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PhD THESIS

EVALUATION OF L-ARGININE/NITRIC OXIDE METABOLIC PATHWAY IN ERYTHROCYTES IN RELATION WITH OXIDATIVE STRESS: FOCUS ON DIFFERENT CARDIOVASCULAR DISEASES

BIO/12

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1. Abstract

Background: A decreased nitric oxide (NO) bioavailability and an increased oxidative stress play a pivotal role in different cardiovascular pathologies. Recent studies have shown that red blood cells (RBCs) participate in NO formation in the bloodstream.

Aim: The aim of this study was to assess the L-arginine (Arg)/NO pathway and the oxidative stress status in RBCs and in plasma of patients with microvascular angina (MVA), investigating similarities and differences with respect to coronary artery disease (CAD) patients or healthy controls (Ctrl).

Materials and Methods: Analytes involved in Arg/NO pathway and the ratio between the oxidized and the reduced forms of glutathione, as index of oxidative stress, were measured by liquid-chromatography tandem mass spectrometry (LC-MS/MS). The arginase and the NO synthase (NOS) expression were assessed by immunofluorescence staining. NOS activity was evaluated by *ex-vivo* experiments through the conversion of L-[$^{15}N_2$]arginine to L-[^{15}N]citrulline.

Results: Both MVA and CAD patients showed alterations in the ability of RBCs to produce NO, based on an increase of NO synthesis inhibitors, parallel to that found in plasma, a reduction of NOS expression and activity and an increased arginase expression. When summary scores of NO synthesis and of oxidative stress were computed, both patient groups were associated with a positive oxidative score and a negative NO score, with the CAD group located in a more extreme position with respect to Ctrl.

Conclusions: This finding points out to an impairment of the capacity of RBCs to produce NO in pathological conditions characterized by alteration at the microvascular bed with/without no significant coronary stenosis.

2. Introduction

Biochemical premises

2.1. Nitric oxide

Nitric oxide (NO) is an intra- and intercellular signaling molecule that plays important roles in many physiological and pathological processes, including vasodilatation, neuronal transmission, immunomodulation, cardiac contraction, inhibition of platelet aggregation, stem cell differentiation and proliferation [1-7]. The discovery of its protective role in the cardiovascular system dates back to 1867 [8] but for over one hundred years NO was considered as a toxic gas. Until the early '80's, the critical role of NO in the cardiovascular system was identified, and the Nobel Prize in Physiology or Medicine was awarded to Drs. Robert F. Furchgott, Louis J. Ignarro and Ferid Murad for their seminal discoveries of NO as a signaling molecule in blood vessels [9]. Since the identification of NO as the endothelium derived relaxing factor, numerous other protective properties of NO in the cardiovascular system have been characterized.

NO is produced endogenously, in equimolar amounts to L-citrulline (Cit), through the action of NO synthases (NOSs) from the substrate L-arginine (Arg). It has a half life of seconds in biological systems and it is highly diffusible, allowing it to quickly target adjacent cells. There are three mammalian NOS isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS), identified on the basis of their relatively distinct primary amino acid sequences (only 50–60% identity), tissue and cellular distribution, and mode of regulation [10, 11]. These enzymes are highly homologous and have a common dimer structure; each monomer contains a reductase and an oxygenase domain, implicating the importance of molecular oxygen [12]. The reductase domain encloses binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and nicotinamide adenine dinucleotide phosphate oxidase (NADPH). The oxygenase domain contains a heme center and binding sites for Arg and tetrahydrobiopterin (BH_4), the principal NOS cofactor.

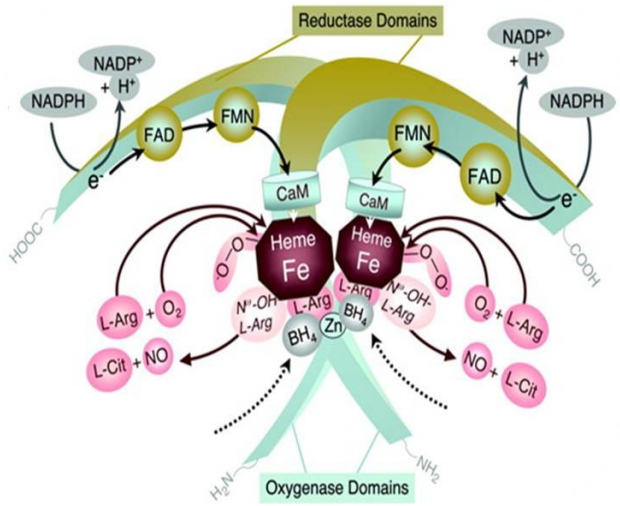


Figure 1: Schematic representation of NOS enzyme (modified from Förstermann U, et al. Circulation 2006;113:1708-1714).

The main difference between the three isoforms is related to their Ca^{2+} dependence: nNOS and eNOS, the constitutive isoforms, are Ca^{2+}-dependent and require elevated levels of intracellular Ca^{2+} to become activated, whereas iNOS does not [13].

The distinct properties of each NOS isoform have important implications since it is the magnitude, duration, and the cellular sites of NO production that determines its overall physiological or pathophysiological effect. For example, the release of NO from the constitutive isoforms of NOS is localized and acts transiently, playing in this way a crucial role in the cardiovascular and renal system, where it controls organ blood flow distribution, inhibits platelet aggregation and adhesion to the vascular wall, limits leukocyte adhesion and smooth muscle cell proliferation, promotes diuresis and natriuresis with the kidney [10, 11] and is involved in neurotransmission [1, 2]. Most of these actions are mediated through the binding of NO to Fe^{2+} in the heme prosthetic group of soluble guanylate cyclase, which catalyzes the conversion of GTP to cyclic GMP [10, 11]. In contrast, iNOS is expressed in a wide variety of host defense and other cells in response to inflammatory stimuli, such as endogenous cytokines and bacterial lipopolysaccharide endotoxin (LPS), resulting in a delayed (in the order of hours) but prolonged synthesis of high levels of NO. It is now well accepted that, while NO released from iNOS is biologically appropriate, it is simultaneously involved in many pathological events.

