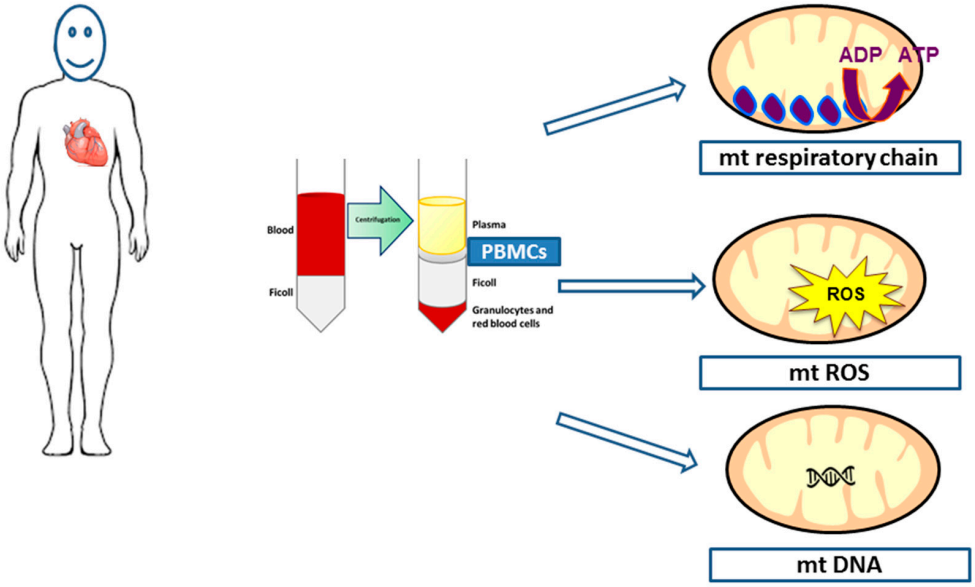

Figure 1. Density gradient centrifugation of whole blood allows peripheral blood mononuclear cells (PBMCs) isolation and then mitochondrial respiratory chain, reactive oxygen species, and DNA analysis.

The PBMC fraction consists of lymphocytes (T, B, and natural killer cells), monocytes, and dendritic cells. Circulating lymphocytes represent a mixed population of cells and ensure cellular or humoral immunity. Many types of lymphocytes can be distinguished: B cells produce antibodies, T cells support cellular immunity, and natural killer cells have their own cytolytic activity. The production of monoclonal antibodies specific for an expressed antigen can be conducted for immunophenotypic lymphocyte classification: Cluster of differentiation (CD) was created to group antibodies that recognize the same antigens. However, to date, no clear data are available on these specific cells' subtypes concerning their mitochondrial respiration. Monocytes, with a uni-lobular nucleus, have an important role in phagocytosis and the innate immune system.

Platelets are un-nucleated cells produced by cytoplasm fragmentation of megakaryocytes in the bone marrow, circulating in the peripheral blood for 7 to 10 days. They play a significant role in homeostasis and are essential for thrombus formation during the hemostatic process and are largely involved in thrombosis, myocardial infarction, stroke, and phlebitis. Platelets thus play an important role in CVDs, both in the pathogenesis of atherosclerosis and in the development of thrombotic events when presenting with qualitative and/or quantitative impairments [11,13,26,27]. Circulating platelets possess numerous mitochondria, can be obtained easily even from critically ill patients, and their isolation is performed routinely with success [28].

2.2. Isolation and Mitochondrial Respiratory Chain Activities' Determination in PBMCs and Platelets (Figure 1)

Mitochondria are the main source of cellular energy, coupling the oxidation of fatty acids and pyruvate to the production of high amounts of ATP through the mitochondrial electron transport chain (ETC) [29]. Briefly, free electrons are transferred from complex I to complexes II, III, and IV of the ETC, thereby allowing complexes I, III, and IV to extrude protons from the matrix. The return of H+ ions from the mitochondrial membrane interspace towards the matrix allows the complex V to phosphorylate ADP into ATP.

The function of each complex is investigated by using a spectrophotometer and can be performed in cellular or tissue samples. Besides the determination of oxygen consumption, ATP synthesis and the mitochondrial membrane potential can also be investigated [30]. Specifically, Hsiao and Hoppel presented an optimal comprehensive method for analyzing the ETC activity in

PBMCs [31]. There are two general techniques that have been used in vitro for the assessment of mitochondrial function and detection of delicate changes in the respiration rate of mitochondria in PBMCs and platelets isolated from peripheral blood by measuring oxygen consumption [12,13,32]. The first method is the extracellular flux analyzer. This technique provides efficient, comprehensive, and highly reproducible results and is commonly used to measure cellular bioenergetics function in intact and permeabilized cells [33]. A distinct trait of this protocol compared to others is that it does not entail mitochondrial isolation and can be operated using a minimal number of cells [33]. The second method is high-resolution respirometry (Oroboros O2K), which permits active investigation of metabolic pathways [12] and requires the availability of sufficient numbers of cells [10].

Although circulating platelets count for small numbers of functional mitochondria, they have high energy consumption levels and have been used widely to study the mitochondrial function in human disease due to their accessibility [34]. This is confirmed by a review by Kramer et al. presenting the maximal mitochondrial oxygen consumption devoted to the bioenergetic function in circulating platelets, monocytes, and lymphocytes. Interestingly, there is a distinct metabolism program between circulating platelets and leukocytes that could act as different sensors of the metabolic and inflammatory stress in many diseases [13].

3. Mitochondrial Respiratory Chain Complex Activities of PBMCs and Platelets in Patients with Cardiovascular Diseases

3.1. PBMCs Mitochondrial Respiratory Chain Activity in Cardiovascular Diseases

Interestingly, when evaluating mitochondrial respiratory chain complexes' activity in PBMCs in heart failure patients, Li et al. demonstrated that mitochondrial oxygen consumption, particularly in complex I and II, was significantly smaller as compared to the control group [29]. Such depressed PBMC mitochondrial function was observed in patients with early-stage congestive heart failure (CHF, asymptomatic patients) [29]. Possible explanations of this reduction in the electron transport chain activity in PBMCs are increased mitochondrial mitophagy and decreased biogenesis per mononuclear cell [29]. Moreover, the mitochondrial respiration was inversely related with inflammatory factors, such as high sensitivity C-reactive protein, IL6, and TNF-α. Thus, impaired mitochondrial respiratory functions of PBMCs characterize heart failure patients. Accordingly, a significant reduction of NDUFC2 expression, a subunit of mitochondrial complex I, has been detected in peripheral circulating mononuclear cells in patients with acute coronary syndrome [35]

More generally, there are several factors that might disrupt the function of the circulating leukocyte mitochondrial respiratory chain in CHF. Increased intracellular oxidants could induce mitochondrial permeability transition and inhibit respiratory coupling, which reflects mitochondrial respiratory chain disruption [36,37]. Kong et al. observed a reduction in the leukocyte, lymphocyte, and monocyte mitochondrial transmembrane potential (MTP) in congestive heart patients, in association with apoptosis and increased inflammation and ROS formation [37]. This decrease was more notable in the edematous CHF group when considering lymphocytes. Additionally, increased ROS led to mitochondrial depolarization [37]. Further, the percentage of apoptotic cells was greater in PMN than PBMCs (42.9% vs. 20%, respectively).

Song et al. found lower MTP and higher ROS levels in lymphocytes of CHF patients at low risk associated with increased serum NT-ProBNP, a diagnosis and prognosis biomarker in heart failure [36]. Furthermore, Coluccia et al., analyzing the mitochondrial membrane potential by cytofluorometric TMRM and JC-1 staining, found significant mitochondrial depolarization in PBMCs among HF patients after the administration of inflammatory stimulus lipopolysaccharide (LPS) [38]. The ultrastructural changes in mitochondria PBMCs showed a decrease in the index associated with the loss of inner mitochondrial membrane (IMM) and with an increase in the percentage of the apoptotic cells and mitophagy in HF-PBMC individuals, both at baseline and after LPS stimulation. The impairment of the inner mitochondrial membrane in PBMCs might reflect the

impairment of the electron transport chain mitochondrial uncoupling [38] (Table 1). Thus, PBMCs' mitochondrial respiration can be considered as an innovative model to investigate the pathophysiology of CVDs.

3.2. Platelets' Mitochondrial Respiratory Chain Activity in Cardiovascular Diseases

Circulating platelets contain small number of functional mitochondria (averaging four mitochondria/platelet), but they are very metabolically active with a high rate of ATP turnover [39]. Platelets have higher oxygen consumption rates compared to leucocytes, since higher levels of ATP are required for the normal functioning of ion channels that maintain the intracellular ionic balance, essential for preventing platelet activation in basal conditions [13]. In platelets, mitochondrial complex III and IV are very low, underlying the severe impact of mitochondrial damage on platelet function. Electron transport chain activity in platelets is altered in many diseases [34]. However, there are few data in CVDs.

In resting platelets, mitochondrial respiration accounts for three-quarters of the energy production, with glycolysis providing the remaining [40]. The metabolic pool of ATP and ADP is located in the cytoplasm whereas non metabolic ATP and ADP are segregated into dense (δ) granules (storage pool); they are secreted during cellular stimulation and are essential for the late phase of aggregation [41]. Another important platelet trait is the fact that mitochondrial complex III and IV proteins are few, leading to increased sensitivity toward mitochondrial dysfunction [13]. Many studies have demonstrated an interest in monitoring platelet mitochondrial respiration in diabetes, Alzheimer's, or Parkinson's disease [11]. Following platelet activation, mitochondrial respiration and glycolysis enhance extra metabolic ATP production, thus sustaining shape change, aggregation, and secretion [11,39,42]. Such increased energy consumption is a main determinant of platelet function.

In cardiogenic shock (when the trigger is hypoperfusion), there is inhibition of platelet mitochondrial respiratory chain enzymes similar to that observed in sepsis. According to some authors, salicylic acid or its derivatives may interfere with platelet mitochondrial function, mainly acting as uncoupling agents. However, this issue still deserves further studies [42–44]. Petrus et al. shed light on the association between hyperpolarization of the mitochondrial membrane, ROS formation, and platelet secretion, and, for instance, diabetic patients had a lower platelet oxygen consumption rate associated with increased ROS generation [11,12]. Furthermore, circulating platelet mitochondria are not restricted to the generation of ATP, but also have an important role in initiating platelet activation through many interlinked mitochondrial processes [11,34]. Impairment of the electron transport chain leads to increased generation of ROS, which triggers platelet activation and, potentially, to a reduced mitochondrial membrane potential and mitochondrial permeability transition pore opening (Figure 2) [11].

On the other hand, Nguyen et al. recently observed that the platelets of patients with pulmonary hypertension secondary to left heart diseases demonstrated an enhanced maximal respiratory capacity despite a normal basal oxygen consumption rate [45]. Increased fatty acid oxidation, together with the metabolic syndrome, likely contributed to this result. Further and interestingly, platelets' bioenergetics correlated with right ventricular dysfunction but not clearly with hemodynamic in these group 2 pulmonary hypertension (PH) patients, suggesting that non-hemodynamic parameters might play a significant role in such a setting.

Table 1. Mitochondrial function, oxidative stress, and apoptosis in circulating blood cells during cardiovascular disease.

Population Characteristics	Study Design/ Cells Analyzed	Mitochondrial Function	Oxidative Stress ROS Production/ Antioxidant Level	Cell Viability/Apoptosis	Results	References
HF pediatric patients with single ventricle (SV) congenital heart disease	PBMCs	-Oxygen consumption rate (Seahorse) -Mitochondrial respiration (oroboros)	ROS (Amplex red dye)	NA	-Respiratory capacity, coupling efficiency and mitochondrial oxygen flux were reduced in SV patients. -ROS was higher in SV patients	Garcia Anastacia et al., 2019, Circulation (Abstract) [46].
-Mild Congestive Heart Failure patient (CHF) (Class I-II) *n* = 15, 14 male, 1 female Age: 63 ± 13 yo EF: 44.3 ± 14.5 % -Moderate-to-severe CHF (Class III) *n* = 16, 15 male, 1 female Age: 61 ± 14 yo EF: 26.9 ± 6%	PBMCs	-Mitochondrial respiration (oroboros) -Maximal electron transfer system capacity (ETS)	Assessment of ROS generation in permeabilized PBMCs before and after addition of mitochondrial oxidative phosphorylation uncoupler (FCCP) urinary 8-OHdG, a biomarker of oxidative DNA damage	N/A	Mitochondrial respiratory capacity of class III HF was lower than class II patients. -ETS capacity was significantly reduced in class III compared to class I–II -Mitochondrial ROS level was higher in class III CHF compared to class I–II patients, before and after FCCP.	Shirakawa et al., 2019, Scientific Report. [22]
Chronic HF patients *n* = 15, 12 male, 3 female Age: 56.6 ± 10.8 yo EF: 28 ± 8% Control group *n* = 10, 8 male, 2 female Age: 49.3 ± 8 yo EF: 65 ± 2%	PBMCs Basal and modulation by LPS	Mitochondrial membrane potential (TMRM and JC-1 staining).	-For cytoplasmic oxidative stress evaluation: PBMCs were incubate with 5 μM 2′,7′-dichlorofluorescein diacetate at 37 °C for 10 min. -For mitochondrial oxidative stress evaluation: (MitoSOX™ Red mitochondrial superoxide) -For antioxidant system (SOD GPx levels)	Assessment of overall cell damage Mitochondrial area percentage of intact cristae, and loss of inner mitochondrial membrane (IMM) -Cell damage (Annexin-v and P1 staining by flow cytometric analysis) -Assessment of mitophagy flux (gene expression by	**Baseline** -Cytoplasmic ROS: no difference between HF-PBMCs and healthy subject. -Mitochondrial ROS: increased in HF-PBMCs as compared to controls -Index associated with the loss of inner mitochondrial membrane was lower in HF patients -mitophagy flux: increased autophagy genes in HF-PBMCs **After LPS** -Mitochondrial membrane potential:	Coluccia et al., 2018, Oncotarget. [38]

				RT-PCR quantitation).	depolarization in PBMCs of HF patients (p < 0.05). -Antioxidant system: reduced SOD (P < 0.05 and <0.01) and GPx (p < 0.05) activity in HF-PBMCs -Cytoplasmic ROS: HF-PBMCs shows marked increase cytoplasmic ROS than control group. (p < 0.05) -Mitochondrial ROS: increased in HF patients (p < 0.05). - Index associated with the loss of inner mitochondrial membrane was more deteriorated after stimulation, and reduction of mitochondrial area with intact cristae in HF-PBMCs than in healthy group (p < 0.01) -Cell damage: apoptotic cell percentage was increased in HF patients. (p < 0.05) -Mitophagy flux: the response in HF-PBMCs was increased much more after stimulation.	
Congestive heart patients (CHF) n = 20, 16 male, 4 female Age: 68.9 ± 8 yo EF: 24.9 ± 5.9% -Control group n = 15, 13 male, 2 female Age:63.3 ± 9.4 yo EF: 60.0 ± 5.3%	Leukocyte were isolated by gradient centrifugation to measure cellular lipid, protein, PARP & AIF Modulation: Activation of PARP	N/A	C-reactive protein, N-terminal probrain-type natriuretic peptide, oxidative nitrative stress, plasma total peroxide level (PRX), total plasma antioxidant capacity (TAC)and oxidative stress index (OSI), Leukocyte lipid peroxidation, and protein tyrosine nitration (NT)were evaluated. PRX was determined by Oxystat and TAC was detected by OxiSelect™ TAC Assay kit	poly (ADP-ribose) polymerase (PARP), and apoptosis inducing factor (AIF) was measured	In CHF patients, plasma PRX level was markedly increased suggesting the increase of oxidative stress in this group. Oxidative stress of leucocytes increased in CHF group. PARP activity and AIF in circulating mononuclear cells of CHF group was higher than in the control group. A positive correlation was demonstrated between oxidative stress (Plasma PRX level, OSI) and PARP activation in circulating leukocytes with pro-BNP levels of CHF.	Bárány et al., 2017 Oxidative Medicine and Cellular Longevity. [8]
Pulmonary	Platelets	Oxygen	ROS level analyzed using	N/A	Maximal oxygen consumption rate was	Nguyen et al.,

hypertension patients (PH group classified as WHO Group 2) *n* = 20, 10 male, 10 female Age: 69 ± 7.4 Control group *n* = 20, 10 male, 10 female Age: 69.4 ± 17.6		consumption (Seahorse) Extracellular acidification rate (Seahorse)	MitoSOX	significantly increased compared to controls Activity of complex II tended to increase in Group 2 PH platelets compared to controls (p = 0.09). Enhanced maximal capacity correlates negatively with right ventricular stroke work index No change with administration of inhaled nitrite, a modulator of pulmonary hemodynamics.	2019, Plos one. [45]
CHF *n* = 54, male Age: 60 ± 10 EF% 33.3 ± 7.7 Control group *n* = 30, male Age: 61 ± 10 EF% 65.1 ± 7.3	PMBCs (peripheral blood Lymphocyte Serum NT-ProBNP level were assessed	Mitochondrial transmembrane potential (MTP) Analyzed by flow cytometry described as JC-1 fluorescence ratio	ROS level of PBMCs were investigated. Described as DCF fluorescence intensity.	CHF patients experienced decreased MTP, (and increase level of ROS of lymphocytes (intensity 11.12) than the control group. -CHF patients had higher Serum NT-ProBNP level -Study conclude that patients with CHF, the MTP and ROS level of PBMCs are correlated with the changes in serum NT-ProBNP level	Song et al., 2016, Heart, Lung and circulation.[36]
Early stage HF patients *n* = 25, 12 male, 13 female Age: 49 ± 3 years EF: 67.40 ± 0.83 Control group *n* = 24, 11 male, 13 female Age: 47 ± 3 years EF: 69.63 ± 0.99	PBMCs sample	Mitochondrial respiration (Oroboros)	Measurement of inflammatory factors: High sensitivity C-reactive protein (hs-CRP), IL6, and TNF-α -Oxidative stress biomarker: MDA Antioxidant system: SOD By using ELISA	Decreased mitochondrial oxygen consumption in HF compared to control group. -Inflammatory factors were significantly higher in patients with early stage HF. -SOD reduced, but MDA stayed unchanged in diseased patients.	Li et al., 2015 Scientific Report. [29]
HF patients with left ventricular assist device *n* = 10, 8 male, 2 female Age, median (range): 65 (57–69) EF% (median (range): 15 (10–20)	PBMCs (Circulating blood leukocyte)	N/A	-Detection of ROS in leukocyte by flow cytometry, and immunofluorescence microscopy -Antioxidant defense system; SOD in erythrocyte was measured by	N/A	-In HF patients, the mean fluorescence intensity (MFI) of DCF-DA exhibited increased level of ROS in peripheral blood leukocyte than in control group. Post-operative value (1 week): Neutrophils ROS (+51%) Lymphocytes ROS (+37%) Monocytes ROS	Mondal et al., 2013, International Journal of Medical Sciences. [47]

Control group *n* = 10, 8 male, 2 female Age, median (range): 63 (26–74) EF %: NA			spectrophotometry. -oxidized low density (oxLDL) lipoproteins were analyzed in plasma, by ELISA. -DNA damage markers were assessed in blood lymphocyte, and measured by immunofluorescence microscopy		(+54%) -Quantity of ROS reach the highest 3 months later (value not specified) -SOD level decreased in HF patient than in control. And continue to decrease to reach the minimum at 3 months post-operative. -oxLDL were markedly higher in HF than in control group. These results suggested increased oxidative stress among HF patients which leads to mitochondria dysfunction. -Markers used to express DNA damage, reveals abnormal DNA repair.	
Congestive heart patients (CHF) *n* = 15 9 Male, 6 female Age: 79 ± 9 EF% =37 ± 17 Control group *n* = 9 6 male, 3 female Age: 49 ± 22 EF% =63 ± 5	WBC and Platelets blood sampling from radial artery, brachial vein and coronary sinus	N/A	Oxidative stress (immunofluorescence microscopy analysis of nitrotyrosine) -cytoplasmic oxidative stress (incubation of resuspended buffy coat with 5-6 CM-DCF). -7 CHF and 6 health individuals were evaluated for Mitochondrial oxidative stress, (Mitotracker red CM-H2 XRos M7513 Probe). -Both cytoplasmic and mitochondrial oxidative stress (live- cells fluorescence microscopy and FACS)	N/A	CHF exhibited increased protein nitosylation in arterial and venous WBC than control. -Cytoplasmic oxidative stress in CHF was increased in venous and arterially localized WBC and platelets. -For coronary sinus sampling, the number of ROS was higher than in venous (946 ± 475 vs. 659 ± 428 per 10,000 cells). -CHF patients had elevated mitochondrial ROS in WBC and platelets than healthy group. The number of ROS-positive venous WBC and platelets is (478 ± 261 per 10,000 cells vs. 162 ± 81 per 10,000 cells for control group). While, ROS-positive arterial WBC and platelets is 471 ± 211 per 10,000 cells vs. 85 ± 42 per 10,000 cells for healthy group. This increased number of circulating ROS suggesting increase oxidative stress in HF patients.	IJsselmuiden et al., 2008, (CardiovascularMedicine. [48]
Acute CHF Edematous *n* = 15 male 9	PBMCs (Peripheral blood leukocyte)	Mitochondrial transmembrane potential (MTP) in leukocyte was	Intracellular oxidants formation was examined by DCF for 20 min at 37°.	-Cell apoptosis was measured by tunnel assay	In CHF, MTP of PBMCs was markedly decreased, with the weakening in edematous HF patients more than in non-edematous specifically in lymphocyte.	Kong et al., 2001, Journal of the American College of Cardiology. [37]

female 7 Age: 72.6 ± 3.7 EF% 36.2 ± 5.1 Non-edematous *n* = 15 male 10 female 5 Age: 78.5 ± 2.8 EF% 35.3 ± 2.7 Control group *n* = 20 male 18 female 2 Age: 68.5 ± 1.6	10 mL venous blood sample was collected, 5 mL was anticoagulated and assayed for fluorescence staining	analyzed by flow cytometry	-Fluorescence was Detected by flow cytometry -Analyzing plasma factors nitrogen metabolites. -Lipid peroxides including (MDA, HNE) -inflammatory factors: IL6, and TNF-α using ELISA.	-Intracellular oxidants of PBMCs were increased, with the highest was in monocytes. -Edematous CHF had higher DCF fluorescence level than the other CHF group. -Apoptotic cells percentage was higher in polymorphonuclear leukocyte (PMN) than PBMCs. -edematous leukocyte presented with higher percentage of apoptosis than another CHF group. -plasma nitrogen level, lipid peroxide, and inflammatory factors was higher in CHF than control.