NO is present in the cell under different forms, and between them as nitrite and nitrate. As regard the first compound, it has been recently demonstrated that nitrite, previously recognized as an inert, stable end-product of NO metabolism, can actually serve as an important substrate for NO production through non enzymatic reduction, via deoxyhemoglobin [14], or through enzymatic reaction of oxidoreductases such as xanthine oxidoreductase [15-17]. Nitrite plasma concentration ranges from 50 to 300 nM but, to achieve a vasodilation effect, a higher dose of this molecule (about 180 μM) is required [14]. Under physiological condition, in fact nitrite does not function as an NO storage pool; but, under hypoxic condition, this molecule reacts with deoxyhemoglobin and releases NO for vessel dilation through the following reaction: NO_2^- + $HbFe^{2+}$ (deoxyhemoglobin) + $H^+ \rightarrow HbFe^{3+}$ (methemoglobin) + NO + OH^- [14]. A high concentration of methemoglobin in plasma will reverse the reaction direction, decreasing NO generation.

As nitrite, also nitrate derive by two major sources: from the reaction of NO with oxyhaemoglobin, and from diet. Normal plasma levels of nitrate are in the 20–40 μM range [18-21], and during regular exercise, the increased eNOS expression and activity also results in higher circulating levels of these compounds [22-24]. Dietary nitrate intake instead is considerable and many vegetables are particularly rich in this anion [25] (for example, a plate of green leafy vegetables such as lettuce or spinach contains more nitrate than that is formed endogenously over a day by all three NOS isoforms combined [26]). Drinking water can also contain considerable amounts of nitrate, although in many countries the levels are strictly regulated. Nitrate can also be found in some food stuffs, most notably as a preservative in cured meat and bacon.

NO largely functions through its interactions with heme moieties also in a variety of enzymes, one of these is soluble guanylyl cyclase (sGC). The binding of NO to the heme in sGC activates the enzyme to produce cGMP which is the second messenger responsible for the vasodilation associated with NO. NO can also bind to cysteines and thiols through nitrosation reactions and these modifications are important post-translational changes that alter and regulate protein function. In pathophysiological states, NO reacts with reactive oxygen species (ROS) to form peroxynitrite ($ONOO.^-$) and other reactive nitrogen molecules, leading to nitration reactions that then mediate cell injury and death. Nitrotyrosine is one such product of nitration from peroxynitrite; it is associated with numerous pathologic conditions and has been found in diseased tissues.

In the cardiovascular system, NO has been shown to exert many vasculoprotective roles: specifically, it shows protective effects on vascular smooth muscle cells (VSMCs), endothelial cells, platelets and inflammatory cells.

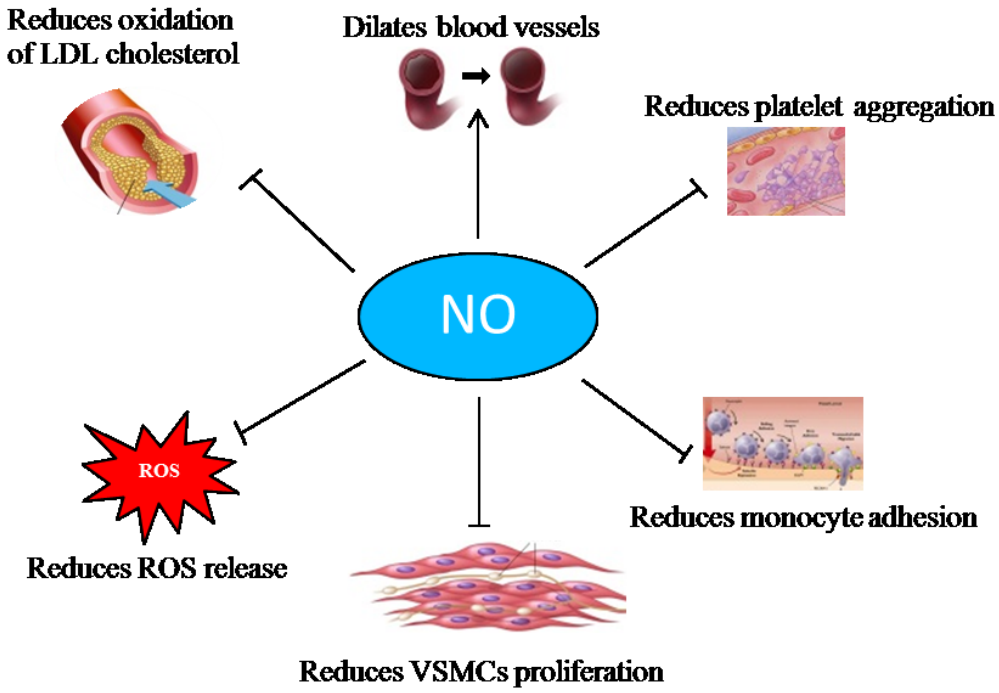


Figure 2: Principal NO functions in the cardiovascular system.

The inhibitory effect of NO on VSMC proliferation was first reported in 1989 by Garg et al., who uses three NO donors to evaluate the process in a dose-dependent manner [27]. The ability of these donors to block proliferation was reversed when hemoglobin (Hb), a strong NO scavenger, was added. This potent vasodilator has also been shown to inhibit VSMC migration through both cGMP-dependent and -independent pathways [28, 29]. As regards endothelial cell proliferation and migration, the first evidence of the involvement of NO has been reported in 1994 [30]. Three NO donors enhanced proliferation and migration of coronary venular endothelial cells. In contrast, these promoting effects were reversed by the NOS inhibitor. In addition in 1991, Kubes et al. reported that NO could inhibit leukocyte and platelet adhesion to vascular endothelium [31].

NO suppresses also platelet aggregation. In 1983, Mellion et al. demonstrated that, when human platelets were stimulated with agents such as ADP, collagen, U46619, or sodium arachidonate, NO donors inhibited platelet aggregation in a dose dependent manner. In

contrast, this anti-aggregatory effect was attenuated when methemoglobin, a hemoprotein with a high affinity for NO, was present [32]. Concomitantly, the NO donors also induced a rapid and marked increase in the cGMP production. This increase was partially reversed by hemoproteins. Meanwhile, cAMP levels remained unchanged. Moreover, sGC purified from platelets was significantly activated by NO donors. This anti-aggregatory effect of NO was also confirmed by McCall et al. [33].