Yo: years old; LPS: Lipopolysaccharide; SOD: Superoxide Dismutase; GPx: Glutathione peroxidase.

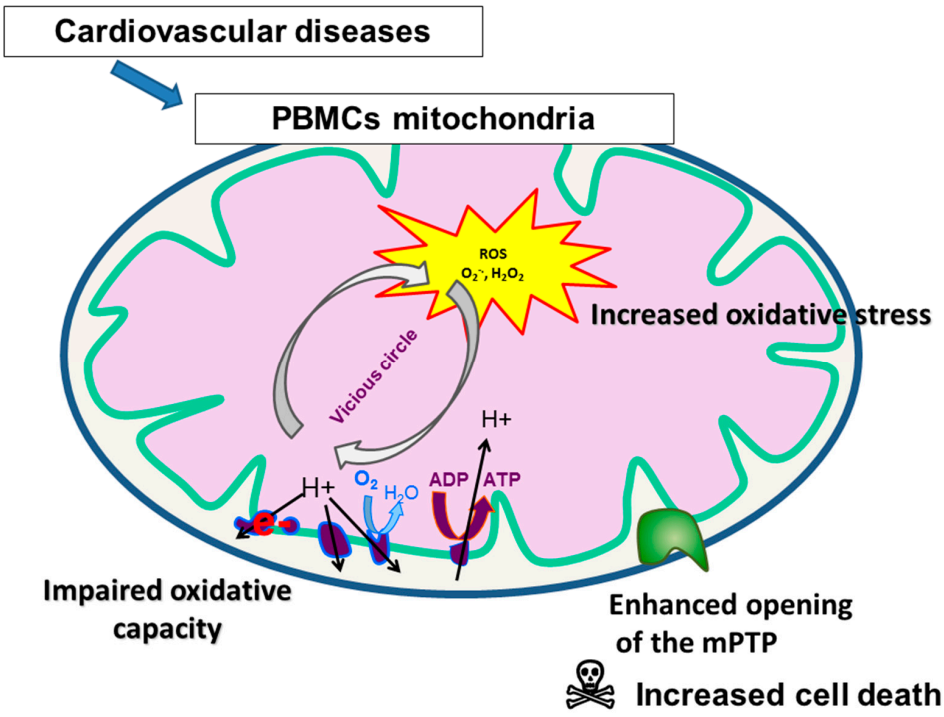

Figure 2. Mitochondrial alterations in PBMCs or platelets during cardiovascular diseases.

4. Mitochondrial ROS Production and Antioxidant Defense of PBMCs and Platelets in Patients with Cardiovascular Diseases

4.1. Measurements of ROS in Circulating Cells

ROS include superoxide, H_2O_2, and peroxynitrite, thought to be the most common and important biological oxidants. In the cardiovascular system, different sources of ROS coexist, and NADPH oxidase, xanthine oxidase, and uncoupled eNOS, together with mitochondrial ROS, participate in endothelial dysfunction in relation to inflammation, leading to a worse prognosis. ROS production results from enzymatic reactions in different cell components, including mitochondria, and, is associated with normal basal metabolic energy generation. In the mitochondria, ROS are physiologically produced mainly across mitochondrial complex I and III of the ETC [22,49–53]. Thus, a normal balance of ROS is essential for cellular functions; however, once the level of ROS surpasses the standard concentration, cellular damage will result, leading finally to apoptosis and cellular death [54,55]. Therefore, an accurate and potent detection method of ROS is crucial for cardiovascular system studies [56], but ROS measurement with high accuracy is still challenging because of ROS' short half-life [57]. Griendling et al. listed all measurement approaches of ROS in detail [58]. In a biological system, the gold standard for measuring ROS in the form of free radicals is thought to be EPR (electron paramagnetic resonance), also recognized as electron spin resonance [55,57,58]. Other measuring techniques of ROS include chemical assays for superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), or peroxynitrite ($ONOO^-$) with fluorescence analysis in the presence of redox sensitive probes or direct chemiluminescent assays [58].

Another frequent method used in clinical setting to measure ROS is the measure of byproducts, such as lipid peroxidation through malondialdehyde (MDA), 4-Hydroxy-Trans-2-Nonenal (HNE), and isoprostanes F_2-IsoPs determination [57,58]. Additionally, oxidative modification of protein and nucleic acid is a classic approach in cardiovascular cells [57,58]. For example, ELISA (enzyme-linked immunosorbent assay) has been recognized as the most common measuring technique used [57]. On the other hand, flow cytometry is the most powerful technique

for single cell analysis of the immune system, in particular for leukocytes and platelets [55]. Many fluorescent probes are used for ROS detection in blood cells via flow cytometry [55]. For illustration, DCFH-DA, DAF-2 DA/DAF-FM DA, DHR123, and DHE are all intercellular probes and are detected as green fluorescence except DHE, which is detected as red fluorescence for both leukocytes and platelets. However, there are multiple artifacts related to the DCFH-DA probe and its use remains discussed [59]. Thus, although progress is still to be performed for oxidative stress evaluation, PBMCs can be incubated with chemiluminescent, bioluminescent, or fluorescent redox active probes to detect cytoplasmic or mitochondrial ROS. Particularly, mitochondrial ROS evaluation is possible with specific probes that can pass through the mitochondrial membrane by the addition of a triphenylphosphonium group to a fluorescent probe, like mitosox, which is an analogue of DHE, and for selective detection of H_2O_2 within the mitochondria, MitoPY1 can be used with imaging techniques [60].

Further, the quantitation of reactive species metabolites, ROS scavengers, and antioxidant enzymes can be obtained from chromogenic and enzymatic assays from culture supernatants. Gene expression analysis of PMBCs also allows assessment of antioxidant systems and of other molecules modulating intracellular oxidative stress, such as the *OXPHOS* genes. Finally, quantitative assessment of the mitochondrial structure and function provide additional information when oxidative stress has mitochondrial genesis.

4.2. Mitochondrial ROS in PBMCs in CVDs

4.2.1. Mitochondrial ROS in PBMCs in Heart Failure

Oxidative stress plays a key role in the development and progression of CVDs and could be used as an indirect marker to predict disease severity and prognosis [61–63]. In this context, mitochondrial dysfunction appears to have increased importance [17,64]. Indeed, high levels of ROS and increased production of superoxide anion by neutrophils have been observed in the blood of HF patients, and white blood cells and platelets producing ROS can amplify oxidative stress and organ damage in HF [48,65]. A recent study showed that circulating PBMCs present structural and functional derangements of mitochondria with overproduction of ROS in HF [38]. Besides, a significant reduction of respiration was associated with a higher mitochondrial ROS production in PBMCs of patients with moderate to severe CHF compared to mild CHF [22]. Furthermore, there was a positive correlation between mitochondrial ROS formation and oxidative DNA damage and plasma BNP levels, which are related to the severity of HF. In CVDs, lymphocytes and monocytes play a key role in atherogenesis, modulating the inflammatory and immune response. Indeed, PBMCs would undergo changes similar to failing cardiomyocytes in HF [36]. Based on these data, the use of circulating leukocytes may become a relevant biomarker in cardiovascular diseases and might serve to better understand its pathogenesis [66].

The mechanisms by which mitochondrial ROS in PBMCs are increased in CVDs are multifactorial. Enhancement of myocardial ROS might stimulate ROS generation in PBMC mitochondria via the mechanism of ROS-induced ROS generation upon the passage of circulating PBMCs through the heart. Indeed, the proportion of mitochondrial ROS-loaded blood cells is higher in the coronary sinus than in the peripheral veins of CHF patients [48]. Another hypothesis is the role of inflammatory factors present in HF, such as circulating cytokines, that trigger ROS generation [29]. Further, in heart failure, tissue hypoxia may trigger an increase in the production of ROS, which is a strong stimulus of pro-inflammatory cytokines, such as IL6 and TNF-α [67]. Li et al. confirmed the involvement of mitochondrial dysfunction of PBMCs in the pathophysiology of heart failure; extreme inflammation and decreased antioxidant capacity were closely associated with heart diseases, especially in early stage heart failure patients [29].

Other markers of oxidative stress have been described, such as myeloperoxidase (MPO), oxidized low density lipoproteins (oxLDL), and F_2Isoprostane [66]. Elevated lipid peroxidation has been shown to be associated with the severity of HF, such as malondialdehyde (MDA) and 4-Hydroxy-2-nonenal (HNE) [68]. In addition, two studies showed a positive correlation between the

total plasma peroxide levels (reflecting oxidative stress index) in leukocytes with serum NT-proBNP [8,36]. Mondal et al. demonstrated that HF patients with implanted left ventricular assist devices exhibit excessive production of ROS as well as DNA damage in circulating leukocytes [47]. Similarly, Garcia Anastacia et al. observed increased ROS level and deteriorated mitochondrial respiratory capacity in circulation PBMCs in pediatric HF patients who underwent cardiac transplant [46].

4.2.2. Mitochondrial ROS in Arterial Hypertension, Coronary Artery Disease, and Stroke

Yasunari et al. measured the oxidative stress of circulating leukocytes in both hypertensive and diabetic patients and concluded that the level of oxidative stress was significantly increased in arterial hypertension [69]. This study used peripheral leukocytes as a biomarker to detect hypertension-related vascular damage [51]. In fact, the role of measuring ROS in leukocytes in hypertensive patients might help monitor the effect of treatments [51].

In PBMCs, the evaluation of oxidative stress and mitochondrial function in coronary artery disease has been attempted via assessment of the gene expression profile of complex I subunit (NDUFc2). Raffa et al. found a significant reduction of complex I subunit with increased levels of ROS and decreased ATP levels [35].

Only a few works in the literature have demonstrated the role of ROS in circulating cells in the development of stroke [51]. Aizawa et al. showed that in stroke patients, the ROS levels of peripheral mononuclear cells (circulating neutrophils) are increased compared to controls [70].

4.3. Mitochondrial ROS in Circulating Platelets in CVDs

It is now clear that mitochondria modulate the pro-thrombotic function of platelets through energy generation, redox signaling, and apoptosis initiation [71–73]. Thus, studies have related increased platelet activation with mitochondrial hyperpolarization and ROS production. Yamagishi et al. demonstrated that hyperglycemia induces hyperpolarization in normal platelets, resulting in the production of mitochondrial ROS and subsequent activation [74]. Furthermore, Avila et al. observed in diabetic patients that platelets had decreased rates of oxygen consumption and hallmark signs of increased ROS production [75]. Preserving platelet mitochondrial function may therefore allow a decrease of the risk of thrombotic events in diabetic patients [76].

5. Circulating Mitochondrial DNA (mtDNA) Originating from PBMCs and Platelets in Patients with Cardiovascular Diseases (Table 2)

5.1. Circulating Mitochondrial DNA (mtDNA) Originating from PBMCs in Patients with Cardiovascular Diseases

Adequate numbers of mtDNA (free-cell mtDNA) or (circulating mtDNA) are important for mitochondrial as well as cellular function. mtDNA are released by cells undergoing stress or having pathological events [77]. MtDNA encodes 2 ribosomal RNAs, 22 transfer RNAs, and 13 polypeptides of the respiratory chain [78]. Mitochondria contain several copies of mtDNA. The number of mtDNA copies in cells correlates with the size and number of mitochondria, which change under different energy demands and oxidative stress and under different pathological conditions. The mtDNA copy number or content reflects the mitochondrial function through the mitochondrial enzyme activity and ATP production [79]. Quantification of the mtDNA copy number of PBMCs using real-time polymerase chain reaction (PCR) was found to produce consistent and reproducible results [80].

Unlike nuclear DNA, mtDNA is vulnerable to ROS damage because of the lack of histone protection and effective DNA repair mechanisms. When mtDNA damage occurs, it results in mitochondrial dysfunction, inflammation, and cell senescence participating in the pathogenesis of CVDs and atherosclerosis. The mtDNA copy number might reflect the level of mtDNA damage, potentially being a biomarker of mitochondrial function and a predictor of CVDs' risk and prognosis [77,79,81].

Studies have tested mtDNA for the evaluation of CVDs [81,82]. High levels of circulating mtDNA behave as a danger-associated molecular pattern molecule (DAMP), enhancing inflammation and organ damage [83]. In addition, the effective release of mtDNA requires antigen-presenting cells, such as mononuclear and lymphocytes cells, to be involved [84]. Bliksøen et al. observed a correlation between increased mtDNA content and the incidence of myocardial infarction, suggesting mtDNA as a diagnostic biomarker for acute myocardial infarction (AMI) [83]. Likewise, previous evidence emphasized that mtDNA damage might promote atherosclerosis through mitochondrial impairment [85]. As an illustration, Fetterman et al. studied mtDNA damage in PBMCs in patients presenting with diabetes mellitus, clinical atherosclerosis, and CVDs through the isolation of lymphocytes and monocytes. They found that mitochondrial DNA impairment was directly related to oxidative phosphorylation impairment, which ends up with oxidative stress and organ dysfunction [86]. However, in this study, the author found no changes in the mtDNA copy number between the three groups. Sudakov et al., indicated an increase in the circulating mtDNA content in the blood of patients with acute coronary syndrome, which could be a biomarker for the probability of death from myocardial infarction [87].

Studies support the notion that a lower level of mtDNA content indicates a high risk for CVD and sudden cardiac death [81] but others suggested that increased circulating mtDNA content was linked with reduced LV diameters and volumes and thus enhanced cardiac function [77]. By the way, at least, peripheral blood mtDNA might be a predictor of heart characteristics. Chen et al. performed a study to reveal the association between the peripheral mtDNA copy number in leukocytes and risk of CHD. A correlation between the circulating mtDNA content and the formation of atherosclerotic plaque suggested a connection among low mtDNA and a high risk of coronary heart disease [88]. Huang et al. conducted studies in heart failure and acute myocardial infarction patients with consistent results. Both patients type showed lower mtDNA content than the control group [89,90]. Discrepancies in these results might be related to the disease severity, aging, or other risk factors factor that may modify directly or indirectly the outcome. Also, the site of mtDNA extraction might be important. Indeed, in one study, the mtDNA was extracted from platelet-poor plasma while other studies have investigated mitochondria from leukocytes.

Taken together, although still needing further analysis, decreased circulating mtDNA might potentially be assumed to be a risk factor for heart failure and used as a biomarker for cardiovascular disease prognosis.

Similarly, in ischemic stroke patients, Lien et al. quantified the mtDNA content in peripheral leukocytes and found a significant reduction compared to the control individuals [91]. Furthermore, Zhang et al., in patients at risk for atherosclerosis, observed an inverse correlation between the mitochondrial copy number and the risk of sudden cardiac death [92].

Table 2. Mitochondrial DNA in peripheral circulating cells and cardiovascular disease.

Population Characteristics	Study Design	Mitochondrial Function/mtDNA Copy Number	Oxidative Stress	Cell Viability/ Apoptosis	Results	Reference
Ischemic stroke patients Total *n* = 350 Age: 60.9 ± 9.1 Male *n* = 246 Female *n* = 104 Control group *N* = 350 Age:60.4 ± 9.1 Male *n* = 246 Female *n* = 104	mtDNA in Peripheral Blood Leukocyte	-mtDNA content (rt PCR) -The ratio of mtDNA to NuclearDNA is used to estimate the number of mtDNA per cell	-oxidized glutathione (GSSG), and reduced glutathione (GSH), (enzymatic (method) -8-hydroxy-2′-deoxyguanosine (biomarker of oxidative DNA damage, ELISA)	NA	mtDNA content in peripheral leukocyte for ischemic stroke patients was significantly lower than the control group. $P < 0.0001$ mtDNA content evaluated for 150 ischemic stroke patients = 0.90, while in 50 control individuals = 1.20 -The level of GSSG and 8-hydroxy-2′-deoxyguanosine were higher in patients with ischemic stroke than on the control group. GSSG Ischemic stroke = 1.83 Control = 0.79 8-hydroxy-2′-deoxyguanosine ischemic stroke = 6.33 Control = 4.87 These results exhibited that oxidative stress was higher in patients with ischemic stroke than in control group	Lien et al., 2017, Journal of American Heart Association [91]
3 cohort study with a risk factor of CVD 1st: Cardiovascular Health Study (CHS) *n* = 4830 Age: >65 years 2nd: Atherosclerosis Risk in Communities (ARIC) *n* = 11153 Age: Between 45 to 65 years 3rd: Multiethnic Study of Atherosclerosis (MESA) *n* = 5887	In CHS: DNA was extracted from the buffy coat using salt precipitation following proteinase K digestion In ARIC: DNA was extracted from the buffy coat of whole blood using (Qiagen) In MESA: DNA was extracted from leukocyte using (Qiagen)	In ARIC and MESA, mtDNA copy number was measured by using prob intensities of mitochondrial single nucleotide polymorphisms (SNP) on the Affymetrix Genome-Wide Human SNP Array 6.0 IN CHS: mtDNA was calculated using multiplexed TaqMan-based PCR	NA	NA	-The effect of mtDNA copy number on the incidence of coronary heart disease was higher than in stroke and in other CVDs In all 3 cohort groups, the mtDNA copy number was inversely associated with CVD events	Ashar et al., 2017, JAMA Cardiology [79]

Age: 45 to 85 years Control: NA						
Coronary Heart Disease (CHD) classified in 4 groups according to Gensini score 1-Gensini score: 0-–22 *n* = 99, Male 72, Age: 57.3 2-Gensini score: 22–55 *n* = 98, Male 73, Age:57.9 3-Gensini score: 55–96 *n* = 102, Male 79, Age: 58.3 4-Gensini score:96–254 *n* = 101, Male 86, Age: 58.8 -Control group *n* = 110 Age: 58.1	mtDNA of Leukocytes for CHF categorized by Gensini score	-genomic DNA was isolated from peripheral blood cells by E.Z.N.A blood DNA Midi Kit. -mtDNA quantification (Quantitative real time PCR).	NA	NA	mtDNA content of PBMCs was lower in CHD patients than in the control group. -mtDNA was reduced significantly, while Gensini score was increased suggesting the level of circulating mtDNA correlates with presence and severity of CHD.	Liu et al. 2017, Atherosclerosis [82]
Acute coronary syndrome (ACS) Total *n* = 14 Divided into 2 groups 1st group: (Survivor) who survive during 30 day of hospitalization *n* = 11, male 9, female 2 Age: 53 2nd group: (deceased) who died due to ACS during time of analysis *n* = 3 female Age: 87	Blood samples were collected from platelet poor plasma	-To evaluate mtDNA. Isolation performed with PROBA-NK reagent kit. -quantitation of mtDNA was performed by PCR	NA	NA	-Deceased group: the level of mtDNA level was higher (5900 copies/mL) than the survived group (36 copies/mL) $p = 0.049$ -increased level of mtDNA in plasma suggest a probability of death of 50% for ACS patients	Sudakov et al., 2017, European Journal of Medical Research [87]
Patients from the Atherosclerosis Risk in Communities (ARIC) *n* = 11093 male *n* = 4971	mtDNA in peripheral blood buffy coat	mtDNA copy number was measured by using prob intensities of mitochondrial single nucleotide polymorphisms (SNP) on the	NA		-Inverse association between mtDNA copy number and sudden cardiac death	Zhang et al., 2017, Eur Heart Journal [92]

female n = 6122 Age: 57.9 ± 6.0		Affymetrix Genome-Wide Human SNP Array 6.0				
Acute myocardial infarction patient undergoing primary angioplasty n = 55 male n = 47 female n = 8 Age: 57.4 ± 11.4 years Control group: n = 54 male n = 44 female n = 10 age: 55.3 ± 7.4	Peripheral blood leukocyte	Leukocyte mitochondrial DNA copy number (MCN) was measured from venous blood using PCR -AMI patients were divided into two groups according to median baseline leukocyte mtDNA copy number = 82/cell 1st group MCN ≥ 82 2nd group MCN < 82	NA	NA	-Baseline characteristics: In AMI patients the plasma leukocyte mtDNA copy number was significantly lower than in the control group. 122.7 ± 109.3 vs. 194.9 ± 119.5/cell p = 0.003 -AMI patients with lower MCN, had higher left ventricle shape sphericity index (SI), at 1,3,6 months after angioplasty and higher left ventricle diastolic and systolic volume at 6 months after angioplasty.	Huang et al., 2017, Circulatiog Journal, [90]
Patients with diabetes mellitus and atherosclerosis cardiovascular disease Total n = 275 -only Atherosclerosis: N = 55 Female 18 Age:60 ± 10 -only DM: N = 74 Female 47 Age: 55 ± 10 -Atherosclerosis and DM N = 48 Female 31 Age: 62 ± 8 Control group n = 98 Female 49 Age: 55 ± 7	PBMCs	Measuring mitochondrial DNA damage in PBMCs by PCR. -Total DNA was separated using QIAmp DNA mini kit and quantification determined by using Pico-green assay kit	Oxidative stress of arterial pulsatility (increased baseline pulse amplitude p = 0.009)	NA	Mitochondrial DNA damage was higher in all 3 diseased group, as compared with controls, with the highest in the group combining atherosclerosis and diabetes. -mtDNA measured in DM alone (0.65 ± 1.0) -mtDNA measured in atherosclerosis alone (0.55 ± 0.65) -mtDNA measured in both atherosclerosis and DM (0.89 ± 1.32) p < 0.05 mtDNA damage correlated with baseline pulse amplitude	Fetterman et al., 2016, (Cardiovascular Diabetology) [86]
General population Total n = 701	Peripheral blood cells	To assess the circulating mtDNA content, PCR was	NA	NA	There is a relation between peripheral blood mtDNA copy number and left ventricular	Knez et al. 2016, International