The main source of NO is the vascular endothelium but, during the last decade, the discovery of the endothelial NOS in RBCs generate a great interest in these circulating cells.

2.2. The red blood cell

It is well established the ability of RBCs to transport oxygen (O_2) between respiratory surfaces and metabolizing tissues by means of their high intracellular concentration of Hb. In addition these cells, when pass through the microcirculation, may sense tissue O_2 conditions via their degree of deoxygenation and couple this information to the release of vasodilator compounds, such as ATP or NO, that enhance blood flow in hypoxic tissues.

To explain how erythrocytes play a role in vessel regulation by preserving endothelial-derived NO bioactivity different models have been proposed [14, 34-40]. They are based essentially to the presence of a RBC-free zone near the endothelium as a consequence of the pressure gradient created by blood flow and of a NO concentration gradient surrounding the erythrocyte, known as unstirred layer, that was created by the rapid reaction between NO and Hb. This latter phenomenon limits the access of new NO generated by the endothelium into the RBC [41, 42]. One of the main players in these models is the erythrocytic Hb, able to sense changes in O_2 concentrations and to modulate NO levels. Regarding this molecule, in 1996 the Stamler's group formulated the "SNOHb Hypothesis" stating that, rather than simply inhibiting NO activity through the dioxygenation reaction, Hb is capable of preserving, transporting, and exporting NO activity [34]. A central point of this hypothesis is that NO, bound to the heme of Hb in the T-state (low-oxygen affinity), is transferred to the β93Cys when the Hb undergoes T to R (high-oxygen affinity) transition, forming SNOHb. This theory helps to explain a dynamic role of RBC in controlling blood vessel tone, with hypoxia releasing NO and causing vasodilation and hyperoxia scavenging NO and constricting blood vessels. However the SNOHb hypothesis, which conceptually links NO

bioavailability to Hb, has been debated intensively some years later [37, 39]. In addition, a more recent work demonstrated that the allosterically controlled transfer of NO from heme to the β93Cys does not occur under normal conditions; thus, a defect in this process does not contribute to pathologic states [43]. Also the loss of the highly conserved β93cys residue does not affect isolated red cell dependent hypoxic vasodilation in two knockin mouse models [40].

Another model proposed hypothesizes that RBCs can reduce naturally occurring nitrite to vasoactive NO via the nitrite reductase activity of deoxyHb [14, 38]. Instead of the red cell picking up of NO produced by the endothelium, this hypothesis takes into consideration the nitrite produced by the oxidation of NO in the plasma compartment. The reaction between nitrite and deoxyHb results in an intermediate with properties of $Hb(II)NO^+$ and Hb(III)NO. The $Hb(II)NO^+$ can react with GSH or produce SNOHb, whereas the Hb(III)NO can release NO. As the blood is oxygenated in the lungs, some of these intermediates decompose, limiting the build-up of reactive NO species. Regarding this hypothesis, a work published by Vitturi and coworkers showed that in human RBCs the deoxyHb mediated transport of nitrite is regulated by the anion exchanger (AE)-1, which represents the 25% of RBC membrane protein [44]. They proposed a model in which the binding of deoxyHb to AE-1 inhibits nitrite export under low oxygen tensions, allowing the coupling between deoxygenation and nitrite reduction to NO along the arterial-to-venous gradient.

In addition to these functions, in order to control systemic NO bioavailability, RBCs are also able to release ATP in response to different stimuli, including exposure to reduced O_2 tension, mechanical deformation, β-adrenergic receptor agonists, and prostacyclin analogs. These stimuli activate a signal transduction pathway mediated by different G proteins and the identity of the final ATP conduit appears to depend on the initiating stimulus. In fact Pannexin 1, a protein known to form a channel capable of serving as an ATP conduit in other cell types, is involved in the release of ATP from erythrocytes in response to exposure of cells to lowered O_2 tension [45-47], while VDAC 1 must serve as a channel for ATP release induced by prostacyclin receptor activation [48]. The finding that different conduits for ATP release are components of discrete erythrocyte signalling pathways insinuates the idea that the selective activation of these pathways is important in vascular regulation, in order to eventually develop new strategies for the treatment of vascular disease.

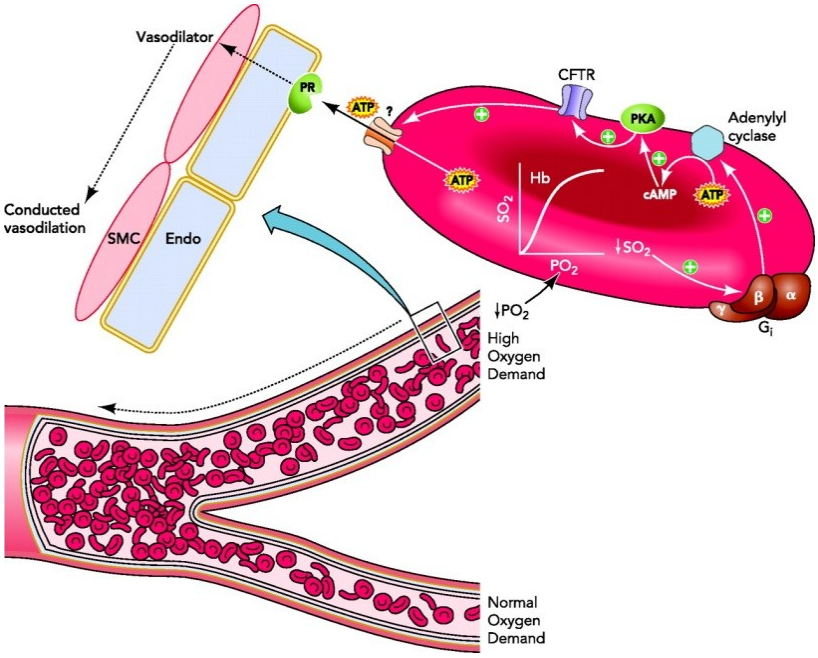


Figure 3: RBC functions as O_2 sensor in the vessel wall.