Divided by 3 tertiles of mtDNA content -Tertile 1 mtDNA content 0.39–0.86 *N* = 233 Female 103 Age: 51.6 ± 16.8 EF% 61.3 ± 7.0 -Tertile 2 mtDNA content 0.86–1.10 *N* = 234 Female126 Age:53.5 ± 14.7 EF%: 63.3 ± 6.56 -Tertile 3 mtDNA content 1.11–3.06 *N* = 234 Female 128 Age:54.3 ± 14.2 EF%: 62.9 ± 6.65		used. Total DNA was extracted from peripheral blood sample using QIAmp DNA Mini Kit.		function. Higher mtDNA content was associated with better systolic and diastolic left ventricular function	Journal of Cardiology [77]
Chronic Heart Failure Total *N* = 1700 -Ischemic HF *N* = 790 Male 543 Age: 62.6 ± 10.4 EF% 57 -Nonischemic HF *N* = 910 Male 572 Age: 53.8 ± 14.3 EF% 40 Control group *n* = 1700	Circulating Leukocyte -Blood sample were drawn, and leukocyte were isolated in K2-EDTA tubes.	Total DNA was extracted by using QG-Mini80 workflow with a DB-S kit. And DNAs of cardiac tissues were isolated by using QIAmp DNA Mini Kit. And copy number ratio was evaluated.	ROS were quantified in heart tissues using Dihydroethidium (DHE) staining. -In lymphocyte intracellular ROS was analyzed by flow cytometry using DCFH-DA -LDL was detected	HF patients presented a low mtDNA content compared to control group. Median 0.83, IQR: 0.60–1.16 vs. median 1.00, IQR: 0.47–2.20) $P < 0.001$. Ischemic HF patients are more susceptible to lower mt DNA copy number (Median 0.77, IQR: 0.56–1.08) than non-ischemic HF median 0.91, IQR 0.63–1.22 -mtDNA content of leukocyte was not correlated with LV diameter $p = 0.988$ -in HF group, LDL was associated with the mtDNA copy number $p = 0.007$	Huang et al., 2016, Medicine [89]

male 1115 Age: 57.7 ± 11.0 EF%: NA					-Lower circulating mtDNA was correlated with increased risk of HF, $p < 0.001$ -In HF patients, the level of ROS was higher than in control group in heart tissues and in lymphocytes.	
Coronary heart Disease Patients *N* = 378 Male 279 Female 99 Age: 57.9 -Control group *n* = 378 male 279 female 99 Age: 58.9	Peripheral Blood Leukocytes -5 mL of venous blood was drawn from each individual and anticoagulated into sodium citrate tube.	-DNA was separated from peripheral blood leukocyte using E.Z.N.A blood DNA Midi Kit. -DNA content was measured using PCR	NA	NA	-mtDNA content was inversely related to increased risk of CHD -CHD group shows marked lower mtDNA content, compared to controls, $p < 0.001$, -CHF had higher neutrophils counts compared to controls (5.10 ± 1.66 vs. 4.50 ± 1.51) but no difference in WBC count $p = 0.154$	Chen et al. 2014 (Atherosclerosis) [88]
Myocardial infarction ST segment elevation MI (STEMI) *n* = 20, 5 female Stable angina pectoris (SAP) *n* = 10, 1 female Both undergoing percutaneous coronary intervention (PCI) and categorized as transmural or non-transmural Age: between 30 and 75 years	Platelet poor plasma	Venous blood sample were gathered, and DNA was extracted from platelet poor plasma using QIAmp DNA blood Mini Kit -Quantification of mtDNA using real time PCR	NA	NA	-Baseline characteristics: Both groups were similar except SAP group which received more PCI treatment than the other group. -After PCI: 3 h later, mtDNA plasma level of NADH dehydrogenase subunit 1 (ND1) were increased in STEMI compared to SAP. $p = 0.01$ -patients with transmural: ND1 levels were greater in STEMI patients *n* = 10, than STEMI patients with non-transmural *n* = 6 -positive correlation between the severity of myocardial damage and the level of mtDNA, mtDNA being increased in myocardial infarction.	Bliksøen et al., 2012, [83]

5.2. Circulating Mitochondrial DNA (mtDNA) Originating from Platelets in Patients with Cardiovascular Diseases

Several physiological stimuli that cause platelet activation at low concentrations could induce platelet apoptosis at higher concentrations. This type of dual signaling is potentially important in the regulation of coagulation. Increased platelet apoptosis has been reported in a number of pathologies, including type 2 diabetes [93]. Activated platelets can release functional mitochondria and mtDNA. Beyond the measurement of mitochondrial function in patients with disease, and due to its lack of a nucleus, platelets provide a unique source of mtDNA [71]. An increasing number of studies support the idea that evaluation of the bioenergetic function in circulating platelets may represent a peripheral signature of mitochondrial dysfunction in metabolically active tissues (brain, heart, liver, skeletal muscle). Indeed, owing to their easy accessibility, there is interest in the use of platelets to study mitochondrial (dys) function in human disease over time. Accordingly, impairment of mitochondrial respiration in peripheral platelets might have potential clinical applicability as a diagnostic and prognostic tool as well as a potential biomarker in treatment monitoring. In sepsis, an alteration in the bioenergetics of platelet mitochondria was directly correlated with the clinical outcome [94].

In CVDs, there are few studies on circulating platelets' mitochondrial dysfunction. Baccarelli et al. suggested that platelet mDNA methylation may be implicated in the etiology of CVDs [95]. Regarding the fact that cardiovascular diseases are strongly influenced by platelet function though acute thrombotic and atherogenic mechanisms, we can expect that evaluation of the bioenergetic function in circulating platelets may represent a potential biomarker of CVD susceptibility, prognosis, or treatment.

An experimental evaluation of atherosclerosis by Yu and co-workers displayed that mtDNA damage was recognized in circulating monocytes, as well as decreased complex I and IV, which were associated with mitochondrial dysfunction [96]. However, this study showed an independent relation between atherosclerosis and reactive oxygen species, as it showed that those at high risk of atherosclerosis have extensive mtDNA damage with no increase in ROS levels [96].

6. Conclusions

In summary, this review outlines the importance of mitochondrial function in circulating blood cells and particularly, its relationship with CVDs. Impaired mitochondrial respiratory chain activity and ATP generation, changes in mitochondrial DNA content, and increased ROS formation in PBMCs and likely in platelets are often associated with several types of cardiovascular diseases. Currently, an evaluation of the mitochondrial function of circulating cells in human blood for cardiovascular disease might be considered as a new noninvasive approach that deserves further studies to improve its diagnosis and prognosis interest. Also, mitochondrial function and ROS and mtDNA involvement in CVD physiopathology support that a better knowledge of these aspects might open new therapeutic perspectives.

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2. Result II: Review of PBMCs and Lung Disease:

Since lung alteration is often associated with heart failure and subsequently with heart transplantation, we also studied published data in the context of lung diseases. While mitochondrial respiration of PBMCs appears to be decreased in chronic obstructive bronchopneumonitis, it is increased in asthma, particularly exacerbated asthma, and in pulmonary arterial hypertension, which can also be observed in connection with heart transplantation. An increase in circulating oxidative stress is observed during these pathologies. These findings in the literature prompted us to concentrate on mitochondrial respiration and oxidative stress in heart transplant patients.

Le Résumé:

Les maladies pulmonaires telles que la bronchopneumopathie chronique obstructive, l'asthme, l'hypertension artérielle pulmonaire ou la fibrose pulmonaire idiopathique sont des causes majeures de morbidité et de mortalité. Complexe, leur physiopathologie est multifactorielle et comprend un dysfonctionnement des mitochondries pulmonaires et une libération accrue d'espèces réactives de l'oxygène (ERO), ce qui mérite une attention accrue. En outre, et c'est important, les cellules sanguines circulantes (cellules mononucléaires du sang périphérique - (PBMC) et plaquettes) participent probablement à ces maladies systémiques. Cette revue présente les données publiées à ce jour et montre que la capacité d'oxydation mitochondriale des cellules sanguines circulantes est probablement réduite dans la bronchopneumopathie chronique obstructive (BPCO), mais augmentée dans l'asthme et l'hypertension artérielle pulmonaire dans un contexte de stress oxydatif accru. Outre ces modifications bioénergétiques des PBMC ou des plaquettes, des modifications de l'ADN mitochondrial (ADNmt) ont également été observées chez les patients. Ces nouvelles connaissances ouvrent des perspectives intéressantes pour déterminer leur rôle en tant que biomarqueurs ou guide potentiel pour une nouvelle approche thérapeutique dans les maladies pulmonaires.

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Review

New Insights into the Implication of Mitochondrial Dysfunction in Tissue, Peripheral Blood Mononuclear Cells, and Platelets during Lung Diseases

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Abstract: Lung diseases such as chronic obstructive pulmonary disease, asthma, pulmonary arterial hypertension, or idiopathic pulmonary fibrosis are major causes of morbidity and mortality. Complex, their physiopathology is multifactorial and includes lung mitochondrial dysfunction and enhanced reactive oxygen species (ROS) release, which deserves increased attention. Further, and importantly, circulating blood cells (peripheral blood mononuclear cells-(PBMCs) and platelets) likely participate in these systemic diseases. This review presents the data published so far and shows that circulating blood cells mitochondrial oxidative capacity are likely to be reduced in chronic obstructive pulmonary disease (COPD), but enhanced in asthma and pulmonary arterial hypertension in a context of increased oxidative stress. Besides such PBMCs or platelets bioenergetics modifications, mitochondrial DNA (mtDNA) changes have also been observed in patients. These new insights open exciting challenges to determine their role as biomarkers or potential guide to a new therapeutic approach in lung diseases.

Keywords: lung diseases; mitochondria; blood; PBMCs; platelets; oxidative stress; COPD; asthma; pulmonary arterial hypertension; pulmonary fibrosis

1. Introduction

Lung diseases, especially chronic obstructive pulmonary disease (COPD), asthma, pulmonary arterial hypertension (PAH), and/or idiopathic pulmonary fibrosis (IPF) are main causes of mortality, resulting in significant health and economic burdens worldwide. Indeed, the prevalence of COPD is increasing with 64 million persons concerned in the world, and according to the latest WHO estimates, it will rise as the third leading cause of death by 2030 [1]. Asthma is the most common chronic disease among children, affecting around 235 million persons [2]. PAH or IPF are less common but are characterized by poor prognosis [3,4]. Based on this evidence, it appears important to gain new insight into lung diseases pathophysiology and to analyze potential biomarkers to better reveal lung function, diagnose, and predict the prognosis of these diseases. As observed during cardiovascular diseases, mitochondrial dysfunctions deserve to be further studied, both at the local and at the circulating levels [5]. Indeed, mitochondrial abnormalities are involved in lung diseases but direct evidence of

mitochondrial dysfunction in human studies is limited, because of the difficulty to biopsy tissues in these frail patients.

Peripheral blood mononuclear cells (PBMCs) or platelets are accessible through a small amount of blood withdrawal and allow mitochondrial function analysis. PBMCs are composed by lymphocytes, monocytes, and dendritic cells, which mainly participate in immunity and inflammation. Platelets are known to modulate hemostasis and are also involved in pulmonary alterations. Isolation of circulating leukocytes is an easy way to represent cardiovascular stress [6–8]. Recent studies in sepsis have suggested that bioenergetics profiling of circulating PBMCs might reflect mitochondrial function in other tissues and are linked with disease grade, immune alterations, and prognosis [9–12]. At rest, PBMCs rely mainly on mitochondrial respiration to match the metabolic demand and show a significant spare respiratory capacity [13]. Compared to biopsy, blood withdrawal is easy, but isolating purified platelets is a time-consuming task and analyzing mitochondria from these platelets needs to be done in a timely and skillful manner [14]. Circulating platelets are rich in mitochondria and could be used to assess bioenergetics and systemic metabolism in pathologies such as diabetes, sepsis, and cardiovascular or sickle cell diseases [15–17]. Reactive oxygen species (ROS) is an important factor in platelet functioning [18], as observed in COPD and adult respiratory distress syndrome [19,20].

The mean bioenergetics profiles in human circulating platelets, monocytes, lymphocytes, and neutrophils were reported in real-time measurements of the mitochondrial oxygen consumption rate (OCR) using the extracellular flux analyzer (Seahorse Bioscience) [21]. In a healthy subject, monocytes are one of the most energetic cell types with high levels of both glycolysis and oxidative phosphorylation. Lymphocytes and platelets are more oxidative and less glycolytic at the basal state; neutrophils show little mitochondrial oxidative capacities [21]. The reserve capacity, difference between basal and maximal mitochondrial respiration, is potentially used by cells in the setting situations needing higher mitochondrial involvement and is about 20% in platelets, whereas it is highest in monocytes or lymphocytes.

Interestingly, despite red blood cells being present with no mitochondria in human, a recent report showed a presence of structurally cell-free competent mitochondria in blood circulation [22]. Circulating peripheral blood cells and plasma characteristics and bioenergetics are presented in Figure 1.

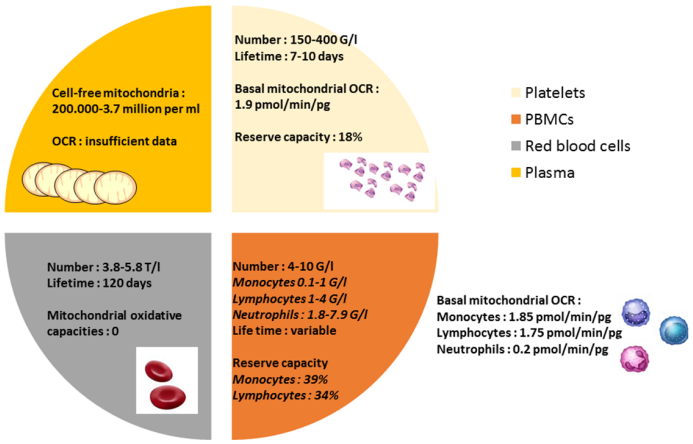

Figure 1. Characteristics of circulating blood cells and plasma: number, lifetime, and mitochondrial oxidative capacity. OCR: mitochondrial oxygen consumption rate.

The study of mitochondrial function in PBMCs and circulating platelets seems to be useful and complementary [13,21]. At present, the link between PBMCs or platelets mitochondrial function and lung diseases is poorly known and the objective of this review is to present the data published so far and to discuss the potential interest of studying circulating blood cells mitochondrial function and ROS production during COPD, asthma, PAH, and IPF.

2. Physiological Mitochondrial Function

Derived from ancient aerobic bacteria, mitochondria are double membrane structures with its own maternally DNA and transcription machinery. Mitochondria are involved in the heme biosynthesis, intracellular calcium regulation, ATP-production, and fatty acid synthesis. Mitochondria are the main source of energy in the cells and oxygen consumption by the mitochondrial respiratory chain (electron transport system—ETS) drives adenosine triphosphate (ATP) synthesis. Defects of mitochondria result in an energy deficiency that can impair cells, and lately the entire organism function. The mitochondrial respiratory system is located in the inner mitochondrial membrane and is formed by five complexes (I-IV and complex V–ATP synthase). Each complex can be analyzed with a spectrophotometer and, besides oxygen consumption, ATP synthesis and mitochondrial membrane potential can be determined [23,24]. Importantly, mitochondrial also generate ROS (i.e., anion superoxide arising from complexes I and III and dismutated to hydrogen peroxide by superoxide dismutase in hydrogen peroxide, which is able to leave the mitochondria), considered as signaling molecules at normal levels but able to generate proteins, lipids, and nucleic acids damages at higher levels [25].

Mitochondria contain DNA (mtDNA) and their own transcriptional mechanisms. MtDNA is very sensitive to oxidative stress and genotoxic agents because of its proximity to the mitochondrial respiratory chain, the major site of ROS production. If mtDNA is damaged, an increasing number of mtDNA copies appears as compensation and is believed as an indicator of mitochondrial function and oxidative stress [26]. After injury, mtDNA fragments accumulate in autolysosomes and are partially degraded by DNAse II.

3. Mitochondrial Dysfunction in Lung Diseases

Many of the lung diseases are thought to be related to aging, and accumulation of dysfunctional mitochondria is considered a marker for the pathological conditions but is also the key factor that drives disease progression. Thus, mitochondrial dysfunction may contribute to the pathogenesis of many human diseases including lung diseases such as COPD, asthma, PAH, and IPF [27,28], often associated with increased ROS and impaired bioenergetics and/or mitochondrial biogenesis, mitophagy, and dynamic, which are critical to maintain cell homeostasis and may result in cellular apoptosis and senescence [29]. Lungs are exposed to the high-oxygen environment and present huge contact areas with the blood to allow hematosis, making them sensitive to oxidative stress and damages. For example, tobacco smoking, a major risk factor for lung diseases, is linked to oxidative stress and induces mitochondrial dysfunction [30]. Mitochondrial ROS can favor pro-inflammatory cytokines secretion and modulate calcium regulation of epithelium and airway muscle cells or extracellular matrix production [31]. Lung parenchymal and immune cells communicate in response to infections, cigarette smoke, and air pollution etc., in order to repair tissues or defend against pathogen, but data on circulating blood cells are sparse.

In addition, some studies have suggested that mtDNA fragments could be implicated in lung diseases pathophysiology, acting as a damage-associated molecular pattern (DAMP) to initiate immunological response [32].

3.1. Chronic Obstructive Pulmonary Disease (COPD)

3.1.1. Mitochondrial Dysfunction and Oxidative Stress in COPD

COPD is a major cause of respiratory failure with a prevalence of 10% in adults over 40 years [1]. The estimates show that it will be the third cause of mortality in 2030. The pathogenesis of COPD is not completely understood. Chronic inflammation is a central feature, leading to airway remodeling, irreversible bronchial obstruction, and destruction of lung parenchyma (emphysema). Acute exacerbations, whatever their origin, increase oxidative stress and induce systemic inflammation both in lung resident and circulating cells of individual patients [33]. Oxidant–antioxidant imbalance is one of the involved mechanisms, but how mitochondrial mediated-inflammation contributes to the

disease progression remains to be determined. Airway epithelium is very sensitive to oxidative stress because of a low expression of anti-oxidative enzymes which are sparsely inducible, mainly after tobacco exposure [34]. In COPD, oxidative stress is enhanced particularly after exacerbation. At the systemic level, plasmatic ROS are enhanced in smokers, whether or not with COPD, and oxidative capacity is lower in plasma of smokers. Wiegman et al. showed an association between mitochondrial dysfunction and excessive mtROS levels in airway smooth muscle cells from COPD patients, which contributes to enhanced inflammation and cell proliferation [31].

In COPD, mtROS are released from activated inflammatory cells or structural cells such as epithelial, endothelial, or smooth muscle cells [35]. In this way, oxidative stress is an adaptive response. It is a mechanism leading to initiate immune response to neutralize infectious agents and to maintain the cellular homeostasis [36]. In excess, ROS damages DNA, lipids, and proteins, resulting in lung cellular death, activation of metalloproteases, inactivation of antiproteases, and degradation of extracellular matrix which result in loss of alveolar units [37]. ROS participate also in cell proliferation and collagen synthesis in smooth muscle.

Moreover, ROS activate redox sensitive transcription factors such as nuclear factor-kappa B (NFκB), resulting in release of pro-inflammatory mediators such as IL 1-like cytokines [36,38]. Levels of IL-1β are increased in lungs of patients with COPD after smoking, suggesting the involvement of inflammasome [38–40].

3.1.2. Mitochondrial Function, Oxidative Stress, and mtDNA in PBMCs or Platelets in COPD

PBMCs have been shown to release more ROS, which may contribute to COPD patient's prognosis [41]. A recent study observed a high level of mitochondrial dysfunction and derived ROS in PBMCs obtained from unstable COPD patients after combustion-generated ultrafine particles exposure [42]. Indeed, exposure to nano-organic carbon particles and soot ultrafine particles (found in environmental pollution) induced the release of pro-inflammatory IL-18 and IL-33 from exacerbated COPD-derived PBMCs, with oxidative stress. Further, a recent work observed that PBMCs in COPD have reduced ability to use glucose, pyruvate, or fatty acids at baseline, which is not observed in PBMCs from healthy smokers which have only impaired glycolysis [43]. Similar results have been observed in sepsis [44].

Because COPD is frequently associated with cardiovascular diseases, activation of blood platelets through inflammation seems to have an important role in the pathophysiology of COPD [19]. After respiratory exacerbation, thrombocytosis is associated with significantly short and long term mortality, supporting an important role of platelets [45]. Antiplatelet therapy may have a protective role in patients after exacerbation of COPD. A study has shown that, during COPD, hypercoagulation (measured through platelet distribution width) is associated with reduced survival [46]. In a guinea pig model, Bialas et al. showed increased proton and electron leaks and decreased mitochondrial respiratory chain capacity in platelets from chronic smoke-exposed animals [47]. Proton and electron leaks in platelets appeared related to ROS production and interactions between oxidative stress and platelets could represent potential therapeutic targets. Indeed, administration of N-acetylcysteine improved the quality of life of stable COPD patients [48].