2.2.1. Nitric oxide synthase in red blood cells

In addition to its central role in regulating vascular homeostasis, the RBC is also able to produce NO endogenously as it express a functional NOS, similar to the endothelial isoform [49]. The presence of a NOS-like activity in RBCs had been a matter of controversy for some time and doubts about its functional significance and isoform identity had been put forward by different authors, probably as a consequence of variable experimental conditions and methodological shortcomings [50-52]. Once demonstrated the presence of a functional NOS in RBCs, the enzyme has been carefully studied and characterized [49, 53, 54]. It responds to a variety of specific regulatory pathways of eNOS, as it is stereospecifically stimulated by the substrate Arg, it is sensitive to insulin and to the common NOS inhibitors. The modulation of its activity depends on the intracellular calcium level and on the phosphorylation at serine 1177 which is regulated by the Akt-phosphatidylinositol-3' -kinase. In addition, RBCs carry important enzymes and molecules involved in the Arg metabolism, such as arginase, transforming the NOS substrate Arg to L-ornithine (Orn) [55], NO synthesis inhibitors asymmetric and symmetric dimethylarginines (ADMA and SDMA) [56], and monomethyl arginine (MMA) and the cationic amino acid transporters [57]. The presence of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme able to convert ADMA to Cit, is still controversial [58, 59].

An important open question is to understand how NO formed by the enzymatic activity in these cells can escape the irreversible and rapid dioxygenation reaction with oxyHb. An attempt to answer this question was made by Gladwin who identified a "metabolon complex" formed by deoxyHb, AE/band3, carbonic anhydrase, aquaporin and Rh-protein channels that could facilitate the export of NO or of its metabolites [60]. The localization of RBC NOS-immunoreactivity on the cytoplasmic side of the RBC membrane [49, 61] supports the important role of this compartment in effectively separating NO production, signaling and scavenging machinery. The RBC submembrane cytoskeleton, in fact, possesses a complex three dimensional structure which may force NO and other small molecules to traverse tortuous channels or pores to access intracellular Hb [62].
The submembrane cytoskeleton barrier is also involved in slowing the reuptake of NO that escapes into the plasma [63, 64].

2.2.2. Arginase enzyme in erythrocytes

RBCs can regulate the NO production by controlling the availability of substrate [62]. Arginase is the final enzyme in the urea cycle responsible for the detoxification of ammonia in the liver of higher animals. It utilizes Arg as a substrate, converting it into Orn and urea by hydrolytic cleavage of the guanidino group from the end of the molecule. There are two enzymatic isoforms, designated AI and AII, that are expressed in the liver and predominantly in kidney and prostate, respectively. The discovery of arginase in human RBCs dates back to '80s [65], but only many years later the evidence of the presence of the isoform I in mature RBCs has been performed [55]. As this enzyme competes with NOS for the common substrate it could be important in the cardiovascular system, where vascular tone and function depend on NO derived from eNOS activity. The direct comparison of the K_m values could suggest that the high K value of arginase (1-20 mM) compared with the low K value of NOS (1-5 μM) should not allow a real competition between these enzymes. However a better evaluation should also take into consideration the rate of catalysis for each enzyme. Arginase has a V_{max} of 1,400 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, while NOS has a V_{max} of 900 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Based on the kinetic analysis of these two enzymes, the relative activity of NOS to arginase, in terms of consuming Arg, is diminished with either increasing Arg concentration or decreasing NOS to arginase molar ratio. Therefore, arginase activity can exceed NOS activity at higher levels of substrate or at higher arginase to NOS molar concentrations. The competition between NOS and arginase for Arg is more pronounced at lower levels of substrate.

As the existence of this mutual influence, the relationship between these enzymes in RBCs has been studied in different pathological conditions. For example, an *in vitro* study conducted in order to elucidate a possible mechanism by which packed RBC transfusions influence immunomodulation by T cell suppression, evidenced increased arginase activity, low Arg concentrations and normal to high Orn levels in the fluid fraction of packed RBCs. These effects were prevented with arginase inhibitor treatment, suggesting this enzyme as responsible to the alteration in T cell proliferation [66].

Up to now, however, few data are reported on cardiovascular disease. The role of arginase 1 in the control of NOS function in RBCs has been recently demonstrated by Yang et al. [67]. They showed, using an animal model of myocardial ischemia reperfusion injury, that the use of arginase inhibitors significantly improved post ischemic functional recovery of heart if administered in whole blood or in plasma added with RBCs but not with buffer solution or plasma alone. Moreover, hearts from $eNOS^{-/-}$ mice were protected when the arginase inhibitor was given with blood from wild type donors. These results strongly support the notion that RBCs contain functional NOS and release NO-like bioactivity and that this process is under tight control by arginase 1 and is of functional importance during ischemia-reperfusion.

2.3. The vascular endothelium and its alterations: endothelial dysfunction

The vascular endothelium, a monolayer of cells between the vessel lumen and the VSMCs, provides not only a physical barrier, but also performs a critical function for the maintenance of blood pressure by releasing vasorelaxing (EDRF) and contracting factors (EDCFs). It is well known, in fact, that the stimulation of endothelial cells by neurotransmitters, hormones, substances derived from platelets, and mechanical shear stress causes the release of different molecules, according to cell conditions. The main vasorelaxing factor is NO; however, it has been recognized that there are several types of EDRFs, including PGI_2, which induce the relaxation of proximal VMSC through its own pathway. EDCFs include endothelin-1, angiotensin II, thromboxane A2 (TXA_2), prostaglandin H2, and ROS, all of which cause vascular contractions as well as various other functions.