MtDNA levels can be modulated by oxidative stress in COPD, as observed in the exhaled breath or urine [49,50]. In leukocytes, mtDNA easily undergo mutations, insertions, or deletions in response to oxidative stress during COPD. Liu et al. showed a decreased peripheral leukocyte mtDNA copy number in COPD patients, suggesting a less mtDNA protection or biosynthesis in these patients [51]. Mitochondrial dysfunction could result in abnormal function of leukocytes in COPD. In this study, the mtDNA copy number was similar in healthy smokers and non-smokers subjects. On the contrary, a study showed an increase of mtDNA/nuclear DNA ratio in the blood from patients with ACOS (asthma-COPD overlap syndrome). Interestingly, there was a correlation between mtDNA and the number of smoked cigarettes [52]. The increase of mtDNA content could compensate the mitochondrial respiratory function decline due to oxidative damage or mutation in this pathology.

In addition, Kim et al. demonstrated that peripheral leukocyte mtDNA copy numbers positively correlated with leukocytes telomeres length in elderly women, suggesting that telomere may relate to mitochondrial function [53]. Indeed, COPD patients have short leukocyte telomeres, associated with an increased risk of total and cancer mortality [54,55].

In summary, mitochondrial dysfunctions in PBMCs of COPD patients result likely in abnormal functionality, and mitochondrial pathophysiology represents an emerging research with potential promising therapeutic avenues (Table 1, Figure 2, modified from [56]).

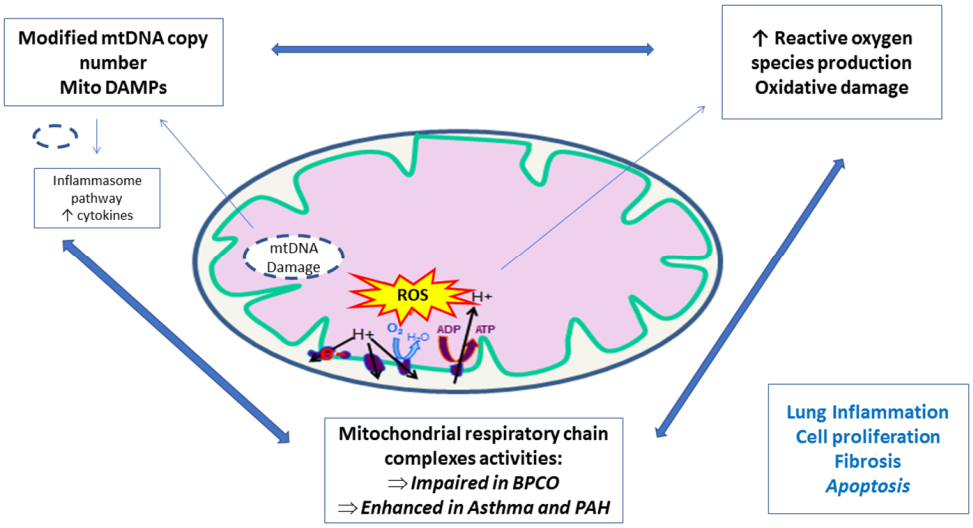

Figure 2. Peripheral blood mononuclear cells and platelets mitochondrial implication in lung diseases.

Table 1. PBMCs or platelets mitochondria respiration is impaired in chronic obstructive pulmonary disease (COPD).

References	Lung Disease Number of Patients	Type of Circulating Blood Cells	Mitochondrial Respiration	Oxidative Stress	mtDNA	Other Results
De Falco. 2017, Front Immunol [42]	Unstable COPD patients	PBMCs treated with combustion-generated ultrafine particles exposure	High level of mitochondrial dysfunction	High level of mtROS High expression of NOD-like receptor 3 in PBMCs in basal conditions in COPD patients		Release of cytokines: IL-18 and IL-33 (dependent on the release of caspase-4)
Bialas. 2018, Int J Chron Obstruct Pulmon Dis [47]	Chronic smoke-exposed guinea pig	Platelets	Increased proton and electron leak Decreased electron transfer system capacity			
Carpagnano. 2016, BMC Pulm Med [52]	ACOS patients (n = 23) COPD patients (n = 13) Asthmatic patients (n = 14) Normal subjects (n = 10)	PBMCs			Increased mtDNA/ nuclear DNA ratio in ACOS patients compared to other groups Increased mtDNA/ nuclear DNA in asthmatic or COPD patients compared to normal subjects	
Agarwal. 2019, Respir Res [43]	Tobacco smoke related COPD patients (n = 14) Non-smokers (n = 16) Healthy smokers (n = 13)	PBMCs	Impaired glucose metabolism in COPD subjects: lower OCR, ATP production, and spare respiratory capacity Impaired pyruvate metabolism in COPD subjects Impaired fatty acid metabolism in COPD subjects			Increase of inflammatory cytokine response (IFN-γ, IL-17, TNF-α, IL-5, IL-9, and IFN-α)
Liu. 2015, PloS One [51]	COPD patients (n = 86) Healthy smokers (n = 33) Non-smokers (n = 77)	PBMCs	Decreased serum glutathione level in COPD		Decreased leukocyte mtDNA copy number of PBMCs in COPD Linear correlation between mtDNA copy number and serum glutathione level	

ACOS: asthma-COPD overlap syndrome; ATP: adenosine triphosphate; COPD: chronic obstructive pulmonary disease; OCR: oxygen consumption rate; PBMCs: peripheral blood mononuclear cells.

3.2. Asthma

3.2.1. Mitochondrial Function and Oxidative Stress in Asthma

Asthma is a frequent disease that affects around 235 million persons [2], showing airflow obstruction, bronchial hyper-reactivity, and inflammation [57]. It is multifactorial, favored by individual/genetic and general/environmental parameters. The progression of bronchial epithelium damage is related to increased inflammation, favored by cytokines release. Mitochondrial dysfunction and enhanced oxidative stress participate in the pathophysiology of asthma through increased mucus secretion and impaired bronchial smooth muscles, as observed both in experimental and clinical studies [58–64]. Accordingly, a decreased antioxidative capacity inferred from reduced superoxide dismutase, gluthation peroxidase, or catalase activity was related to the gravity of asthma [34,61–63]. Interestingly, the role of ROS is not unequivocal depending on the cells involved. Thus, if it is generally accepted that ROS arising from epithelial and smooth muscle cells participate in lung injury and aggravate inflammation [64], ROS might be protective when present in the blood.

3.2.2. Enhanced Mitochondrial Function and ROS Production in PBMCs or Platelets in Asthma

Currently, during asthma, there are some data on the mitochondrial implication of peripheral blood circulating cells. In school-aged children with atopic asthma, antigen-specific IgE receptor expression was revealed on PBMCs [65]. In addition, future exacerbations were associated with the number of basophils during childhood acute wheeze/asthma [66]. Recently, Ederle et al. showed, in severe exacerbated asthmatic patients, an enhanced PBMCs mitochondrial respiration and increased ROS production compared to healthy volunteers [67]. Interestingly, the plasma of asthmatic patients stimulated similarly the PBMC's of control subjects, suggesting a mechanism of protection as proposed during septic shock [68]. On the contrary, PBMCs impaired mitochondrial function was observed early (6 hours) in patients with local allergic rhinitis after an acute nasal allergen challenge [69]. Likewise, PBMCs oxidative capacity was often reduced in cardiovascular diseases [7]. In the study of Ederle et al., obesity might have played a role. Indeed, PBMCs mitochondrial respiration tend to be enhanced in asthmatic patients presenting with a BMI ≥30 kg/m^2 [67] and differential bioenergetics in airway epithelial cells and platelets between lean and obese asthmatics was observed [70,71]. Of note, steroids mediate eosinophils apoptosis via a mitochondrial pathway, and such a link needs to be further studied since systemic steroid treatment longer than 24 hours did not influence the ROS production [72].

Increasing evidence suggests an important participation of platelets and their secretions (thromboxane, serotonin ...) in the pathophysiology of allergic diseases and a potential role as key modulators of immunity. In patients with asthma, alterations in platelets secretion, expression of surface molecules such as IgE receptors, aggregation, and adhesion were observed [73,74]. Platelets activation in bronchoalveolar lavage was associated with airway hyperreactivity [75–77]. Platelets contribute also to the secretion of cytokines and can activate eosinophils [78,79]. Xu et al. showed increased Krebs cycle enzyme activity and less dependence on glycolysis in platelets of asthmatic patients [80]. Taken together, these data suggest a capacity for greater oxygen consumption and more efficient energy production in platelets of asthmatic patients (Table 2).

Table 2. PBMCs or platelets mitochondria respiration is enhanced in asthmatic or in patients with pulmonary arterial hypertension (PAH).

References	Lung Disease Number of Patients	Type of Circulating Blood Cells	Mitochondrial Respiration	Oxidative Stress	mtDNA	Other Results
Ederle. 2019, J Clin Med [67]	Severe asthmatic patients with severe exacerbation (n = 20) Healthy volunteers (n = 20)	PBMCs	Increased PBMCs mitochondrial respiratory chain complexes activity in asthmatic patients Mitochondrial respiratory chain complexes activity in PBMCs is related to plasma constituent	Increased ROS production in the blood of asthmatic patients ROS production is related to plasma constituent		
Winnica. 2019, Antiox Redox Signal [71]	Lean and obese, mild to moderate, asthmatic patients (n = 16) Lean and obese healthy volunteers (n = 21)	Platelets	Similar basal OCR in lean healthy and asthmatic subjects Increased basal OCR in asthmatic obese Enhanced maximal OCR in lean and obese asthmatic patients	Enhanced ROS production in lean and obese asthmatics		
Xu. 2015, Plos One. [80]	Asthmatic patients (n = 12) Healthy controls (n = 13)	Platelets	Similar OCR in both groups Decreased glycolytic rate and greater tricarboxylic acid cycle activity in asthmatic platelets		No change in mtDNA content	No change in mitochondrial number and morphology
Nguyen. 2017, JCI Insight [81]	Group 1 PAH patients (n = 28) Control patients (n = 28)	Platelets	Increased glycolytic rate in PAH patients: decrease of pyruvate dehydrogenase activity No change in basal respiration Enhanced respiratory reserve capacity in PAH dependent on increased fatty acid oxidation Increase in complex II enzymatic activity and decrease in complex I enzymatic activity. No change in enzymatic activity of complex IV.	No change in mitochondrial superoxide production		Positive correlation between respiratory reserve capacity and hemodynamic severity (mean PAP, PVR and right ventricle stroke work index) No change following phosphodiesterase 5 inhibitions, prostacyclin analogue and endothelin receptor antagonist
Nguyen. 2019, Plos one. [82]	Group 2 PH patients (n = 20) Control patients (n = 20)	Platelets	No significant difference in basal oxygen consumption rate. Increased maximal oxygen consumption rate (increased contribution of fatty acid and glucose oxidation).	No difference in mitochondrial superoxide production.		Negative correlation between maximal mitochondrial respiration and right ventricular stroke work index No change following nitrite inhalation.

ETC: electron transport chain; OCR: oxygen consumption rate; PAH: pulmonary arterial hypertension; PAP: pulmonary arterial pressure; PVR: pulmonary vascular resistance.

3.3. Pulmonary Hypertension

3.3.1. Mitochondrial Dysfunction, ROS, and mtDNA in Pulmonary Hypertension

Pulmonary hypertension (PH) is classified in five groups, depending on hemodynamic characteristics [83,84]. Post-capillary PH (group 2) is secondary to left heart diseases [83]. In left heart diseases, PBMCs undergo changes similar to failing cardiomyocytes and the degree of PBMCs mitochondrial dysfunction and increased ROS can be related to the disease severity [6–8,85–87]. Classically, group 1 of PH, which includes idiopathic pulmonary arterial hypertension (PAH), is characterized by pulmonary artery remodeling with intimal fibrosis, medial smooth cells hypertrophy, and in situ thrombosis with plexiform lesions. This arterial remodeling is associated with elevated pulmonary vascular resistance, potentially leading to right ventricular failure and death [88] and we can expect a potential implication of mitochondrial impairment and ROS in PAH.

Particularly, they favor vessels constriction progression through wall thickening via growth factor stimulation and endothelin-1-related smooth muscle proliferation [89–93]. Hypoxic response, inflammation, apoptosis, and vasoconstriction in PAH might also use endothelial mitochondria pathways. In animals and humans, endothelial and pulmonary arterial smooth muscle cells exhibit metabolic switch from mitochondrial oxidative phosphorylation toward cytoplasmic glycolysis even in the presence of oxygen, conferring apoptosis resistance and cellular hyper proliferation [94,95]. This is accompanied by altered mitochondrial ETC activity [94]. These abnormalities concern also cardiac tissue and cells as well as skeletal muscle of PH patients [96–101]. Further, altered *BMPR2* expression, gene implicated in PAH predisposition, was linked to pulmonary arterial endothelium mitochondrial dysfunction [102] and ROS upregulate hypoxia-inducible transcription factors [103,104].

Oxidative stress further enhances vasoconstriction through endothelin-1 and thromboxane A2, increased hypoxic cytosolic calcium concentration, and reduces vasodilatation through decreased prostacyclin production [105–107]. Accordingly, anti-oxidative therapies are beneficial in experimental PAH [108,109]. ROS are produced by inflammatory and vascular cells, and NADPH oxidases are localized in macrophages or polynuclear as well in pulmonary arterial endothelial, smooth muscle cells, and fibroblasts [110–112].

Moreover, studies suggested that mtDNA injury participates in PAH development. The mitochondrial Sirtuin 3, involved in mtDNA repair, is decreased in PAH patients and monocrotaline-induced PH in rats [113,114].

3.3.2. Mitochondrial Function in Platelets during Pulmonary Hypertension

In PAH, platelets participate in vascular thrombosis through different mechanisms [115,116]. Very interestingly, Nguyen et al. showed that circulating platelets from PAH patient's exhibit impaired bioenergetics characterized by increased glycolysis compared to healthy controls [81], Table 2. In these patients, increased glycolysis was associated with a switch toward fatty acid oxidation, and increased respiratory reserve capacity correlated with the hemodynamic severity assessed by right heart catheterization. This suggests a relationship between platelet mitochondrial function and PAH severity. In this study, PAH pulmonary vasodilators modulating endothelin, nitric oxide, and/or prostacyclin activities did not affect the mitochondrial bioenergetics observed in PAH platelets, but this deserves to be confirmed in a greater subset of patients.

It might be useful to investigate a potential link between brain natriuretic peptide (BNP), an established biomarker of PAH severity and prognosis, and platelet bioenergetics alteration which could also reflect abnormalities of pulmonary vascular cells or cardiomyocytes and could be useful to assess PAH gravity and progression. Further, since BNP has been shown to protect cardiac and skeletal muscles mitochondria from ischemia-reperfusion damages [117,118], it might be interesting to investigate whether BNP might also modulate circulating cells oxidative capacities.

Interestingly unlike in PAH (group 1), platelets from subjects with HF with preserved ejection fraction (group 2 PH) did not show change in the glycolytic rate compared to normal subjects [82].

However, similarly to PAH patients, they showed an enhanced maximal respiratory capacity. Besides HF, older age and other comorbidities such as obesity, diabetes, or systemic hypertension might explain in part the observed differences in platelet biology.

3.4. Idiopathic Pulmonary Fibrosis and Interstitial Lung Diseases

Mitochondrial Dysfunction, Oxidative Stress, and Inflammation in Idiopathic Pulmonary Fibrosis

Interstitial lung diseases are characterized by progressive scarring of the lungs. Mechanisms underlying the pathogenesis of these diseases remain incompletely understood and idiopathic pulmonary fibrosis (IPF) is the most common fibrotic interstitial lung disease. Despite anti-fibrotic treatments, IPF has a poor prognosis with a life expectancy of 3–5 years after the diagnosis, which is generally made in patients older than 60 years [3]. A characteristic feature of IPF is the accumulation of myofibroblasts arising from normal lung fibroblasts, largely involved in extracellular matrix remodeling, in response to biochemical courses such as TGF-β (a pro-fibrotic cytokine) or environmental/genetic factors [119].

Recent studies suggested a role of mitochondrial dysfunction in term of biogenesis, dynamic (fusion or fission), and mitophagy in the physiopathology of IPF. There is evidence for mitochondrial dysfunction leading to cellular senescence and apoptosis in alveolar epithelial cells (AECs), fibroblasts, and immune cells, participating in the fibrotic process. Besides a potential role of age per se, mitochondrial dysfunction being a recognized hallmark of aging, the alveolar epithelium is sensitive to injury/apoptosis induced by cigarette smoke and other pollutants, which may affect AECs mitochondrial function. TGF-β also regulates the alterations of mitochondrial function [120–122].

There is an enhanced mtROS production in fibrotic lungs leading to type II AECs apoptosis, but lung macrophages and fibroblasts are resistant to apoptosis. Mitochondria from fibrotic type II AECs change their shape and become enlarged but mitophagy and biogenesis are reduced. In lung macrophages, altered mitochondria are removed via mitophagy and undergo fission. In fibrotic myofibroblasts, mitochondrial dysfunction has been termed "mitochondrial dysfunction-associated senescence," characterized by a distinctive senescence-associated secretory phenotype. Fibrotic fibroblasts present with reduced mitophagy and mitochondrial biogenesis but with enhanced fission. MtROS generated at complex III could be responsible for excessive TGF-β in IPF since they are involved in TGF-β mediated gene expression [123].

Regarding these data, antioxidants could be beneficial in IPF. However, a randomized clinical trial testing N-acetylcysteine failed to improve lung function, rate of death, or acute exacerbations ratio in IPF patients, suggesting the role of other associated factors in IPF development [124]. Mitochondrial targeted antioxidant could reduce TGF-β induced pro-fibrotic gene expression and NOX-4 expression, which is necessary for TGF-β induced-myofibroblast differentiation [123].

To date, no work has studied the mitochondrial function in PBMCs or platelets in IPF. A study of circulating fibrocytes oxidative capacity, which are derived from monocytes lineage and thought to be precursors of fibroblasts, could be interesting [125]. Because epidemiological studies showed a link between IPF and cardiovascular diseases or venous thromboembolism, studying mitochondrial function in platelets could also be helpful in these diseases; platelet hyperactivity in patients with IPF being recently demonstrated [126,127].

4. Connections between Mitochondrial Dysfunction, ROS, and Inflammatory/Fibrotic Pathways in Lung Diseases. Pathophysiological Hypothesis

Such connections are numerous and besides other pathways, mitochondrial membrane polarization changes, altered mitophagy, and damage-associated molecular patterns (DAMPS)-related signaling can be involved in alveolar or pulmonary vascular remodeling observed in lung diseases. For clarity, these pathways are presented separately but they often share mutual mechanisms in COPD, PAH, asthma, and/or IPF (Figure 3).

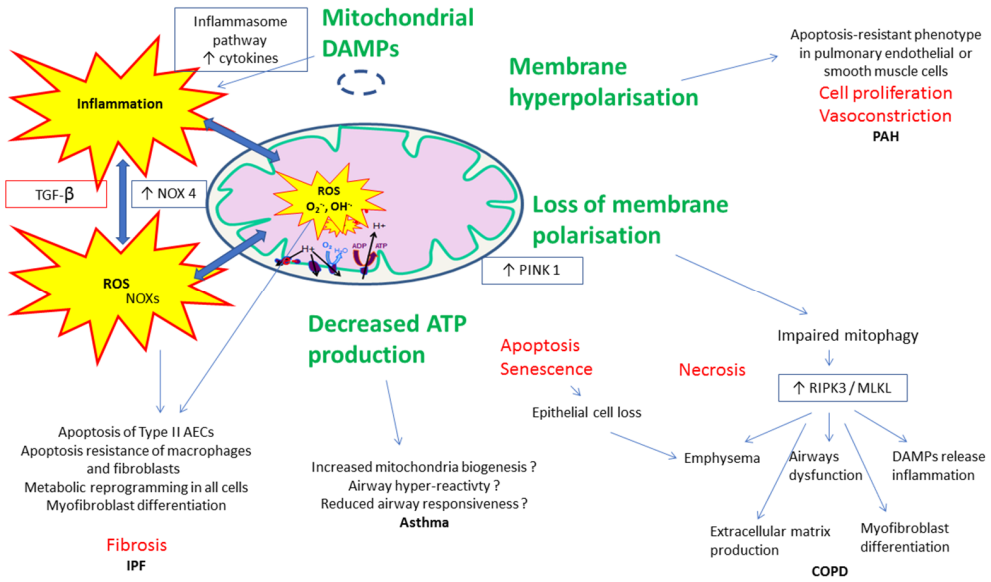

Figure 3. Examples of proposed mechanisms involved in the connections between mitochondrial dysfunction, reactive oxygen species (ROS), and inflammatory/fibrotic pathways in lung diseases.

In PAH, for instance, mitochondrial dysfunction and impaired ATP production promote glycolysis and thus hyperpolarization of the inner mitochondrial membrane, preventing the release of proapoptotic factors and leading to an "apoptosis-resistant" phenotype in pulmonary endothelial or smooth muscle cells. This results in cell proliferation and vasoconstriction [128,129]. Additionally, inhibitors of apoptosis are released from mitochondria when cells are under stress.

In COPD, loss of membrane polarization and elevated expression of the mitophagy regulator protein PINK1 (phosphatase and tensin homolog-induced putative kinase 1), a serine/threonine kinase, in epithelial cells in emphysematous regions of human lungs coincides with increased expression of the protein RIPK3 (receptor-interacting protein kinase 3) which modulates the programmed necrosis and many features characterizing this chronic lung disease [130]. Programmed necrosis (necroptosis) is a cellular program regulated by RIPK1 (receptor-interacting protein kinase 1) and RIPK3, and MLKL (mixed lineage kinase domain-like pseudokinase) in chronic lung diseases.

In asthma, conflicting results have been reported. Thus, decreased ATP production has been reported either to reduce airway constriction which is an energy dependent mechanism, or to be associated with airway smooth muscle thickening [131].