It is well established that vascular endothelium has a pivotal role in the modulation of vascular function and structure, mainly through the formation of NO. Thereby, many

pathophysiological states, mainly by reducing endothelium-dependent vasodilation, evoke endothelial dysfunction (ED), a pathological condition highly related with the development of atherosclerosis and with cardiovascular risk factors [68-70]. What is generally referred to as ED should more appropriately be defined in an early phase as endothelial activation, a switch from a quiescent phenotype toward one that involves an host defense response. The fundamental change involved in this process is a switch in signaling from an NO-mediated silencing of cellular processes toward activation by redox signaling. More specifically, ED is caused by an increase in ROS generation (free oxygen radicals, oxygen ions and peroxides) and a reduction of NO bioavailability in vascular endothelium. At moderate concentrations in fact ROS act as signaling molecules and play an important role in the regulation of vascular tone, oxygen sensing, cell growth and proliferation, apoptosis, and inflammatory responses [71]. In contrast to these regulatory functions under physiological conditions, excessive or sustained ROS production, when exceeding the available antioxidant defense systems, leads to oxidative stress, a process mediated by several mechanisms. Initially, superoxide anions react with existing NO producing peroxynitrite. In addition, ROS reduce the enzymatic activity of eNOS through the oxidative degradation of its essential cofactor, BH_4. This phenomenon is known as eNOS "encoupling" and is characterized by an electron transfer to oxygen instead of Arg, generating superoxide rather than NO [72]. Another mechanism responsible for the reduction of NO is via the regulation of the levels of the NO synthesis inhibitor ADMA. ROS reduce also the enzymatic activity of dimethyl arginine dimethyl aminohydrolase (DDAH), which is crucial for the catabolism of ADMA, and up-regulate gene expression of protein methyl transferases, enzymes responsible for the methylation of Arg and for its conversion to ADMA, resulting in increased ADMA levels [73].

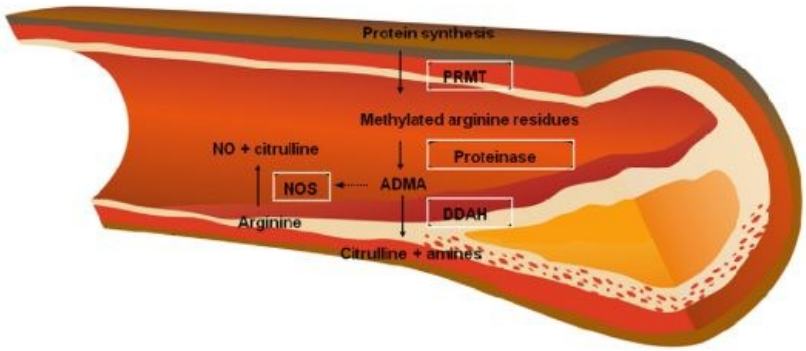


Figure 4: Mechanisms involved in decreased NO bioavailability (from Aldámiz-Echevarría L Int. J. Mol. Sci. 2012, 13(9), 11288-11311).

Furthermore, ROS can downregulate gene expression of eNOS and thus reduce even more NO synthesis [74]. Last, NO bioavailability can also be reduced by the nitration of G-proteins by peroxynitrite [75]. Furthermore, it is well established that oxidative stress leads to endothelial cell apoptosis in cell culture models because of the inhibition of antioxidant enzymes, e.g. superoxide dismutase (SOD), by a ROS-mediated mechanism [76, 77]. The excessive oxidative stress can harm even more the endothelial function [78] and some types of ROS, such as hydrogen peroxide, enhance endothelial cell apoptosis via the destruction of thioredoxin-1 [79].

Also vascular inflammation is highly associated with ED and plays a major role in the development of atherosclerosis, by increasing the expression of vascular adhesion molecule-1, intracellular adhesion molecule-1, E and P-selectin, which are all redox sensitive molecules [80]. Furthermore, it augments the secretion of many cytokines and other inflammatory molecules, which are controlled by redox-sensitive mediators (i.e. nuclear factor kappa-light-chain-enhancer of activated B cells) [80]. Therefore, there is an augmented accumulation and adhesion of leukocytes, mainly macrophages and T lymphocytes, in the sites of impaired endothelial function [81].

The vascular inflammation and the increased generation of ROS further promote the modification of circulating low density lipoproteins (LDLs) in oxidized LDLs (oxLDLs) [82, 83], which exert a crucial role in the pathophysiology of atherosclerosis [84, 85]. After oxLDLs are phagocytosed by macrophages, they turn them into foam cells and promote even more the generation of ROS [86]. OxLDLs also contribute to the development of ED by damaging endothelial cells and enhancing the expression of adhesion molecules (i.e. P-selectin) [87] and cytokines (i.e. chemoattractant protein-1 and macrophage colony stimulating factor) [88, 89].

2.3.1. Assessment of endothelial function

As ED is crucial in the pathophysiology of atherosclerosis and is a prognostic marker both in subjects at high cardiovascular risk and in those with established coronary artery disease (CAD), a wide range of methods are used to evaluate endothelial function. Between them, the most widely used non-invasive technique is the flow-mediated dilation (FMD). It is based on the physiology of blood flow regulation in large arteries: increased blood flow in conduit arteries causes an increase in shear stress and thus, by increasing NO production, leads to vasodilatation. This response is known as FMD or endothelium-dependent vasodilatation, because it is provoked by the capacity of vascular endothelium to produce

NO [90] and is expressed as the percentage change of the artery's diameter from the baseline size. Although FMD seems to be highly reproducible, there are some difficulties that need to be overcome in order to be used in routine clinical practice [91-93]. FMD is impaired by cardiovascular risk factors from the first ten years of life and decreases in proportion to risk factor burden [94]. Therefore, it has prognostic value in patients with CAD irrespectively from the angiographic severity of the disease [95-97]. In addition, it has a predictive role in cardiovascular risk in subjects with peripheral arterial disease [98] and in the general population as well [99]. FMD impairment can be quickly reversed by medical therapy, such as statin use, and by beneficial lifestyle changes [100].

2.4. Causes of endothelial dysfunction: oxidative stress

Oxidative stress, defined as the imbalance between oxidant and antioxidant factors, is mainly involved in different pathological processes and, between them, also in cardiovascular diseases. The important role of oxidative stress in cardiovascular pathophysiology has encouraged quantification of ROS as a promising biomarker reflecting the disease process. However, this has proven to be a complex challenge given the evanescent nature of these molecules. The short half-life of these species makes them excellent signaling molecules but confounds their measurement in the circulation of complex biological systems by standard approaches [101]. Instead the focus has been on measuring stable markers in the circulation that may reflect systemic oxidative stress, such as alteration in xanthine oxidase, eNOS and NADPH oxidase activities [102]. In this contest, the ratio between the oxidized and the reduced form of glutathione (GSSG/GSH), which is a recognized index of oxidative stress, can be used as a stable marker [103].