In IPF, the mitochondrial quality control system plays various roles depending on pulmonary cell types. While type II alveolar epithelial cells, lung macrophages, and fibroblasts show increased mtROS production in fibrotic lungs, their response differs. Type II AECs undergo apoptosis but lung macrophages and fibroblasts display apoptosis resistance. In response to increasing oxidative stress, all three cell types undergo a metabolic reprogramming which leads to the development and progression of lung fibrosis. Particularly, fibroblasts are transformed into a myofibroblast state, the effector cells of IPF [132–134].

Damage-associated molecular patterns (DAMPs), generated and released by cellular injury, may trigger inflammation, apoptosis, and innate immune responses by activating pattern recognition receptors. Mitochondria-associated DAMPs include mtDNA, which through auto-, paracrine and/or systemic effects activate the inflammasome pathway, resulting in increased cytokine release by immune cells as well resident cells favoring this inflammation, cell proliferation, and apoptosis. Mitochondrial DAMPs can be considered as proinflammatory inductors of the pulmonary remodeling observed in lung diseases and have been correlated to mortality in diseases such as sepsis and IPF. Their precise role in COPD, asthma, or PAH still deserves further studies [29,135].

Of note, ROS do not only arise from mitochondria in lung tissue. The NADPH oxidase homolog NOX4 is overexpressed in the lungs, primarily in myofibroblasts in fibroblastic foci and remodeled blood vessels, but also in epithelial cells associated with aberrant bronchiolization. NOX4 induction is largely mediated by production of the pro-fibrotic growth factor TGF-β. NOX 4 also induces mitochondrial ROS production. The production of ROS promotes mitochondrial DNA damage by reducing the mitochondrial expression of mitochondrial sirtuin 3 and OGG1 to mediate alveolar epithelial cells apoptosis [136,137]. It is the same in PAH, NADPH oxidase homologs NOX 2 and NOX 4 are key producers of ROS in vasculature. Like in IPF, NOX 4 mediates TGF-β1 dependent pulmonary vascular remodeling [138]. NOX 4 also mediates the effect of platelet-derived growth factor (PDGF) and HIF-1α which are critical to the pathogenesis of PAH.

Hypoxia, a frequent condition in lung diseases, induces the production of mitochondrial-derived oxygen-free radicals and ROS by the mitochondrial ETC. Of the molecules involved in this process, H_2O_2 can activate the transcription factor and lower oxygen-induced factor HIF-1α, which is implicated in pathophysiology of the lung diseases like PAH. Chen et al. showed that HIF-1α promoted pulmonary arterial smooth muscle cell proliferation and inhibited hypoxia-induced apoptosis, possibly through the regulation of mitochondrial dynamics [139].

Another mechanism which leads to mitochondrial dysfunction in PAH is the nitic oxide (NO)-cyclase guanylate- cyclic GMP pathway. NO regulates cellular respiration and mitochondrial biogenesis. In PAH, decreased NO level is associated with mitochondrial impairment with decreased ATP levels and dysregulated endothelial angiogenesis [140].

5. Antioxidative Therapies

Despite a clear involvement of ROS in lung diseases physiopathology, and albeit antioxidants appeared beneficial in experimental models, treatment with antioxidants has been largely unsuccessful and is not part of standard care in humans. Indeed, although improving some functional parameters, N-acetylcysteine or nutrients rich in antioxidants showed generally no beneficial effect on the rate of adverse events or death rates [124,141,142]. The failure of trials might have resulted from an incomplete understanding of the role of mitochondrial ROS in lung diseases development. Indeed, mitochondrial ROS are not always detrimental. They could play a protective role at lower levels and act differently according to the redox microenvironment, which varies spatially and temporally in different subcellular compartments and in different cell types. Thus, only specific subsets of patients might benefit from antioxidant therapies, an individual's susceptibility potentially depending on variation in their antioxidant genes.

6. Conclusions

Mitochondrial dysfunction associated with lung inflammation and oxidative stress contributes to COPD, asthma, PAH, and idiopathic pulmonary fibrosis. To allow adequate function and maintenance of intracellular homeostasis, mitochondria rely on quality control pathways (mitochondrial biogenesis, fusion/fission, mitophagy ...), and their alteration disrupts organelle metabolism and biogenesis, inflammation adequacy, and even innate immunity. The general concept that enhanced oxidative metabolism drives toward an anti-inflammatory response and that shifting toward a glycolytic metabolism favor an inflammatory response varies depending on the cells. Mitochondria are also platforms for pattern recognition receptors signal transduction and mediators in effector responses. Thus, mitochondrial DAMPS can activate the NRLP3 inflammasome, resulting in proinflammatory cytokine release. They act as the danger signal recognized by immune receptors [131,143,144]. Accordingly, for instance, TLR9 is involved in mtDNA induced in acute and chronic lung inflammation, through the TLR9-p38 MAPK pathway and STING pathway, respectively [145]. All pulmonary cell types are involved in the triad mitochondrial dysfunction–inflammation–enhanced ROS production.

Besides the role of mitochondrial dysfunction and oxidative stress at the tissue levels, studying PBMCs or the platelet's redox state takes roots on the fact that these cells play a key role in the

inflammatory and immune mechanisms involved in lung diseases. Likely, an altered redox state of dysfunctional circulating blood cells could enhance oxidative stress at the tissue level, potentially worsening the progression of the disease. However, to date, it appears that despite a generally increased ROS production, mitochondria respond quite differently depending on the lung disease. Thus, an impaired mitochondrial oxidative capacity is observed in the case of COPD, but PBMCs and/or platelets mitochondrial respiration is enhanced in the setting of asthma or PH. Whether such stimulated oxidative capacities might be protective, deserves to be determined.

Further studies are needed to reinforce the current evidence and to identify the potential of circulating PBMCs or platelets as biomarkers, which might allow a better understanding of the mechanisms involved in lung diseases, a better follow-up of patients, and possibly might help to identify novel original therapies.

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3. Results III: PBMCs Mitochondrial Respiration and Heart Transplant : Original Data (Revised Version)

Introduction: La fonction mitochondriale des cellules mononucléaires circulantes du sang périphérique (PBMCs) est une nouvelle approche intéressante des maladies cardiaques. Ainsi, la respiration mitochondriale des PBMCs diminue en relation avec la sévérité de l'insuffisance cardiaque. Cependant, aucune donnée n'est disponible chez les patients transplantés cardiaques (Htx).

Population et méthodes : nous avons déterminé la respiration mitochondriale des PBMCs par respirométrie haute résolution (Oroboros Instruments) et la production d'anions superoxydes par résonance paramagnétique électronique (Bruker-Biospin) chez 20 sujets sains et 20 Htx appariés et avons étudié les caractéristiques cliniques, biologiques, échocardiographiques, coronarographiques et biopsiques.

Résultats : La respiration mitochondriale du complexe II de la chaîne respiratoire des PBMCs était diminuée chez les Htx (4,69 ± 0,84 vs 7,69 ± 1,00 pmol/s/million de cellules chez les contrôles et les patients Htx, respectivement ; p=0,007) et la respiration du complexe IV était augmentée (24,58 ± 2,57 vs 15,68 ± 1,67 pmol/s/million de cellules ; p=0,0035). La production d'anions superoxydes était également accrue chez les Htx (1,47 ± 0,10 vs 1,15 ± 0,10 µmol/min ; p=0,041). Le rapport leucocytes/lymphocytes était augmenté chez Htx, dont le complexe II était corrélé au nombre de leucocytes (r=0,51, p =0,02) et à l'imagerie Doppler tissulaire (r= -0,62, p=0,005). Le complexe IV était augmenté chez les deux patients présentant un rejet aigu et corrélé négativement avec le temps de relation isovolumétrique de Htx (r= -0,45, p=0,045).

Discussion : bien que présentant une fonction systolique normale, le Htx a démontré une respiration mitochondriale anormale des PBMC. Contrairement aux thérapies immunosuppressives, un dysfonctionnement diastolique subclinique pourrait être impliqué dans ces changements. De plus, la lymphopénie pourrait réduire la respiration du complexe II et le rejet aigu augmenter celle du complexe IV.

Conclusion : La respiration mitochondriale des PBMC semble modifiée dans le Htx, potentiellement liée à un changement cellulaire, une légère dysfonction diastolique et/ou un rejet aigu.

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Peripheral Blood Mononuclear Cells Mitochondrial Respiration and Superoxide Anion after Heart Transplantation

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Abstract: Introduction: The mitochondrial function of circulating peripheral blood mononuclear cells (PBMCs) is an interesting new approach of cardiac diseases. Thus, PBMC's mitochondrial respiration decreases in relation with heart failure severity. However, no data are available in heart-transplanted patients (Htx). **Population and Methods**: we determined PBMCs mitochondrial respiration by high-resolution respirometry (Oroboros Instruments) and superoxide anion production using electron paramagnetic resonance (Bruker-Biospin) in 20 healthy subjects and 20 matched Htx and investigated clinical, biological, echocardiographic, coronarography and biopsy characteristics. **Results:** PBMCs mitochondrial respiratory chain complex II respiration was decreased in Htx (4.69 ± 0.84 vs 7.69 ± 1.00 pmol/s/million cell in controls and Htx patients, respectively; p=0.007) and, complex IV respiration was increased (24.58 ± 2.57 vs 15.68 ± 1.67 pmol/s/million cell; p=0.0035). Superoxide anion production was also increased in Htx (1.47 ± 0.10 vs 1.15 ± 0.10 µmol/min; p=0.041). Leucocyte to lymphocyte ratio was increased in Htx, whom complex II correlated with leucocyte number (r=0.51, p =0.02) and with tissue Doppler imaging (r= -0.62, p=0.005). Complex IV was increased in the two patients with acute rejection and correlated negatively with Htx's isovolumetric relation time (r= -0.45, p=0.045). **Discussion:** although presenting with normal systolic function, Htx demonstrated abnormal PBMC's mitochondrial

respiration. Unlike immunosuppressive therapies, subclinical diastolic dysfunction might be involved in these changes. Additionally, lymphopenia might reduce complex II and acute rejection enhance complex IV respirations. **Conclusion:** PBMC's mitochondrial respiration appears modified in Htx, potentially linked to cellular shift, mild diastolic dysfunction and/or acute rejection.

Keywords: PBMCs; mitochondrial respiration; heart transplantation; oxidative stress; cardiac function

1. Introduction

Despite the significant progress made in recent years in the treatment of patients presenting with preserved -or reduced- ejection fraction, many of them till reached terminal heart failure characterized by a poor short-term prognosis [1]. In this context, heart transplantation remains the treatment of choice, allowing major increases in life quality and duration. Nevertheless, a better knowledge of heart transplant pathophysiology appears interesting in order to improve cardiac function and to reduce rejection of the graft, which remains a significant issue. The usual clinical, biological, and echocardiographic follow-up does not always allow an early diagnosis, urging therefore to perform regularly cardiac biopsy and coronary artery angiography [2].

Recent data demonstrated that myocardial mitochondrial function was impaired in Htx presenting with cardiac allograft vasculopathy, supporting interest in mitochondrial functions analysis after heart transplantation [3]. Mitochondrion is the main producer of cell energy, coming from the oxidative phosphorylation, at 95% [4]. This organelle is the interface between physiological and pathophysiological mechanisms. The functions of mitochondria are multiple including metabolic control and apoptosis, accompanied by the generation of reactive oxygen species (ROS) [4]. Under basal or pathological conditions, the electron transport chain and proton motive force control the electron leakage for ROS production. This is at the origin of a proton gradient and the membrane potential [5]. The controllers of ROS production in mitochondria are numerous, presented in the different complexes, like complex I, and III. In the case of cardiovascular pathologies, characterized by a reduced cardiac function, a decreased energy production can be observed, inducing cell damages, and apoptosis. Considering, myocardial ischemia-reperfusion, a mitochondrial respiratory dysfunction of complex I and II, lead to an increase of mitochondrial superoxide anion [5].

Furthermore, avoiding cardiac biopsy, a metabolic approach focused on the peripheral blood mononuclear cells (PBMCs) allowed improve the knowledge of heart failure pathophysiology [6]. Thus, heart failure rely not only on cardiac defect per se, but it also includes systemic repercussions and particularly reduced PBMCs mitochondrial respiration. The higher the degree of heart failure, the greater the reduction in PBMCs mitochondrial oxidative capacities. Such alterations appeared also linked to inflammation [7–9].

To date there is no data concerning PBMCs mitochondrial respiration in Htx. Heart transplantation generally normalize the systolic function of the transplanted heart, but this occurs in an inflammatory context modulated by the immunosuppressive therapies. Circulating and local immune cells playing a major role in heart transplantation success [10], we challenged the

hypothesis that PBMCs mitochondrial respiration might be impaired depending on patient's cardiac and/or systemic characteristics.

We therefore investigated PBMCs mitochondrial respiratory chain complexes respiration together with superoxide anion, a major reactive oxygen species (ROS) in heart-transplanted patients and matched healthy controls.

2. Materials and Methods

Population and Parameters Determined

2.1. Population

Twenty Htx aged over 18 years were included during their follow up and compared to 20 healthy volunteers, matched for sex and age. To avoid cardiac bypass surgery-related systemic inflammation, no patient was included around the perioperative period. The delay since transplantation ranged from 1 to 31 years.

The patients who had a cardiac transplant less than a year ago are excluded.

The participants gave their informed consent, and the Ethical Committee of the Strasbourg University (CE-2016-91, 15 December 2016) approved the study.

2.2. Parameters Determined

Usual clinical, biological and cardiovascular explorations that particularly included echocardiography, coronary artery angiography and right heart catheterization with right ventricular biopsy -using conventional well-controlled methods- were performed in Htx. In addition, 30 mL of venous blood was sampled for PBMCs mitochondrial respiration and 1 mL was stored on ice for superoxide generation determinations in both Htx and control subjects. The Htx biological characteristics were determined by the hospital laboratory.

2.3. Extraction of Circulating Peripheral Blood Mononuclear Cells (PBMCs)

Briefly, blood was gently deposited over a Ficoll density gradient (Eurobio, Lymphocytes separation medium, Courtabeauf France, France) and centrifuged (2100 rpm, 25 min, 18 °C, without brakes). PBMC were recovered, washed in a DPBS solution (Dulbecco's Phosphate Buffer Saline 0067M, Hyclone, South Logan, UT, USA) and centrifuged (1600 rpm, 10 min, 18 °C). If erythrocytes be observed with PBMC, cells were immersed in a Versalyse- type solution for 20 minutes allowing the remaining red blood cells to be lysed without affecting the other cell line. Then a new wash with DPBS was carried out. Finally, isolated PBMCs were counted using flow cytometry (Muse Cell Analyser, Merck Millipore, Darmstadt, Germany).

2.4. Mitochondrial Respiration of PBMCs

Oxygen consumption of 2.5×10^6 PBMCs /mL was determined using a high-resolution oxygraph (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) at 37 °C with a continuous stirring. Cell membranes were permeabilized with saponine (125 µg/mL) and complex I was activated with glutamate (5 mM), and malate (2 mM). This step allows obtain the basal dioxygen consumption of CI at the leak stage, without activation of ATP synthase. Subsequently, different substrates and inhibitors were introduced in the oxygraph's chamber: (1) ADP (2 mM) induces

the activation of ATP synthase (OXPHOS CI) and the oxidative phosphorylation, via the electron transport coming from complex I. (2) Succinate (25 mM), an activator of the mitochondrial complex II, is added and allows observation of oxidative phosphorylation via the complexes I and II (OXPHOS CI+II). (3) Rotenone (0.5 μM) inhibits the Complex I, and we observe then oxidative phosphorylation via the complex II (OXPHOS CII). (4) TMPD/ascorbate (0.5 mM/0.5 mM) gives electrons directly to the Complex IV, and activates it (figure 1). To be more precise, Complex IV activity was calculated by the subtraction between step (4) and step (3). All the results are expressed in pmol/s/10^6cells.

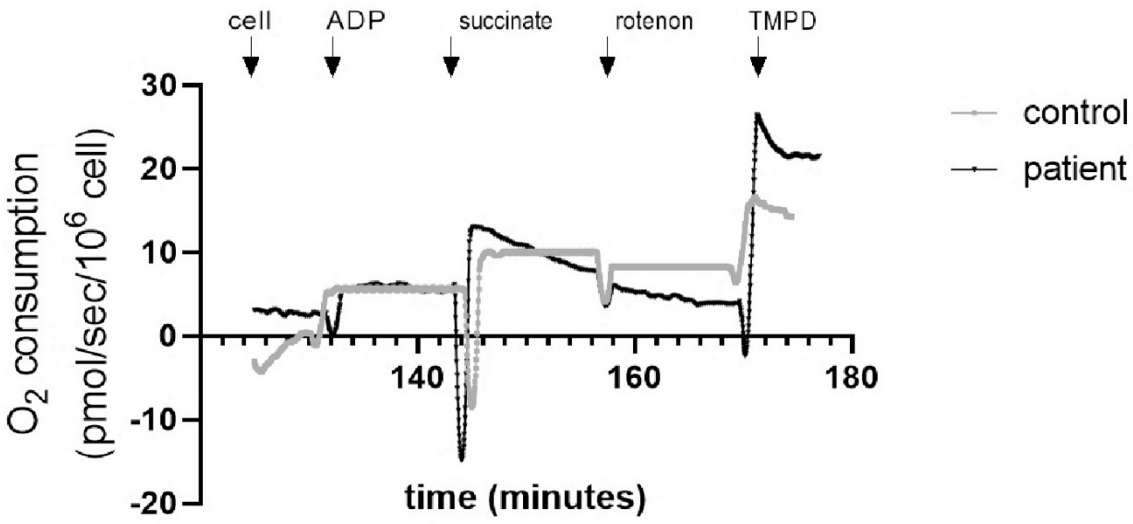

Figure 1: An illustrative curve of mitochondrial respiration of Peripheral Blood Mononuclear Cells (PBMCs) in controls and heart transplanted patients, obtained by Oxygraph Oroboros.

2.5. Measurement of Superoxide Anion

The production of superoxide anion ($O_2^{\cdot-}$) was determined using electronic paramagnetic resonance (EPR) (E-scan, Bruker-Biospin, Rheinstetten, Germany) at 37° C [11]. One hour after sampling, blood was mixed with CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpytrolidine HI, 200 μM). 40 μL of the mixture obtained was introduced into a glass EPR capillary tube (Noxygen Science Transfer & Diagnostics, Elzach, Germany) and placed inside the cavity of e-scan spectrometer. A free radical trapper was used to stabilize $O_2^{\cdot-}$. The EPR signal obtained is proportional to the superoxide concentration in the blood sample. The parameters used were: center field g = 3477,452; sweep width 60 G; microwave power 22.21 mW; 2.3 G amplitude modulation; constant time 40.96 ms; Conversion time 10.24 ms; number of lag curve points 6. Result are expressed in μmol/min.

2.6. Statistical Analysis

Statistical analyses were carried using GraphPad Prism 8 software (version 3.0) and quantitative data were expressed as mean ± standard error of the mean (SEM). Qualitative

variables were described as numbers and percentages. The normality of the value distribution was analysed according to a Shapiro-Wilk test. Patient and Control characteristics were compared according to a non-parametric Mann–Whitney test was used to compare the control and the heart transplant groups. The observed correlations were analysed with a non-parametric spearman test. The p-value has been set at 0.05.

3. Results

3.1. Clinical Characteristics of the Subject

Age and sex were similar in the two groups (Table 1). The delay since transplantation was 9.3 ± 1.8 years and Htx heart rate, systolic and diastolic pressures were respectively 82 ± 3 bpm, 136 ± 4 and 84 ± 3 mm Hg. The main causes leading to transplantation was ischemic cardiomyopathy, followed by dilated and non-obstructive cardiomyopathy. Htx received a usual immunosuppressive treatment including ciclosporine and/or everolimus, cellcept, corticoids. They also received antihypertensive drugs ARBs and/or diuretics, cholesterol lowering medication, anti-diabetic or other therapy as needed.

Table 1. Clinical characteristics of the subject.

	Healthy Controls	Htx
Gender (M/F)	17/3	18/2
Age (years)	59.6 ± 2.4	59.5± 2.5
BMI (kg/m^2)	25.9 ± 0.98	24.4 ± 1.0
Comorbidity (n, %)		
Hypertension	0	14, 70%
Diabetes	0	6, 30%
Dyslipidaemia	0	15, 75%
Initial cardiomyopathy (n, %)		
Cardiac ischemia	0	7, 35%
Dilated cardiomyopathy	0	3, 15%
Non obstructive cardiomyopathy	0	3, 15%
Valvular	0	2, 10%
Congenital	0	2, 10%
Rhythmic	0	1, 5%
Toxic	0	1, 5%
Genetic	0	1, 5%

3.2. Biological Characteristics of the Patients

The main biological characteristics of the Htx are presented in Table 2, as compared to laboratory normal range. Globally, Htx presented with normal or near normal values considering glycemia, lipids and the hepatic function. Creatininemia, as a surrogate of the renal function, appeared moderately increased.

Table 2. Biological characteristics of the heart transplanted patients.

	Normal values (range)	Htx (mean ± SEM)
Glycemia (mmol/L)	4.55-6.38	6.28 ± 0.44
Creatinine (umol/L) ± SEM	64-104	137 ± 16
Cholesterol (g/L) ± SEM	1.50-2.00	1.83 ± 0.1 n=19
LDLc (g/L)± SEM	<1.60	0.95 ±0.1 ,n=19
Triglycerides (g/L) ± SEM	0.35-1.50	1.97 ± 0.24 ,n=19
BNP (pg/mL) ± SEM	10-150	221.42 ± 104.42 , n=19
HB (haemoglobin level (g/dl)	12 – 16	12.81 ± 0.39 ,n=20
Blood Cells Count (*10$^{9/}$/L)		
Leucocytes	4-11	6.72± 0.47, n=20
Lymphocytes	1–4	1.65 ± 0.21, n=20

Neutrophils	1.40-7.70	4.29 ± 0.39
Inflammation		
CRP (mg/L) ± SEM	<5.0	6.31 ± 1.25, n=20
NLR (Neutrophil-to-lymphocyte ratio)	1.53 ±0.01, n=20	3.56 ± 0.65, n=20
LLR (Leukocyte to Lymphocyte ratio)	3.09±0.02, n=20	5.16 ± 0.73, n=20

Heart transplanted patients (Htx), LDLc: Low Density Lipoprotein cholesterol, BNP: brain natriuretic peptide.

Neutrophils to lymphocyte ratio (NLR), and leukocyte to lymphocyte ratio (LLR), are proposed as markers of inflammation. Neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio as predictors of survival after heart transplantation [12]. They were increased in Htx as compared to the control group (1.53 ± 0.01 vs 3.56 ± 0.65, respectively, p = 0.0002 and 3.09 ± 0.02 vs 5.16 ± 0.73, respectively, p = 0.0025) (Figure 2).

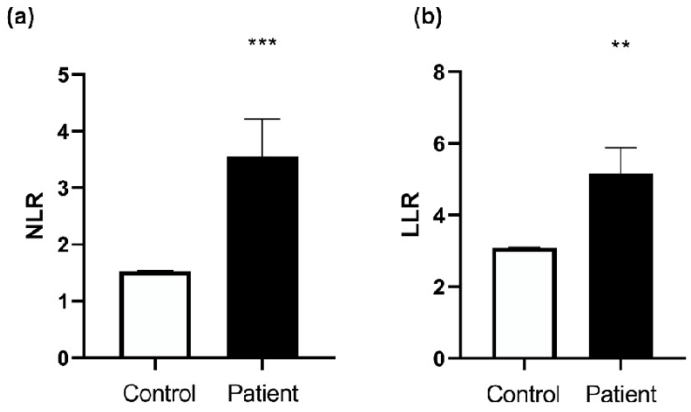

Figure 2. Controls and heart transplant group ratios. (a) Neutrophils to lymphocyte ratio (NLR), (b) Leukocyte to lymphocyte ratio (LLR). Values are means ± SEM, **p<0.01, ***p<0.001. n=20/ group.

3.3. Htx's Cardiovascular Explorations

3.3.1. Echocardiography

Table 3 shows main left and right heart echocardiographic characteristics of the Htx. Considering the transplant group, left heart systolic and diastolic functions were normal with filling pressures within the normal range. Similarly, right ventricular fractional shortening, cardiac index and systolic pulmonary artery pressure remained in the normal range in Htx.

Table 3. Echocardiographic characteristics of Htx.

	Normal values	HTx
Left ventricular Ejection Fraction (%)	> 55	63 ± 1.6
Right ventricular fractional shortening (%)	> 32	47 ± 1
Cardiac index (L/min/m²)	2.5-3.5	3.16 ± 0.11
Systolic pulmonary artery pressures (PAPs) (mmHg)	< 35	32.7 ± 1.8
E/A	1-2	1.71 ± 0.09
IVRT (Isovolumic Relaxation Time) (ms)	60-100	99.05 ± 1.19

HTx: Heart transplant patient. E/A: Mitral E and A wave's ratio.

3.3.2. Coronary Artery Angiography

The coronarography was normal in nine patients. Localized irregularities or stenosis less than 30% were observed in five patients and significant stenosis was present in the remaining six patients, three of them having either angioplasty or stenting in their history.

3.3.3. Right Heart Biopsy

The systematic right heart biopsy was normal in all but two patients who demonstrated for one humoral rejection (pAMR1h) and for the second cellular rejection (+ to ++).

3.4. Decreased Complexe II and Increased Complexe IV Mitochondrial Respiration in Htx's PBMCs.

Figure 3 shows the mitochondrial respiratory chain activities in controls and Htx. Non-phosphorylating respiration with activation of complex I alone (CI leak) was similar in controls and heart transplant patients (2.37 ± 0.37 vs 1.61 ± 0.18 pmol/sec/million cell, respectively).

Similarly, the O_2 consumption of oxidative phosphorylation by complex I (CI OXPHOS) shows no significant difference between controls and heart transplant patients (6.16 ± 0.60 vs 5.12 ± 0.54 pmol/sec/million cell, respectively). And, the O_2 consumption of oxidative phosphorylation by complex I and II (CI+II OXPHOS) shows no significant difference between controls and heart transplant patients (11.89 ± 1.26 vs 11.25 ± 1.31 pmol/sec/million cell, respectively). The respiratory control (RCR), calculated as the ratio between oxydation and OXPHOS by complex I was not significantly different in the two groups.

Nevertheless, the O_2 consumption of oxidative phosphorylation by Complex II (CII OXPHOS) was significantly decreased in heart transplant patients (7.68 ± 0.99 vs 4.68 ± 0.83 pmol/sec/million cell, respectively; p= 0.0067).

And, on the other hand, CIV OXPHOS: the O_2 consumption of oxidative phosphorylation by complex IV alone with subtracting CII, is significantly increased in heart transplant patients (15.68 ± 1.67 vs 24.58 ± 2.56 pmol/sec/million cell, respectively; **p=0.0035).

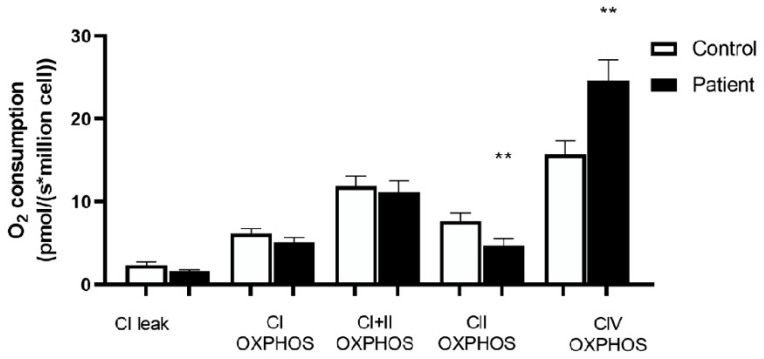

Figure 3. Mitochondrial respiration of Peripheral Blood Mononuclear Cells (PBMCs) in controls and heart transplanted patients. . CI: mitochondrial complex I; CI+II: mitochondrial complexes I and II; OXPHOS: mitochondria ADP-activated state of oxidative phosphorylation, CII: mitochondrial complex II. CIV: Mitochondrial complex IV oxidative phosphorylation; Values are means ± SEM, **: p<0.01, n=20 per group.

3.5. Increased Superoxide Anion Production after Heart Transplantation

The superoxide anion production was significantly increased in the heart transplant patients' group (1.15 ± 0.10 vs 1.47 ± 0.10 µmol/min, for controls vs Htx patients, p=0.04, figure 4).

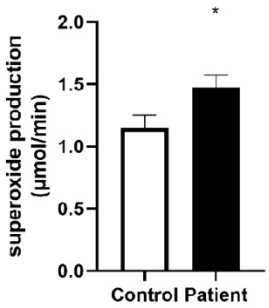

Figure 4. Superoxide anion production in controls and heart transplanted patients. Values are means ± SEM, *: p<0.05, n=20 in control and 19 in Htx group.

3.6. Correlations Related to Complex II Mitochondrial Respiration

Concerning blood cell counts, we observed a significant positive correlation between OXPHOS CII and the number of leukocytes (r= 0.51, p= 0.02, n= 20, figure 5a). The correlation between the number of lymphocytes and OXPHOS CII tended to be significant (r= 0.42, p= 0.07, n= 20, figure 5b). Additionally, OXPHOS CII and the posterior wall peak early diastolic myocardial velocity obtained using tissue doppler imaging were negatively correlated (r= - 0.60, p=0.05, n=20, figure 5c).

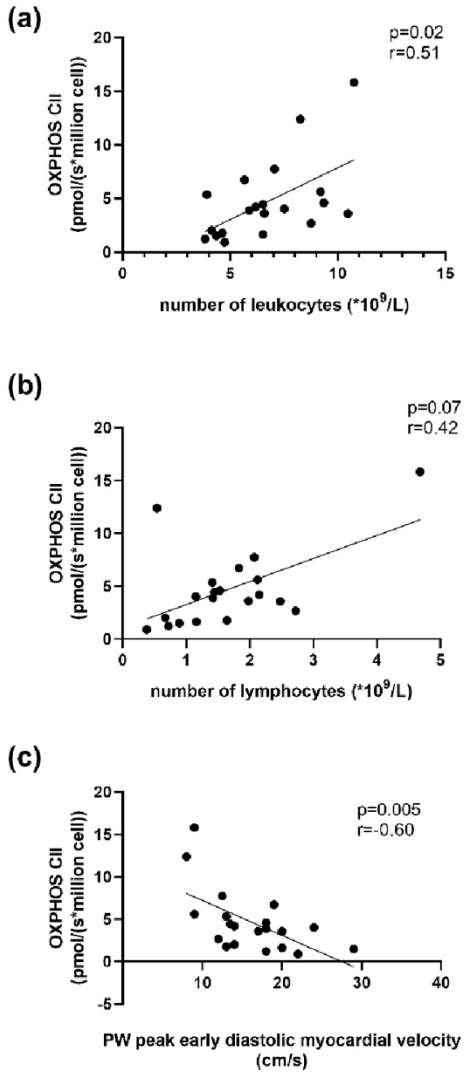

Figure 5. Correlation between OXPHOS CII and number of leukocytes (a), number of lymphocytes (b) and Posterior wall (PW) peak early diastolic myocardial velocity (cm/s), (c).

3.7. Correlations Related to Complex IV Mitochondrial Respiration

We observed a significant negative correlation between Htx's OXPHOS CIV and IVRT (r= -0.45, p= 0.04, n= 20). (Figure 6).

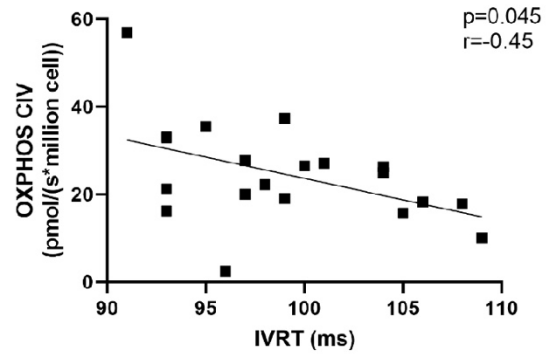

Figure 6. Correlation between OXPHOS CIV and IVRT. IVRT: Isovolumic Relaxation Time.

We then investigated potential relationship between mitochondrial respiration changes in Htx and their immunosuppressive therapies (Figures 7-8). We observed no significant difference related to the different therapy used. This was also true when analysing CMV infection, rejection degree or the other parameters.

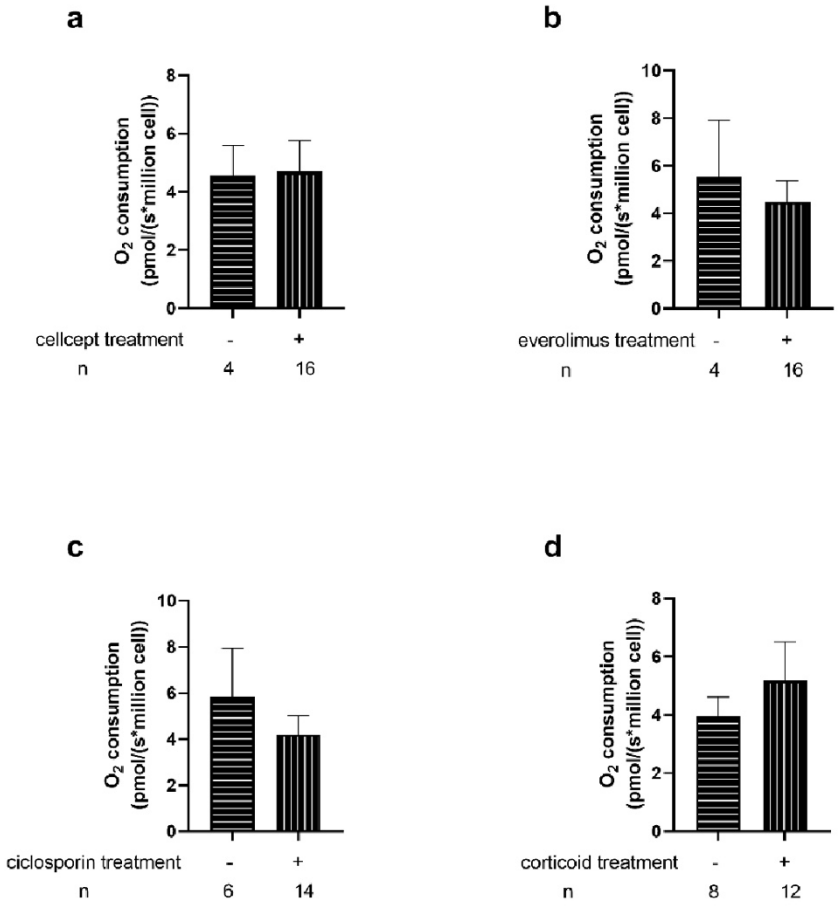

Figure 7. Effects of immunosuppressive therapies on transplant patients PBMCs mitochondrial respiration by complex II (OXPHOS CII). -/+ represents the group without or with specific treatment. n: number of patients in each group. Values are means ± SEM (a) Cellcept. (b) Everolimus (c) Ciclosporin (d) Corticoid.

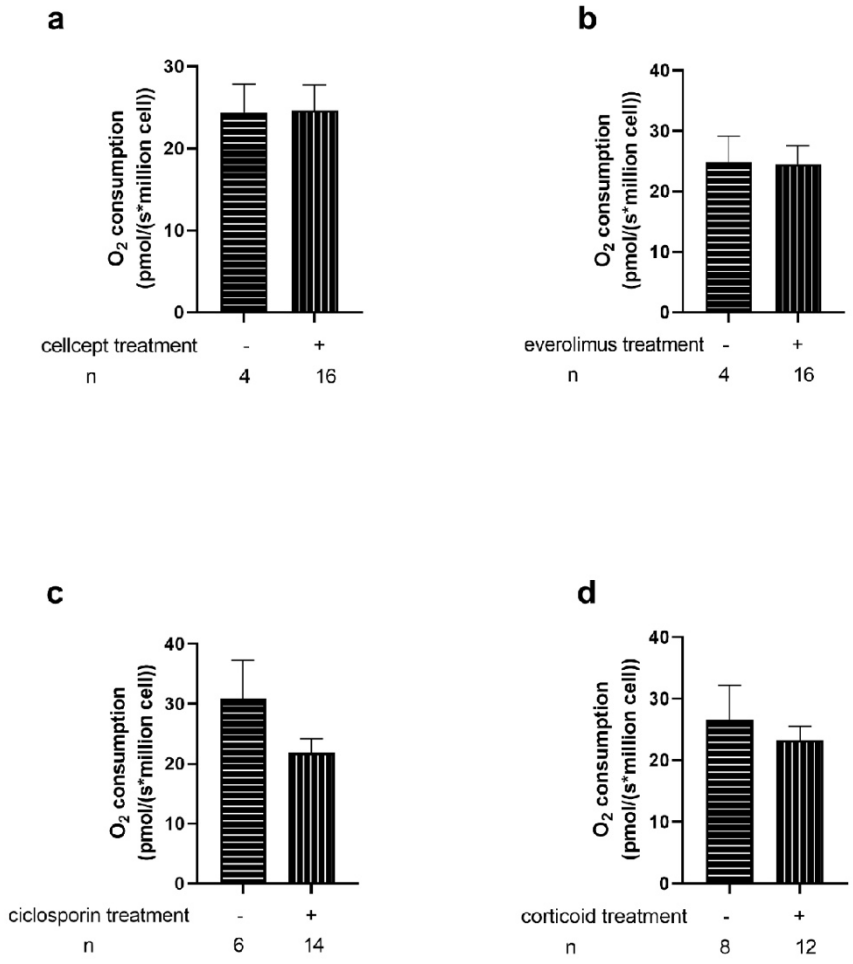

Figure 8. Effects of immunosuppressive therapies on transplant patients PBMCs mitochondrial respiration by complex IV (OXPHOS CIV). -/+ represents the group without or with specific treatment. n: number of patients in each group. Values are means ± SEM. (a) Cellcept. (b) Everolimus (c) Ciclosporin (d) Corticoid.

4. Discussion

The main results of this study are that after heart transplantation, PBMC's mitochondrial respiratory chain complexes show significant decrease in complex II and increase in complex IV mitochondrial respirations. This was relatively unexpected in well-being Htx, but subclinical diastolic dysfunction might be involved in these changes. Additionally, lymphopenia and mild inflammation might favor complex II respiration decrease and acute rejection might be involved in complex IV stimulation.

The mitochondrial respiratory chain is composed by five complex and besides calcium handling and participation in apoptosis, its role is to create energy for the cells. This is a major issue, particularly in the heart, an organ needing high oxidative capacity allowing for permanent

systolic and diastolic activities. Cardio myocytes mitochondrial alterations are considered as part of heart failure pathophysiology and authors consistently reported decreases in cardiac mitochondrial complex respiration in several setting including dilated and ischemic cardiomyopathy [13,14]. However, the need for cardiac biopsy limits this approach, suggesting investigations in a surrogate marker.

In this view, PBMCs mitochondrial respiration appears particularly interesting since it just necessitates blood withdrawal and may reflect cardiac muscle alterations. Studies indicate that PBMCs may function as a feasible non-invasive novel biomarker of heart failure and surrogate for myocardial mitochondrial respiratory function [15,16].

PBMCs mitochondrial dysfunction was observed in heart failure patients, in relation with inflammation and the severity of the disease [7,9,17]. Interestingly, mitochondrial respiration of cardiomyocyte was reduced by 40 % in acute cellular rejection following heart transplantation [18], further supporting studies on PBMCs mitochondrial respiration in Htx. Indeed, impaired cardiac mitochondrial bioenergetic might be associated with impaired mitochondrial bioenergetic in PBMC [6], thus identifying novel check points in cardiac immune metabolism as potential therapeutic targets in post-transplant care.

To explore the mechanisms involved in the mitochondrial respiration changes observed in our cohort of Htx, we took into account clinical, biological and cardiovascular parameters, including the underlying pathology responsible for heart transplantation, the delay since transplantation, cardiac, coronary, rejection investigations and the different categories of drug given to the patients.

4.1. Decreased PBMCs Mitochondrial Respiratory Chain Complex II Respiration after Heart Transplantation.

Complex II, called succinate dehydrogenase (SDH), is the sole complex that does not pump protons across the inner mitochondrial membrane and has all of its subunits encoded by nuclear DNA [19,20]. In the electron transport chain, complex II reduces ubiquinone to ubiquinol and alterations might be related to mutations, which have been observed in cardiomyopathy [21]. Complex II phosphorylating activity decrease has been shown in patients with early-stage HF and might be related to reduced mitochondrial biogenesis or increased mitophagy per mononuclear cell [7].

Complex II deficiency being associated with cancer [22,23] and viral infection [19], we investigated a possible relationships between complex II respiration and the presence of cancer and the viral status in our Htx population. Complex II respiration was not specifically decreased in patients having developed cancer or CMV. Similarly, complex II respiration decline was not associated with increasing age [24], nor with the immunosuppressive regimen although both ciclosporin and MMF might impair mitochondrial respiration [25–27]. On the other hand, ciclosporin can be protective after binding to cyclophilin D, improving thus mitochondrial function and reducing ROS production and inflammation [28]. Further, potential deleterious effect might have been counterbalanced by mTOR inhibitors that rather improve the mitochondrial function of PBMCs and decrease the level of inflammatory markers [29]. This might also explain the lack of relationship between increased superoxide anion and decreased complex II respiration in our Htx's PBMC.Incidence of oxidative stress is likely mild in these

patients [30], but studies investigating whether the increase in superoxide anion production in Htx might mainly be related to mitochondrial dysfunctions and/or to enzymatic sources like NADPH or xanthine oxydases will be useful.

Interestingly, study of mitochondrial respiration of endomyocardium in Htx with cardiac allograft vasculopathy showed that maximally coupled respiration of mitochondrial complex I and II was significantly reduced [3]. Our data on PBMCs are in line with these results, albeit we did not find a clear correlation between vasculopathy and complex II respiration in our patients. This might be because many Htx were well-being with conserved left ventricular ejection fraction and no or only few vasculopathy signs. On the other hand, the significant negative correlation between Complex II respirations and the posterior wall peak early diastolic myocardial velocity suggest a role in diastolic function in Htx's PBMC mitochondrial respiration decrease.

Changes in blood cell count might also likely participate in the complex II alterations observed in Htx. Indeed, as observed in heart failure [6], the increased neutrophil/lymphocytes ratio might have led to decreased PBMC mitochondrial respiration. Such cellular switch with a relative lymphocytopenia related to inflammation and down-regulation of the immune system [31] could lead to a decrease in global PBMC mitochondrial respiration, since neutrophils poorly contribute to the oxygen consumption rate and cellular bioenergetics, as compared to lymphocytes [32]. Accordingly, we observed a correlation between complex II respiration and leucocyte number and, low number of lymphocytes tended to be associated with a low complex II-related mitochondrial respiration in Htx.

4.2. Increased PBMCs Mitochondrial Respiratory Chain Complex IV Respiration after Heart Transplantation.

Cytochrome C oxidase (COX) also known as complex IV, is the final enzyme of the electron transport chain system in mitochondria as it is the last electron acceptor [33,34]. This protein is considered as an important modulatory location for oxidative phosphorylation (mitochondrial respiration)"OXPHOS" since this is the location where over 90% of oxygen is consumed without the formation of ROS [35–37]. In addition, complex IV is known to modulate ROS production and diminish oxidative damage [38].

Thus, increased complex IV activity can be viewed as a compensatory mechanism for the decreased complex II respiration observed in Htx. It might also be related to statin treatment (generally associated with decreased inflammatory markers) since simvastatin increased complex IV mitochondrial respiration in PBMC, as compared to untreated controls in association with an increase in superoxide production [39].