2.4.1. Glutathione

γ-L-glutamyl-L-cysteinyl-glycine, chiefly known as glutathione (GSH), is required for several cell processes interconnected with alterations in the maintenance and regulation of the thiol-redox status, due to its capability to exist in different redox species [104]. Under physiological conditions the reduced GSH is the major form, with a concentration 10 to 100-folds higher than the oxidized species (oxidized GSH, GSSG and mixed disulphide, GSSR). GSSG is predominantly produced by the catalysis of GSH peroxidase (GPX) as well as from the direct reactions of GSH with electrophilic compounds, e.g., radical species. The majority of GSH (almost 90%) is in the cytosol, which also represents the

main place for its synthesis; from cytosol, GSH is distributed into organelles such as mitochondria, nucleus and endoplasmic reticulum [104, 105].

GSH is the most important hydrophilic antioxidant that protects cells against exogenous and endogenous toxins, including ROS and reactive nitrogen species (RNS) [106, 107]. The resulting oxidized form of GSH (GSSG), characterized by a disulphide bond between two molecules of GSH, is efficiently reduced back to GSH by the NADPH-dependent catalysis of the flavoenzyme GSH reductase. Indeed, the GSH and GSH-related enzymatic systems are efficient tools that cells have exploited in detoxification and, at the same time, represent the most ancient notice on the physiological role played by the tripeptide.

As regard to its biosynthesis, GSH is generated in vivo by the consecutive action of two ATP-dependent enzymes, from the precursor aminoacids cysteine, glutamate and glycine. The first enzyme, glutamate–cysteine ligase (GCL) is the rate-limiting enzyme, while the second one, required for the novo GSH biosynthesis, is glutathione synthase (GS). Even if theorically all cell types synthesize GSH, the main source of the tripeptide is liver, where the bulk of cysteine, the rate limiting amino acid, derived from diet is metabolized. After its synthesis, GSH is delivered to some intracellular compartments, including mitochondria, endoplasmic reticulum, nucleus, and to the extracellular space (e.g., blood plasma and bile) for utilization by other cells and tissues [104]. In contrast to GSH synthesis, which occurs intracellularly, GSH degradation occurs exclusively in the extracellular space, and, in particular, on the surface of cells that express the enzyme γ- glutamyl transpeptidase (also called γ-glutamyltransferase, GGT) [108, 109]. The GGT is the only enzyme that can initiate catabolism of GSH and GSH-adducts (e.g., GSSG, glutathione S-conjugates, and glutathione complexes). GGT is an heterodimeric glycoprotein located on the external plasma membrane of specific cells present in kidney tubules, biliary epithelium and brain capillaries where it hydrolyses GSH into glutamic acid and cysteinyl-glycine; this dipeptide is further hydrolysed by cell surface dipeptidases and the resulting aminoacids taken up by cells for regeneration of intracellular GSH [110]. The intra- and extracellular GSH levels are determined by the balance between its production, consumption, and transportation. Due to important physiological functions of GSH, these processes are tightly regulated. The activities of the enzymes involved in GSH metabolism are controlled at transcriptional, translational, and post-translational levels. The investigation on the field related to production of ROS/RNS during metabolic processes, even under physiological conditions, unlocks another branch of research focusing on the role of GSH ranging from antioxidant/radical scavenger to redox signaling modulator. GSH effectively scavenges free

radicals and other ROS and RNS (e.g., hydroxyl radical, lipid peroxyl radical, superoxide anion and hydrogen peroxide) directly and indirectly through enzymatic reactions. The chemical structure of GSH determines its functions, and its broad distribution among all living organisms reflects its important biological role [111]. In particular, it has long been established that the thiol moiety of GSH is important in its antioxidant function in the direct scavenge of radical species. Indeed, the one-electron reduction with radicals is not chemically favorable, because it would generate the unstable thiyl radical GS; however, the reaction is kinetically driven in the forward direction by the removal of GS through reactions with thiolate anion (GS-) and then with oxygen. The first reaction leads to the generation of GSSG-, which in the presence of O_2, generates GSSG, and superoxide. On the other hand, GSH does not react directly non- enzymatically with hydroperoxides. In fact, its role as a cosubstrate for the selenium-dependent GPX has been recognized as the most important mechanism for reduction of hydrogen peroxide and lipid hydroperoxides. Additional roles for the antioxidant function of GSH have emerged that a restrictly related to signal transduction: (i) the interaction of the tripeptide with NO or with RNS;(ii)the involvement of GSH in the process of protein S-glutathionylation.

Clinical premises

The endothelium is a thin layer of cell surrounding all cardiovascular system.

2.5. The cardiovascular system

The cardiovascular system consists of heart, blood vessels, and approximately five liters of blood that the blood vessels transport.

As a liquid connective tissue, blood transports many substances through the body and helps to maintain homeostasis of nutrients, wastes, and gases. Blood is made up of RBCs, white blood cells, platelets, and plasma.

RBCs are the most common type of blood cell and make up about 45% of blood volume. Erythrocytes are produced inside of red bone marrow from stem cells at the rate of about 2 million cells every second. The shape of erythrocytes, biconcave disks with a concave curve on both sides of the disk, gives these cells a high surface area to volume ratio and allows them to fit into thin capillaries. Immature erythrocytes have a nucleus that is ejected from the cell when it reaches maturity to provide it with its unique shape and flexibility. The lack of a nucleus means that RBCs contain no DNA and are not able to repair themselves once damaged. The main function of these cells is to transport oxygen in the blood through the red pigment Hb, an iron-containing protein able to greatly increase their oxygen carrying capacity. The high surface area to volume ratio of erythrocytes allows oxygen to be easily transferred into the cell in the lungs and out of the cell in the capillaries of the systemic tissues.

White blood cells, also known as leukocytes, make up a very small percentage of the total number of cells in the bloodstream. They are divided into two major classes: granular leukocytes (neutrophils, eosinophils, and basophils, on the basis of the type of chemical-filled vesicles in their cytoplasm) and agranular leukocytes (lymphocytes and monocytes. They carry out their functions in the body's immune system.

Also known as thrombocytes, platelets are small cell fragments responsible for the clotting of blood and the formation of scabs. They derive from megakaryocytes in the red bone marrow who periodically release thousands of pieces of membrane that become platelets. This type of circulating cells does not contain a nucleus and only survive in the body for up to a week before macrophages capture and digest them. The main function of platelets is to control hemostasis, or the clotting of blood and formation of scabs. These cells normally

remain inactive in the blood until they reach damaged tissue or leak out of the blood vessels through a wound. Once active, platelets change their shape and become very sticky in order to latch on to damaged tissues. Then they release chemical clotting factors and begin to produce the protein fibrin to act as structure for the blood clot.

Between these classes of blood cells, plasma is the non-cellular or liquid portion of the blood that makes up about 55% of the blood's volume. It is a mixture of water, proteins (principally antibodies and albumin), and dissolved substances, including glucose, oxygen, carbon dioxide, electrolytes, nutrients, and cellular waste products. The plasma functions as a transportation medium for these substances as they move throughout the body.

The cardiovascular system is powered by the body's hardest-working organ — the heart, which is only about the size of a closed fist. The heart is a muscular pumping organ located medial to the lungs along the body's midline in the thoracic region. Its bottom tip, known as apex, is turned to the left, so that about 2/3 of the heart is located on the body's left side with the other 1/3 on right. The top of the heart, known as the heart's base, connects to the great blood vessels of the body: the aorta, vena cava, pulmonary trunk, and pulmonary veins. Even at rest, the average heart easily pumps over five liters of blood throughout the body every minute by a complex network of arteries, arterioles, and capillaries and returned to heart through venules and veins, through a process called circulation, consisting of two loops with different functions: the pulmonary and the systemic circulation. The first loop transports deoxygenated blood from the right side of the heart to the lungs, where the blood picks up oxygen and returns to the left side of the heart. It is supported by the right atrium and ventricle of the heart. In the pulmonary circulation, the artery brings oxygen-poor blood into lungs and the pulmonary vein carries oxygen-rich blood back to heart.

Systemic circulation, instead, transports highly oxygenated blood from the left side of the heart to all tissues of the body and removes wastes from body tissues, returning deoxygenated blood to the right side of the heart. The left atrium and left ventricle of the heart are the pumping chambers for the systemic circulation loop. In this system arteries carry oxygen-rich blood away from heart, and veins carry back oxygen-poor blood.

Twenty major arteries make a path through tissues, where they branch into smaller vessels called arterioles, which further branch into capillaries, the true deliverers of oxygen and nutrients to cells. Once the capillaries deliver oxygen and nutrients and pick up carbon dioxide and other waste, they move the blood back through wider vessels called venules. Venules eventually join to form veins, which deliver the blood back to heart to pick up oxygen.

All blood vessels are the body's highways that allow blood to flow quickly and efficiently from the heart to every region of the body and back again and their size corresponds with the amount of blood that passes through the vessel. They are lined with a thin layer of endothelium, that keeps blood cells inside of the vessels and prevents clots from forming. There are three major types of blood vessels: arteries, capillaries and veins.

Arteries are blood vessels that carry highly oxygenated blood away from the heart. To withstand the high levels of pressure, the wall of the arteries is thicker, more elastic, and more muscular than those of other vessels. Smaller arteries are more muscular in the structure of their wall and they contract or expand to regulate the blood flow through their lumen, controlling blood pressure in this way. Arterioles are narrower arteries that branch off from the ends of arteries and carry blood to capillaries; they face much lower blood pressures than arteries due to their greater number, decreased blood volume, and distance from the direct pressure of the heart. Thus arteriole wall is much thinner than that of arteries. Arterioles, like arteries, are able to use smooth muscle to regulate blood flow and blood pressure.

As regards capillaries, they are the smallest and thinnest blood vessels in the body, being present in almost every tissue. Capillaries connect arterioles on one end and venules on the other end and carry blood very close to the cells of the tissues in order to exchange gases, nutrients, and waste products. The wall of capillaries consist of only a thin layer of endothelium, which acts as a filter to keep blood cells inside of the vessels while allowing liquids, dissolved gases, and other chemicals to diffuse along their concentration gradients into or out of tissues. At the arteriole ends of capillaries there are precapillary sphincters, bands of smooth muscle able to regulate blood flow on the basis of energy and oxygen requirements.

The connection between capillaries and veins is embodied by venules. Venules are similar to arterioles as they are small vessels that connect capillaries, but unlike arterioles, they connect to veins instead of arteries, picking up blood from many capillaries and depositing it into larger veins for transport back to the heart.

Finally, veins are the large return vessels of the body and act as the blood return counterparts of arteries. Because the arteries, arterioles, and capillaries absorb most of the force of the heart's contractions, veins and venules are subjected to very low blood pressures, allowing the veins' wall to be much thinner, less elastic, and less muscular than the arteries' one. Veins rely on gravity, inertia, and the force of skeletal muscle contractions to help push blood back to the heart. To facilitate the movement of blood,

some veins contain many one-way valves that prevent blood from flowing away from the heart. As skeletal muscles in the body contract, they squeeze nearby veins and push blood through valves closer to the heart; when the muscle relaxes, the valve traps the blood until another contraction pushes the blood closer to the heart.