The inverse correlation observed between isovolumic relaxation time and the activity of complex IV also suggest its implication in cardiac diastolic function but this will need confirmation albeit increased complex IV activity has been observed in cardiac ischemia [40]. Interestingly, the two patients presenting with cellular or humoral rejection during the study showed an increase in the activity of complex IV. Although, their number is insufficient to conclude definitively, this might be a compensatory activation of the immune response toward an anti-inflammatory effect [41].

Potentially giving coherence to the mitochondrial changes obserbed in this study, EL Mills et al. proposed that inhibition of succinate oxidation promotes an anti-inflammatory outcome [42].

Limitations of the study.

Although the number of patients included allow demonstrate changes in PBMC respiration after heart transplantation, a larger population would be useful to determine further the mechanisms involved. Particularly, the hypothesis of a potential link between complex IV stimulation and acute rejection need investigations likely through a multicenter study in view of its relatively low frequency.

5. Conclusions

After successful heart transplantation, PBMC's demonstrated a significant decrease in complex II and an increase in complex IV mitochondrial respirations, together with increased superoxide anion production. Although confirming data observed in cardiovascular diseases [43], these changes occur in well-being Htx. Subclinical diastolic changes might be involved and further, complex II respiration alteration likely relate to relative lymphopenia. Complex IV increase might also potentially relate to acute rejection. Both mitochondrial respiration changes might favor anti-inflammatory pathways and thus, studies are needed to determine whether PBMC's mitochondrial respiration might be potential markers of acute rejection and/or mild diastolic dysfunction after heart transplantation.

Author Contributions:

Funding:

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Strasbourg (CE-2016-91, 15 December 2016).

Informed Consent Statement: Informed consent has been obtained from the patients to publish this paper.

Data Availability Statement:

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Discussion

I. Cardiac Mitochondrial Function after Heart Transplantation

Researches implemented in mitochondrial function in human after transplantation are rare [135]. Cardiac mitochondrial respiration following heart transplant showed deteriorated oxidative capacity, with enhanced number of CD3 + lymphocytes in heart tissue [135].

In addition, Scheiber et al. performed a recent study on the effect of mild acute rejection (ACR) on mitochondrial function [136]. This study found that there is an association between mild ACR and mitochondrial respiration dysfunction[136]. Also, it has been shown that instabilities of mitochondrial bioenergetics of the heart plays a role in allograft rejection [135]. A study undertaken by Lichscheidt et al. evaluated the association between cardiac allograft vasculopathy (CAV) and mitochondrial function and conclude that mitochondrial respiration is reduced in patients with CAV [137]. All these studies performed endomyocardial biopsy to evaluate the mitochondrial function of the transplanted heart.

To date, analysis of mitochondrial function in PBMCs in human's adult following Htx does not exist. Therefore, our study sought to determine if alterations in mitochondrial respiration in PBMCs might be present and associated or not with heart transplant characteristics.

II. PBMCS and mitochondrial respiration.

Expected Characteristics of Biomarkers:

Biomarkers are defined as a biological molecule found in the blood stream, body fluids, or tissues which works as hallmarks or indicators of normal or pathological medical conditions that can be measured objectively, accurately and reproducibly [104,138,139]. Also, the association of biomarkers with biological characteristics and phenotypes in terms of prognosis, severity, and clinical aspects is one of the most important uses of biomarkers [140].

They are classified into:

a) diagnostic biomarkers, used to identify the presence of the disease or to recognize subjects with a disease subtype;
b) predictive biomarkers, used to detect which therapy a patient or a group of patients will respond to;
c) prognostic biomarkers, used to predict disease development

It is crucial to investigate for biomarkers that can help understanding the mechanism of disease, distinguishing the goal of treatment and evaluating success or failure of therapies [140].

Peripheral Blood Mononuclear Cells (PBMCs) as Biomarkers:

Peripheral blood mononuclear cells (PBMCs) represent a promising approach in research. They appeared as a functional and potential source of biomarkers as they are easy to collect, and because of the likelihood of detecting lower expressed proteins as well as they are a reliable model for patients' stratification [140].

Between the biological specimens used in the field of biomarker discovery, blood-derived samples have received special attention. Blood collection is straightforward and affordable, thus, it is making it an excellent source of biomarkers. Moreover, blood can be easily fractionated into several components, including plasma (or serum), buffy coat, which contain white blood cells (WBCs) and platelets, and red blood cells (RBCs). Plasma, serum and peripheral blood mononuclear cells (PBMCs), obtained from buffy coat, are the most often utilized blood fractions in biomarker discovery research [141].
Furthermore, PBMCs have two additional advantages: they are able to be cultured indefinitely and allows researcher to do stimulation tests such as a study of inflammatory response [140].

Many studies have shown that PBMCs, mimic and reflect the conditions of the surrounding tissue and an indicator of overall mitochondrial health, making them an excellent non-invasive are suitable source of biomarkers [139,142–144]. PBMCs can also be a source of

miRNA, mRNA, circRNA, and methylation markers [139]. The studies that have been proved PBMCs as a biomarker include Alzheimer's disease (AD) [145], amyotrophic lateral sclerosis (ALS) [140], obesity and weight loss studies [146], inflammatory disease [147,148], cancer [149–153] , cardiovascular disease [154–158], idiopathic pulmonary fibrosis [159].

Key studies that support the use of PBMCs mitochondrial respiration as a biomarker:

a) Cardiovascular disease:

On the recent review on PBMCs in cardiovascular disease, it has been shown that PBMCs could be used as a biomarker in cardiovascular disease [160].

In evaluating mitochondrial respiration of PBMCs on heart failure population, three studies have been recognized to support the use of PBMCs as a viable marker that linked to systemic inflammation , oxidative stress and/or severity of the disease [161–164].

Interestingly, only two studies on small group of pediatrics with single ventricle congenital heart disease (SV-CHD) patients prior to the transplantation accomplished by Garcia Anastacia et al. and Zegelbone et al ., noticed increased ROS level and decreased mitochondrial respiration in PBMCs [154,155,160]. Another study on pediatric patients with the same kind of disease undertaken Fontan palliation procedure, but mitochondrial function showed elevated respiration in PBMCs which correlated with heart failure in post-Fontan SV-CHD [165]. The small number of patients (less than five patients) may explain such discrepancies, urging for larger scale studies.

Nevertheless, these results signify that PBMCS could be used to stratify the risk of heart failure in single ventricle congenital heart disease [165].

b) Respiratory (lung) disease:

In a study of severe asthmatic patients, there was an increase in PBMCs mitochondrial respiration and the authors suggested this increase to participate in patients defence mechanism. However, further studies to be considered in order to determine if PBMCs could

be used as a biomarker in asthma [64]. Another recent study shows that mitochondrial respiration in PBMCs negatively correlate with the disease severity in pulmonary arterial hypertension (PAH) [166], however, the study also suggested further investigations on mitochondrial biogenesis of PBMCs in PAH patients.

c) Muscle Disease

Recent research in skeletal muscle investigated whether PBMC mitochondrial respiration might be applied as a biomarker of skeletal muscle mitochondrial respiration in young healthy men. The study concluded that in young healthy individuals, PBMCs seems to be not appropriate biomarker for muscle mitochondrial function. However, it suggested that PBMCs mitochondrial respiration might be beneficial as a biomarker to investigate PBMC mitochondrial function of muscle in pathological populations [167].

Additionally, Rose at al. conducted a comparative study of mitochondrial respiration of PBMCs and skeletal muscle fibres in female population. This study supports that the bioenergetic phenotype of PBMCs cannot summarize muscle mitochondrial function but may give an idea on the overall metabolic health. However, it need to be validated in a larger population [168].

d) Neurodegenerative disorders

Similarly, Schirinzi et al (2022), found that PBMC mitochondria in Parkinson's disease patients had an unusual pattern of respiration, with increased maximal and spare respiratory capacities. Respiratory changes are most likely a result of the increased energetic demand caused by the disease's clinical-pathological progression and the resulting compensatory adaptations. Such changes vary according to disease stage and neuropathological substrate; they are more prevalent in patients with biochemical markers of weakness. This study recognize PBMCs as a marker of central neuropathology as it allows for a better understanding of mitochondrial function in Parkinson's disease[169].

In addition, recent examination in spinal cord injury provides proof that less invasive clinical techniques for assessing mitochondrial respiratory capacity measured with PBMCs may be nearly equivalent to the gold standard method that uses skeletal muscle fibers for people with Spinal cord injury [170]. Thus, it confirms that PBMCs could be used as a surrogate biomarker for mitochondrial health in those patients.

In depressive patients, a study found that the mitochondrial respiratory activity in PBMCs was negatively correlated with the progression of depression and could define different descriptions of the depressive phenotype[171].

III. PBMCS, Mitochondrial Respiration, Inflammation and Oxydative stress after Heart Transplantation.

PBMCs mitochondrial respiratory chain respiration and heart transplantation.

Heart transplantation has launched as the treatment of choice for heart failure population who have significant symptoms despite receiving the most aggressive medical treatment. Survival among heart transplant recipients has increased because of advancements in immunosuppressive medicines that prevent rejection. Even after a successful transplant, mortality remains high. Mitochondrial dysfunction, generation of reactive oxygen spices (ROS) and inflammation play a critical role as an outcome after HTx.The main objective of this study was to better understand the pathophysiology of mitochondrial peripheral blood mononuclear cells PBMCs through mitochondrial respiration in heart-transplanted patient (Htx). Indeed, the oxidative phosphorylation (OXPHOS) appears to function as a cellular checkpoint. Normal cellular functions are represented by appropriate OXPHOS performance, while changed, non-adequate OXPHOS performance is a danger signal for the host cell [19].

Accordingly, during this study and for the first time to our knowledge, we found different responses in respiratory chain complexes in mitochondrial respiration of PBMCs in adult heart transplantation compared to control individuals matching age and gender.

The main results show that in comparison to control, in PBMCs of heart transplant patients, oxygen consumption by CytOx (complex IV) is significantly increased as well as ROS production while the activity of complex II is markedly decreased. This was relatively unexpected in well-being Htx, but subclinical diastolic dysfunction might be involved in these changes. Additionally, lymphopenia and mild inflammation might favor complex II respiration decrease and acute rejection might be involved in complex IV stimulation.

The mitochondrial respiratory chain is composed by five complexes and besides calcium handling and participation in apoptosis, its role is to create energy for the cells. This is a major issue, particularly in the heart, an organ needing high oxidative capacity allowing for permanent systolic and diastolic activities. Cardio myocytes mitochondrial alterations are considered as part of heart failure pathophysiology and authors consistently reported decreases

in cardiac mitochondrial complex respiration in several setting including dilated and ischemic cardiomyopathy [172,173]. However, the need for cardiac biopsy limits this approach, suggesting investigations in a surrogate marker.

In this view, PBMCs mitochondrial respiration appears particularly interesting since it just necessitates blood withdrawal and may reflect cardiac muscle alterations. Studies indicate that PBMCs may function as a feasible non-invasive novel biomarker of heart failure and surrogate for myocardial mitochondrial respiratory function [154,155].

PBMCs mitochondrial dysfunction was observed in heart failure patients, in relation with inflammation and the severity of the disease [163,164,174]. Interestingly, mitochondrial respiration of cardiomyocyte was reduced by 40 % in acute cellular rejection following heart transplantation [175], further supporting studies on PBMCs mitochondrial respiration in Htx. Indeed, impaired cardiac mitochondrial bioenergetic might be associated with impaired mitochondrial bioenergetic in PBMCs [161], thus identifying novel check points in cardiac immune metabolism as potential therapeutic targets in post-transplant care.

To explore the mechanisms involved in the mitochondrial respiration changes observed in our cohort of Htx, we took into account clinical, biological and cardiovascular parameters, including the underlying pathology responsible for heart transplantation, the delay since transplantation, cardiac, coronary, rejection investigations and the different categories of drug given to the patients.

Decreased PBMCs mitochondrial respiratory chain complex II respiration after heart transplantation.

Complex II, called succinate dehydrogenase (SDH), is the sole complex that does not pump protons across the inner mitochondrial membrane and has all of its subunits encoded by nuclear DNA [19,176]. In the electron transport chain, complex II reduces ubiquinone to ubiquinol and alterations might be related to mutations, which have been observed in cardiomyopathy [177]. Complex II phosphorylating activity decrease of PBMCs has been shown in patients with early-stage HF and might be related to reduced mitochondrial biogenesis or increased mitophagy per mononuclear cell [164].

In Addition, complex II deficiency being associated with cancer [52,178] and viral infection [19]. In our population, there are some patients who experienced those type of infections about 33 % CMV, 75% EBV, and 29% cancer. Thus, we investigated a possible relationship between complex II respiration and the presence of cancer and the viral status in our Htx population. Complex II respiration was not specifically decreased in patients having developed cancer or CMV. Similarly, complex II respiration decline was not associated with increasing age [179], nor with the immunosuppressive regimen although both ciclosporin and MMF might impair mitochondrial respiration [180–182]. On the other hand, ciclosporin can be protective after binding to cyclophilin D, improving thus mitochondrial function and reducing ROS production and inflammation [183]. Further, potential deleterious effect might have been counterbalanced by mTOR inhibitors that rather improve the mitochondrial function of PBMCs and decrease the level of inflammatory markers [184]. This might also explain the lack of relationship between increased superoxide anion and decreased complex II respiration in our Htx's PBMCs. Incidence of oxidative stress being likely mild in these patients [185].

Interestingly, study of mitochondrial respiration of endomyocardium in Htx with cardiac allograft vasculopathy showed that maximally coupled respiration of mitochondrial complex I and II was significantly reduced [186]. Our data on PBMCs are in line with these results, albeit we did not find a clear correlation between vasculopathy and complex II respiration in our patients. This might be because many Htx were well-being with conserved left ventricular ejection fraction and no or only few vasculopathy signs.

Next, we evaluated the secondary objective, which is to study the relationship between these circulating bloods and the different clinical parameters including biological, and echocardiography (Cardiac ultrasound) obtained during usual follow up. There was a significant negative correlation between Complex II respirations and tissue doppler imaging suggest a role in diastolic function in Htx's PBMC mitochondrial respiration decrease.

Changes in blood cell count might also likely participate in the complex II alterations observed in Htx. Indeed, as observed in heart failure [161], the increased neutrophil/lymphocytes ratio might have led to decreased PBMC mitochondrial respiration. Such cellular switch with a relative lymphocytopenia related to inflammation and down-regulation of the immune system [187] could lead to a decrease in global PBMC mitochondrial

respiration, since neutrophils poorly contribute to the oxygen consumption rate and cellular bioenergetics, as compared to lymphocytes [188]. Accordingly, we observed a correlation between complex II respiration and leucocyte number and, low number of lymphocytes tended to be associated with a low complex II-related mitochondrial respiration in Htx.

Increased PBMCs mitochondrial respiratory chain complex IV respiration after heart transplantation.

Cytochrome C oxidase (COX) also known as complex IV, is the final enzyme of the electron transport chain system in mitochondria as it is the last electron acceptor [12,24]. This protein is considered as the major regulatory location for oxidative phosphorylation (mitochondrial respiration)"OXPHOS" since this is the location where over 90% of oxygen is consumed without the formation of ROS [189–191]. In addition, complex IV is known to modulate ROS production and diminish oxidative damage [192].

Thus, increased complex IV activity can be viewed as a compensatory mechanism for the decreased complex II respiration observed in Htx to preserve function and reduce damage. It might also be related to statin treatment (generally associated with decreased inflammatory markers) since simvastatin increased complex IV mitochondrial respiration in PBMC, as compared to untreated controls in association with an increase in superoxide production [193].

Another explanation, it might be due to the low ATP/ADP ratio in heart transplant patients that happened due to the difference in energy demand and the supply. Because of this decreased ATP, CytOx structure switches from dimeric (relaxed state) to monomeric (active state) to fulfill the demand which results in an increased oxygen consumption. On the other hand, when ATP would be high enough, then it would have been bound to CytOx to keep the structure in dimeric form and thus a decreased respiration (called ATP inhibition of the enzyme) [194].

This switching in of dimeric to monomeric exposes the binding sites on each of the monomer of the enzyme as well, eventually an increased oxygen consumption. Since CytOx also pumps protons that take part in establishing the membrane potential, so this increase in respiration would result in increase in membrane potential. Another fact of

switching of the dimeric structure to monomeric could be apparent by TMPD. This compound has the ability to donate electrons to cytochrome oxidase not only via cytochrome c but also through its direct binding to cytochrome oxidase. So, when the enzyme is in monomeric condition due to less ATP/ADP ratio, this TMPD is stimulating and manifesting its effect (double fold) because the sites are exposed on each of the monomer.

The inverse correlation observed between isovolumic relaxation time and the activity of complex IV also suggest its implication in cardiac diastolic function but this will need confirmation albeit increased complex IV activity has been observed in cardiac ischemia [195].

Interestingly, the two patients presenting with cellular or humoral rejection during the study showed an increase in the activity of complex IV. Although, their number is insufficient to conclude definitively, this might be a compensatory activation of the immune response toward an anti-inflammatory effect [196].

Generally, increased mitochondrial respiration is observed in the compensatory adaptation phase fighting against disease progression, as detected by the mitochondrial respiration of PBMCs in patients with Parkinson disease.

Circulating Cell Ratios as Inflammatory Markers in Heart Transplant

Peripheral blood biomarkers such as blood cell ratios (MLR, NLR, PLR) are novel markers of inflammatory reactions activation and physiological stress and tend to play a significant role as a prognostic marker in oncological and cardiovascular diseases[197–199].

In cardiovascular disease , the majority of studies examining NLR and PLR for cardiac surgery found that these indicators had prognostic value prior to the surgery [198]. Thus, there are growing studies validating that high NLR values pre/post operation were linked with artrial fibrillation in patients undergoing coronary artery bypass grafting [199,200]. Another study, after Fontan surgery, indicated that increased NLR and PLR was associated with a greater degree of lymphatic malformations and may thus serve as a useful supplementary biomarker during follow-up[201].

Research in patients having trans-catheter aortic valve replacement found that a greater rise in NLR after valve implantation was linked with a worse survival rate [202]. In lung transplantation, during the follow-up, patients with a greater NLR had a higher incidence of acute rejection than others [203].

Recently, a study performed in patients after HTx investigated the role of NLR and PLR [198]. It concluded that the value of both NLR and PLR before HTx were associated with worse outcomes after heart transplantation [198]. However, the study explains this result by the use of steroids that augment neutrophils while decreasing lymphocytes, thus altering both NLR and PLR.

In our study, neutrophils to lymphocyte ratio (NLR), and leukocyte to lymphocyte ratio (LLR), are proposed as markers of inflammation, and they were increased in Htx as compared to the control group. These finding add to our understanding of mitochondrial dysfunction in circulating leukocytes that might suggest inflammatory action and immune dysfunction in the activation of mitochondrial respiration after heart transplant.

The Role of Oxidative Stress / Antioxidant System in Heart Transplant

Normally under physiological conditions, there is a balance between the generation of ROS and the antioxidant system [204]. Oxidative stress is described as an imbalance between the generation of free radicals and their removal by the antioxidant defence system [204]. When this happens, it leads to the damage of the vascular endothelium, low-grade vascular inflammation, and thus oxidative stress causes progressive endothelial injury in the coronary arteries[204]. All these features constitute the main point of cardiac allograft vasculopathy (CAV) progression.

Following HT, the antioxidant reserve becomes unstable, because of the significant increase in free radical generation and their metabolites associated with surgery, extracorporeal circulation, bouts of acute rejection, and CMV infection [204]. It should be noted that the long-term decline in antioxidant defence mechanisms post HT, can be partially attributed to the pro-oxidant effects of immunosuppressant's [204].

It is believed that after cardiac transplantation oxidative stress is reduced by improving heart function. Pérez et al. (2002) conducted a study to evaluate oxidative stress after heart transplantation. He found that the antioxidant enzymatic activity of superoxide dismutase (SOD) was decreased in HTx patients and there is still permanent oxidative stress in those patients [205]. Another study was consistent with this conclusion and demonstrated a significant long-term decline of antioxidant reserves in patients after successful cardiac transplantation [206].

In addition, Witman et al. (2012) performed a study on patients after heart transplantation to look for the vascular function and the oxidative stress associated with it. The study found that vascular function is decreased with greater time post-HTx (but not soon after transplantation) with free radicals implicated in this progression [207]. Increased ROS generation by cells, together with a reduction in antioxidant enzyme activity, may result in covalent oxidative alteration of lipids, proteins, and DNA[204].

Similarly, a very recent study performed in a group of patients with evidence of cardiac allograft vasculopathy identified that the increase in formation of ROS accompanied by a decrease in the capacity of the antioxidant defence was linked with the presence of CAV [204].

In our population, consistently, we found an increase in superoxide anion, but it might be interesting to determine whether this was associated with a decrease in antioxidant defense and/or with lipid, protein or DNA alterations.

Conclusion and Perspective

Conclusion and Perspective

After successful heart transplantation, PBMC's demonstrated a significant decrease in complex II and increase in complex IV mitochondrial respirations, together with increased superoxide anion production. Although confirming data observed in cardiovascular diseases, these changes occur in well-being Htx. Subclinical diastolic changes might be involved and further, complex II respiration alteration likely relates to relative lymphopenia. Complex IV increase might also potentially relate to acute rejection. Studies are needed to determine whether PBMC's mitochondrial respiration might be potential markers of acute rejection and/or mild diastolic dysfunction after heart transplantation.

It will be of interest in future investigations to study if the mitochondrial respiratory capacity differs between the various leucocyte species. Moreover, in future studies, mitochondrial morphology could be investigated in PBMCs to gain further knowledge about the underlying causes of the differences in the mitochondrial respiration observed in the present study. Investigating in more details the pro- antioxidant balance in link with mitochondrial respiration might also be interesting, particularly performing the same type of study in patients presenting with different degrees of rejection.

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Long Summary in French

Introduction :

Les maladies cardiovasculaires, et en particulier l'insuffisance cardiaque, sont la principale cause de mortalité dans le monde, leur prévalence augmentant progressivement avec l'allongement de l'espérance de vie. Par conséquent, la transplantation cardiaque reste le traitement de choix et le moyen le plus efficace de remédier aux défaillances terminales des organes, ce qui permet d'améliorer considérablement la qualité et la durée de vie. Malgré son efficacité, la transplantation est cependant vulnérable à une morbidité importante et toute amélioration thérapeutique serait la bienvenue.
Des études montrent que la pathophysiologie des maladies cardiovasculaires comprend un dysfonctionnement des mitochondries entraînant une production insuffisante d'énergie cellulaire et une libération accrue d'épices réactives de l'oxygène . Il est intéressant de noter que les PBMC sont susceptibles de participer à cette maladie systémique. Des recherches récentes sur le sepsis suggèrent que le profil bioénergétique des PBMC circulantes peut représenter l'activité mitochondriale dans les organes et est associé à la gravité de la maladie, aux changements immunologiques et au pronostic . En général, les PBMCs dépendent de la respiration mitochondriale pour répondre aux besoins métaboliques et sont facilement accessibles. Un minuscule volume de sang est prélevé pour avoir accès aux cellules mononucléaires du sang périphérique (PBMC), qui permettent d'étudier la fonction mitochondriale. Les cellules PBMC sont composées de lymphocytes, de monocytes et de cellules dendritiques, qui sont principalement impliqués dans l'immunité et l'inflammation . De plus, le dysfonctionnement du cœur de la production d'ATP (qui est le système de phosphorylation oxydative OXPHOS) a une conséquence cruciale dans la progression d'une maladie, particulièrement dans le cœur qui a des demandes énergétiques élevées. A ce jour, il n'existe aucune donnée concernant la respiration mitochondriale des PBMCs chez les patients transplantés cardiaques (Htx), ce qui finance notre approche.

Problématique, objectifs, et Hypothèse de l'étude :

Mes recherches préliminaires, basées sur des revues de la littérature, ont démontré un lien entre les maladies cardiovasculaires et le dysfonctionnement mitochondrial des PBMC associé à une formation accrue de ROS, mais les données sont relativement limitées.

Pour la première fois, notre étude examinera s'il existe un changement dans la fonction mitochondriale au niveau des cellules mononucléaires du sang périphérique chez les patients ayant subi une transplantation cardiaque et, les mécanismes derrière ces éventuels changements. Nous avons émis l'hypothèse que la dysfonction diastolique cardiaque subclinique et/ou le rejet, modulés par les thérapies immunosuppressives, pourraient être impliqués dans la modulation mitochondriale.

Les objectifs :

1. Cette étude vise à déterminer la fonction mitochondriale dans les cellules immunitaires du sang périphérique, ainsi que la formation d'espèces réactives de l'oxygène dans le sang veineux de patients ayant subi une transplantation cardiaque.

2. L'objectif secondaire est d'étudier la relation potentielle entre la respiration mitochondriale des PBMC et les caractéristiques cliniques, biologiques, échocardiographiques et coronarographiques des Htx obtenues lors du suivi habituel après la transplantation cardiaque.

Hypothèse :
"Une altération de la respiration mitochondriale des cellules sanguines circulantes, associée à une augmentation de la production d'espèces réactives de l'oxygène dans la population des transplantés cardiaques, pourrait dépendre principalement des caractéristiques cardiaques et/ou systémiques du patient.

Cette étude apportera donc un nouvel éclairage sur la respiration mitochondriale des PBMCs (OXPHOS dans les cellules eucaryotes) et une meilleure connaissance physiopathologique de la fonction mitochondriale dans la population des transplantés cardiaques.

Population et méthodes : nous avons déterminé la respiration mitochondriale des PBMCs par respirométrie haute résolution (Oroboros Instruments) et la production d'anions superoxydes par résonance paramagnétique électronique (Bruker-Biospin) chez 20 sujets

sains et 20 Htx appariés et avons étudié les caractéristiques cliniques, biologiques, échocardiographiques, coronarographiques et biopsiques.

L'analyse de la fonction mitochondriale se fait dans des chambres d'oxygraphie avec une température controlée à 37°C, grâce à un effet peltier et sous agitation continue. L'appareil est un oxymètre à haute résolution (Oroboros Instruments, Innsbruck, Autriche) présentant des électrodes de Clark dans chaque cuve d'analyse. Un logiciel spécifique (Oroborosdatlab) permet la mesure de la concentration d'oxygène au cours du temps et l'analyse de la vitesse de consommation d'oxygène dans chacune des chambres, reflétant ainsi l'activité de la chaîne respiratoire.

Les électrodes de Clark sont d'abord calibrées. Un protocole de « titration de substrats, découpleurs, et inhibiteurs » (SUIT) est utilisé pour activer les différents complexes de la chaîne respiratoire.

5×10^6 PBMC ont été introduits dans la chambre de l'Oxygraph-2k contenant 2,1 mL d'une solution tampon de Miro5+ Créatine.

La concentration en oxygène a été exprimée en µmol. L^{-1} et la consommation d'oxygène par les mitochondries en pmol $O_2.s^{-1}.10^6$ cellules .

La consommation de dioxygène a été analysée à l'aide du logiciel DatLab 8.4.3 (Oxygraph-2k; Oroboros Instruments, Innsbruck, Autriche). Un étalonnage en présence d'oxygène (air ambiant) était nécessaire avant chaque mesure.

Les membranes cellulaires ont été perméabilisées avec de la saponine (125 µg/mL), et le complexe I a été activé avec du glutamate (5 mM), et du malate (2 mM). Cette étape est destinée à soutenir le flux d'électrons à travers le complexe I (CI) du système de transport d'électrons (STE).

Ensuite, différents substrats et inhibiteurs ont été introduits dans la chambre de l'oxygraphe pour étudier les différents complexes de la chaîne respiratoire mitochondriale. Les différents réactifs ont été ajoutés dans l'ordre suivant :

- L'ADP (2 mM) a induit l'activation de l'ATP synthase (OXPHOS par CI). A cette étape, on parle de phosphorylation oxydative soutenue par les complexes I, III, IV, et V.
- le succinate (25 mM) a été ensuite introduit pour étudier l'OXPHOS par CI&II. Cet état permet de voir l'activité de tous les complexes I, II, III, IV, et V.
- La roténone (0,5 µM), qui inhibe le complexe I, permet d'analyser l'OXPHOSCII, c'est-à-dire l'activité des complexes II, III, IV et V.
- L'ascorbate/ TMPD (0,5 mM/0,5 mM) ont enfin été ajoutés pour activer le complexe IV= Cytochrome C oxydase.
Le résultat a été exprimé en pmol/s/10^6 cellules.

La mesure de l'anion superoxyde dans le sang veineux total a été réalisée par résonance paramagnétique électronique (RPE), E-scan, Bruker-Biospin, Rheinstetten, Allemagne) à 37 °C [62]. En bref, 1 mL de sang veineux a été conservé sur la glace afin de réaliser l'analyse 1 heure après le prélèvement. 25 µL de sang ont été mélangés avec la sonde de spin CMH (1-hydroxy-3-méthoxycarbonyl-2,2,5,5-tétraméthylpyrrolidine HCl, 200 µM). Le mélange a ensuite été introduit dans un tube capillaire RPE en verre (Noxygen Science Transfer & Diagnostics, Elzach, Allemagne), puis placé dans la cavité du spectromètre e-scan (Bruker, Rheinstetten, Allemagne) pour l'acquisition des données. La détection de la production de ERO a été réalisée avec les paramètres RPE suivants: champ central g = 3477,452 ; largeur de balayage 60 G ; puissance micro-ondes 21,85 mW ; amplitude de modulation 2,4 G ; constante de temps 40,96 ms ; temps de conversion 10,24 ms ; nombre de points de courbe de retard 6. Le signal RPE est proportionnel au nombre d'électrons non appariés. Le résultat a été exprimé en µmol/min.

Résultats :

Les **principaux résultats** sont de montrer que dans un contexte d'augmentation du stress oxydant circulant, on observe une altération de la respiration mitochondriale du complexe II, associée à une stimulation de la respiration mitochondriale du complexe IV. Ainsi la modulation de l'activité de la chaîne respiratoire est complexe dépendante.

L'analyse de l'ensemble des paramètres cliniques, biologiques et cardiovasculaires invasifs ou non (coronarographie et quantification d'un éventuel rejet par biopsie intra-myocardique)

montre peu de corrélation significative entre tous ces paramètres. Cependant, il s'avère que l'altération du complexe II est cohérente avec l'atteinte cardiaque observée dans l'insuffisance cardiaque ce qui pourrait correspondre à une inflammation systémique voire à une atteinte minime de la fonction diastolique des cœurs transplantés. La stimulation du complexe IV au contraire pourrait être vue comme un mécanisme compensateur permettant de maintenir une production énergétique adéquate chez les patients transplantés cardiaques.

La respiration mitochondriale du complexe II de la chaîne respiratoire des PBMCs était diminuée chez les Htx (4,69 ± 0,84 vs 7,69 ± 1,00 pmol/s/million de cellules chez les contrôles et les patients Htx, respectivement ; p=0,007) et la respiration du complexe IV était augmentée (24,58 ± 2,57 vs 15,68 ± 1,67 pmol/s/million de cellules ; p=0,0035). La production d'anions superoxydes était également accrue chez les Htx (1,47 ± 0,10 vs 1,15 ± 0,10 µmol/min ; p=0,041). Le rapport leucocytes/lymphocytes était augmenté chez Htx, dont le complexe II était corrélé au nombre de leucocytes (r=0,51, p =0,02) et à l'imagerie Doppler tissulaire (r= -0,62, p=0,005). Le complexe IV était augmenté chez les deux patients présentant un rejet aigu et corrélé négativement avec le temps de relation isovolumétrique de Htx (r= -0,45, p=0,045).

Discussion :

Les principaux résultats de cette étude sont qu'après une transplantation cardiaque, les complexes de la chaîne respiratoire mitochondriale des PBMC montrent une diminution significative des respirations mitochondriales du complexe II et une augmentation du complexe IV. Ceci était relativement inattendu dans le cadre d'une Htx de bien-être, mais une dysfonction diastolique subclinique pourrait être impliquée dans ces changements. De plus, la lymphopénie et une légère inflammation pourraient favoriser la diminution de la respiration du complexe II et le rejet aigu pourrait être impliqué dans la stimulation du complexe IV.

La chaîne respiratoire mitochondriale est composée de cinq complexes et, outre la gestion du calcium et la participation à l'apoptose, son rôle est de créer de l'énergie pour les cellules. C'est un problème majeur, en particulier dans le cœur, un organe nécessitant une capacité oxydative élevée permettant des activités systoliques et diastoliques permanentes.

Les altérations mitochondriales des cardiomyocytes sont considérées comme faisant partie de la physiopathologie de l'insuffisance cardiaque et les auteurs ont régulièrement rapporté des diminutions de la respiration du complexe mitochondrial cardiaque dans plusieurs contextes, y compris la cardiomyopathie dilatée et ischémique.

Cependant, la nécessité d'une biopsie cardiaque limite cette approche, ce qui suggère des recherches sur un marqueur de substitution.

Dans cette optique, la respiration mitochondriale des PBMCs semble particulièrement intéressante puisqu'elle ne nécessite qu'un prélèvement sanguin et pourrait refléter les altérations du muscle cardiaque. Les études indiquent que les PBMCs peuvent fonctionner comme un nouveau biomarqueur non invasif de l'insuffisance cardiaque et comme un substitut de la fonction respiratoire mitochondriale du myocarde.
Une dysfonction mitochondriale des PBMCs a été observée chez les patients souffrant d'insuffisance cardiaque, en relation avec l'inflammation et la sévérité de la maladie. Il est intéressant de noter que la respiration mitochondriale des cardiomyocytes a été réduite de 40 % dans le cas d'un rejet cellulaire aigu après une transplantation cardiaque, ce qui soutient les études sur la respiration mitochondriale des PBMCs dans l'Htx. En effet, l'altération de la bioénergétique mitochondriale cardiaque pourrait être associée à une altération de la bioénergétique mitochondriale dans les PBMC, identifiant ainsi de nouveaux points de contrôle dans le métabolisme immunitaire cardiaque comme cibles thérapeutiques potentielles dans les soins post-transplantation.

Afin d'explorer les mécanismes impliqués dans les modifications de la respiration mitochondriale observées dans notre cohorte de Htx, nous avons pris en compte des paramètres cliniques, biologiques et cardiovasculaires, notamment la pathologie sous-jacente responsable de la transplantation cardiaque, le délai depuis la transplantation, les investigations cardiaques, coronaires, de rejet et les différentes catégories de médicaments administrés aux patients.
Diminution de la respiration de la chaîne respiratoire mitochondriale du complexe II des PBMC après une transplantation cardiaque.

Le complexe II, appelé succinate déshydrogénase (SDH), est le seul complexe qui ne pompe pas de protons à travers la membrane mitochondriale interne et dont toutes les sous-unités sont codées par l'ADN nucléaire . Dans la chaîne de transport des électrons, le complexe II réduit l'ubiquinone en ubiquinol et les altérations pourraient être liées à des mutations, qui ont été observées dans la cardiomyopathie. Une diminution de l'activité phosphorylante du complexe II a été mise en évidence chez les patients atteints d'HF à un stade précoce et pourrait être liée à une biogénèse mitochondriale réduite ou à une mitophagie accrue par cellule mononucléaire.

La déficience du complexe II étant associée au cancer et aux infections virales, nous avons étudié les relations possibles entre la respiration du complexe II et la présence de cancer et le statut viral dans notre population Htx. La respiration du complexe II n'était pas spécifiquement diminuée chez les patients ayant développé un cancer ou un CMV. De même, le déclin de la respiration du complexe II n'était pas associé à l'augmentation de l'âge ni au régime immunosuppresseur, bien que la ciclosporine et le MMF puissent altérer la respiration mitochondriale. D'autre part, la ciclosporine peut être protectrice après s'être liée à la cyclophiline D, améliorant ainsi la fonction mitochondriale et réduisant la production de ROS et l'inflammation. De plus, l'effet délétère potentiel pourrait avoir été contrebalancé par les inhibiteurs de mTOR qui améliorent plutôt la fonction mitochondriale des PBMC et diminuent le niveau des marqueurs inflammatoires. L'incidence du stress oxydatif est probablement légère chez ces patients, mais il serait utile de mener des études pour déterminer si l'augmentation de la production d'anions superoxydes chez les patients atteints de Htx est principalement liée à des dysfonctionnements mitochondriaux et/ou à des sources enzymatiques telles que la NADPH ou les xanthine oxydases.

De manière intéressante, l'étude de la respiration mitochondriale de l'endomyocarde chez les Htx atteints de vasculopathie d'allogreffe cardiaque a montré que la respiration maximalement couplée des complexes mitochondriaux I et II était significativement réduite . Nos données sur les PBMCs sont en accord avec ces résultats, bien que nous n'ayons pas trouvé de corrélation claire entre la vasculopathie et la respiration du complexe II chez nos patients. Cela pourrait

s'expliquer par le fait que de nombreux Htx étaient bien avec une fraction d'éjection ventriculaire gauche conservée et pas ou peu de signes de vasculopathie. D'autre part, la corrélation négative significative entre la respiration du complexe II et la vitesse myocardique maximale diastolique précoce de la paroi postérieure suggère un rôle de la fonction diastolique dans la diminution de la respiration mitochondriale des PBMC des Htx.

Les changements dans le nombre de cellules sanguines pourraient également participer aux altérations du complexe II observées dans l'Htx. En effet, comme observé dans l'insuffisance cardiaque, l'augmentation du ratio neutrophiles/lymphocytes pourrait avoir conduit à une diminution de la respiration mitochondriale des PBMC. Un tel changement cellulaire avec une lymphocytopénie relative liée à l'inflammation et à la régulation négative du système immunitaire pourrait conduire à une diminution de la respiration mitochondriale globale des PBMC, puisque les neutrophiles contribuent faiblement au taux de consommation d'oxygène et à la bioénergétique cellulaire, par rapport aux lymphocytes. En conséquence, nous avons observé une corrélation entre la respiration du complexe II et le nombre de leucocytes et, un faible nombre de lymphocytes tendait à être associé à une faible respiration mitochondriale liée au complexe II chez les Htx.

Augmentation de la respiration de la chaîne respiratoire mitochondriale du complexe IV des PBMC après une transplantation cardiaque.

La cytochrome C oxydase (COX), également connue sous le nom de complexe IV, est la dernière enzyme du système de la chaîne de transport des électrons dans les mitochondries, car elle est le dernier accepteur d'électrons. Cette protéine est considérée comme un emplacement modulateur important pour la phosphorylation oxydative (respiration mitochondriale) "OXPHOS" car c'est l'endroit où plus de 90% de l'oxygène est consommé sans la formation de ROS . En outre, le complexe IV est connu pour moduler la production de ROS et diminuer les dommages oxydatifs.

Ainsi, l'augmentation de l'activité du complexe IV peut être considérée comme un mécanisme compensatoire de la diminution de la respiration du complexe II observée chez les Htx. Elle pourrait également être liée au traitement par statine (généralement associé à une diminution des marqueurs inflammatoires) puisque la simvastatine a augmenté la respiration mitochondriale du complexe IV dans les PBMC, par rapport aux contrôles non traités, en association avec une augmentation de la production de superoxyde.

La corrélation inverse observée entre le temps de relaxation isovolumique et l'activité du complexe IV suggère également son implication dans la fonction diastolique cardiaque, mais cela devra être confirmé, bien qu'une activité accrue du complexe IV ait été observée dans l'ischémie cardiaque.

Il est intéressant de noter que les deux patients présentant un rejet cellulaire ou humoral au cours de l'étude ont montré une augmentation de l'activité du complexe IV. Bien que leur nombre soit insuffisant pour conclure définitivement, il pourrait s'agir d'une activation compensatoire de la réponse immunitaire vers un effet anti-inflammatoire.

Donnant potentiellement une cohérence aux changements mitochondriaux observés dans cette étude, EL Mills et al. ont proposé que l'inhibition de l'oxydation du succinate favorise un résultat anti-inflammatoire .

Conclusion:

Mon travail, orienté par les revues de la littérature, a montré pour la première fois une altération de la fonction mitochondriale des cellules circulantes mononuclées chez les patients transplantés cardiaques par rapport à des sujets sains appariés.

Alors que les traitements immunosuppresseurs ne semblent pas avoir un effet majeur sur la diminution de la respiration mitochondriale du complexe II et la stimulation de la respiration mitochondriale du complexe IV, le contexte général inflammatoire objectivé par une production augmentée de radicaux libres et par des ratios neutrophiles/lymphocytes

augmentés pourraient participer à ces résultats. Bien que les deux patients en rejets lors de l'étude montrent une augmentation de l'activité du complexe IV, leur nombre est insuffisant pour conclure définitivement.

Il serait intéressant à l'avenir de réaliser une étude similaire chez des patients caractérisés par la présence d'un rejet cellulaire ou humoral. Cela pourrait supporter encore plus l'intérêt de l'analyse de ce marqueur dans l'évolution cardiovasculaire des patients transplantés et être un signe de rejet supplémentaire, facilitant ce diagnostic qui reste difficile de nos jours.

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Peripheral Blood Mononuclear Cells (PBMCs) Mitochondrial Respiration after Heart Transplantation (HTx) Potential links with Cellular Shift, Mild Diastolic Dysfunction Diagnosed using Echocardiography and/or Acute Rejection

Summary

The mitochondria are the main source of ATP and oxygen free radicals. A chronic cardiac pathology is accompanied by an alteration of the mitochondrial tissue respiration, linked to the severity of the disease. This diagnosis requires an invasive biopsy, but mitochondrial analysis on circulating Peripheral Blood Mononuclear Cells (PBMCs) could be an alternative, due to the reflection of organ damage on PBMCs. In heart failure, this analysis is little explored, but is not for heart transplantation.

In this prospective study carried out in heart transplant patients, superoxide anion is increased compared to the controls accompanied by an alteration of mitochondrial respiration linked to complex II, and a stimulation of complex IV. These results might have potential links with **cellular shift, mild diastolic dysfunction diagnosed using echocardiography and/or acute rejection.**

My work shows for the first time, an alteration in the mitochondrial function of PBMCs in heart transplant patients.

Abrar ALFATNI

Respiration Mitochondriale des Cellules Mononucléaires du Sang Périphérique (PBMCs) après une Transplantation Cardiaque (HTx) Liens Potentiels avec le Déplacement Cellulaire, le Dysfonctionnement Diastolique léger Diagnostiqué par Echocardiographie et /ou Rejet Aigu

Résumé

Les mitochondries sont la principale source d'ATP. Une pathologie cardiaque chronique s'accompagne d'une altération de la respiration du tissu mitochondrial, liée à la sévérité de la maladie. Ce diagnostic nécessite une biopsie invasive, mais l'analyse des mitochondries sur les cellules mononucléaires circulantes du sang périphérique (PBMC) pourrait être une alternative, en raison du reflet des lésions organiques sur les PBMC.

Dans cette étude prospective réalisée chez des patients transplantés cardiaques, l'anion superoxyde est augmenté par rapport aux témoins accompagné d'une altération de la respiration mitochondriale liée au complexe II, et d'une stimulation du complexe IV. Ces résultats pourraient avoir des liens potentiels avec le déplacement cellulaire, une légère dysfonction diastolique diagnostiquée par échocardiographie et/ou un rejet aigu.

Mon travail montre pour la première fois, une altération de la fonction mitochondriale des PBMCs chez les patients transplantés cardiaques