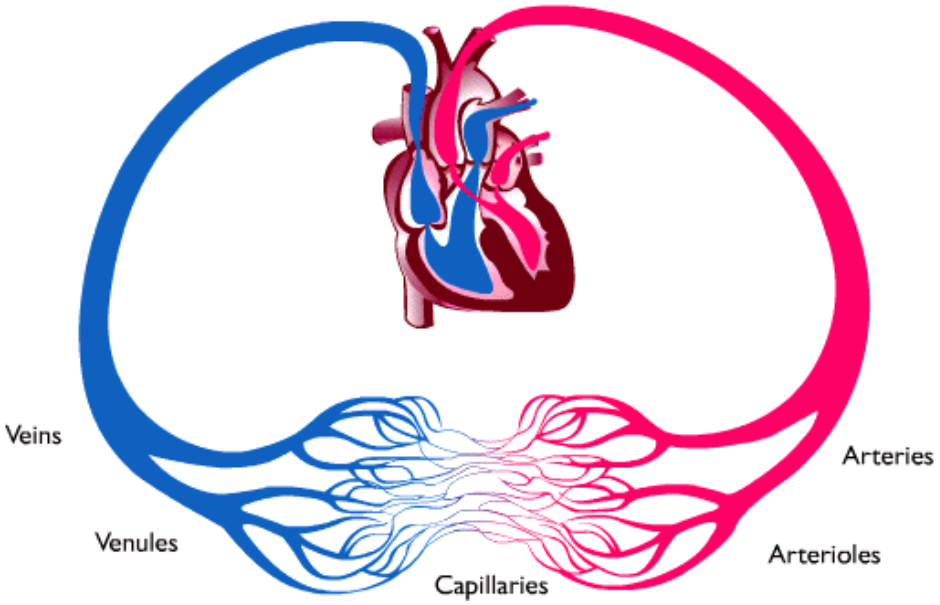


Figure 5: Schematic representation of cardiovascular system.

All this system of vessels forms the macro and microcirculation. The first is the circulation of blood to and from the organs while the second is the circulation present in the vasculature embedded within organ tissues. The circulation that supplies the myocardium is named coronary circulation and is a part of macrocirculation.

2.5.1. Coronary circulation

The heart has its own set of blood vessels that provide the myocardium with the oxygen and nutrients necessary to pump blood throughout the body, the human coronary arteries. They are conduit and capacitance vessels that usually deliver 90-180 mL/min of blood to the myocardium at a mean pressure of 90 mm Hg; they arise from the aortic root, and provide blood to the left and right sides of the heart. Between the, the coronary sinus is a group of veins joined together to form a large vessel on the posterior side of the heart that returns deoxygenated blood from the myocardium to the vena cava.

2.5.2. Microcirculation

Morphologically, the microcirculation is constituted from vessels <300 μm in diameter [112], including arterioles, capillaries, and venules. Alternatively, a physiological definition, based on vessel function rather than diameter or structure, has been proposed [113]. By this definition, vessels that respond to an increase of pressure by a myogenic reduction in lumen diameter are considered part of the microcirculation [113]. Consequently, besides endothelial cells, also VSMCs and pericytes must be included in the microvascular cell population. Although the primary function is to optimise the nutrient and oxygen supply, microcirculation is relevant in order to avoid large hydrostatic pressure fluctuations causing disturbances in capillary exchange and an overall peripheral vascular resistance [114]. An important role in regulating tissue fluid balance and in maintaining osmotic and hydrostatic pressures is played by the lymphatic system, that comprises a oneway transport for fluid and proteins by collecting them from the interstitial space and returning them to the blood circulation [115].

Different parameters are known to influence the microcirculation and, between them, temperature, systemic blood pressure, stress status, physical activity and age.

Consequently, several methods, both invasive and noninvasive techniques, are able to reliably quantify microcirculatory function.

2.5.3. Functions of the Cardiovascular System

The cardiovascular system has three major functions: transportation of materials, protection from pathogens, and regulation of the body's homeostasis.

•Transportation: The cardiovascular system transports blood to almost all body tissues. The blood delivers essential nutrients and oxygen and removes wastes and carbon dioxide to be processed or removed from the body. Hormones are transported throughout the body via the blood's liquid plasma.

•Protection: The cardiovascular system protects the body through the activity of white blood cells. These type of cells clean up cellular debris and fight pathogens that have entered the body. Platelets and RBCs form scabs to seal wounds and prevent pathogens from entering the body and liquids from leaking out. Blood also carries antibodies that provide specific immunity to pathogens that the body has previously been exposed to or has been vaccinated against.

•Regulation: The cardiovascular system is instrumental in the body's ability to maintain homeostatic control of several internal conditions. Blood vessels help maintain a stable body temperature by controlling the blood flow to the surface of the skin. Blood vessels near the skin's surface open during times of overheating to allow hot blood to dump its heat into the body's surroundings. In the case of hypothermia, these blood vessels constrict to keep blood flowing only to vital organs in the body's core. Blood also helps balance the body's pH due to the presence of bicarbonate ions, which act as a buffer solution. Finally, the albumin in blood plasma help to balance the osmotic concentration of the body's cells by maintaining an isotonic environment.

2.5.3.1. Regulation of Blood Pressure

A physiological role of endothelium-derived NO in the control of vascular tone has been demonstrated several years ago. Furthermore, other components of the cardiovascular system can control blood pressure; between them, hormones affect the rate and strength of heart contractions and blood vessels decreases their diameter by contracting the smooth muscle in the arterial wall. The sympathetic (fight or flight) division of the autonomic nervous system causes vasoconstriction, which leads to increases in blood pressure and decreases in blood flow in the constricted region. Vasodilation is the expansion of an artery as the smooth muscle in the arterial wall relaxes after the fight-or-flight response wears off or under the effect of certain hormones or chemicals in the blood. The volume of blood in the body also affects blood pressure. A higher volume of blood in the body raises blood pressure by increasing the amount of blood pumped by each heartbeat. Thicker, more viscous blood from clotting disorders can also raise blood pressure.

2.6. Endothelial and coronary pathologies

2.6.1. Coronary artery disease

Coronary artery disease (CAD), the principal cause of mortality and morbidity worldwide [116, 117], is a complex chronic inflammatory disease, characterized by remodeling and narrowing of the coronary arteries supplying oxygen to the heart. It can have various clinical manifestations, including stable angina, acute coronary syndrome, and sudden cardiac death. It has a complex etiopathogenesis and a multifactorial origin related to environmental factors, such as diet, smoking, and physical activity, and genetic factors that modulate risk of the disease, both individually and through interaction [118].

Atherosclerosis is the main etiopathogenic process that causes CAD and its progression is related to an interplay between environmental and genetic factors, with the latter exerting their effects either directly or via cardiovascular risk factors. Atherosclerosis is a silent progressive chronic process characterized by accumulation of lipids, fibrous elements, and inflammatory molecules in the wall of the large arteries [119-123], which begins with the efflux of low-density lipoprotein (LDL) cholesterol to the subendothelial space, which can then be modified and oxidized by various agents. Oxidized/modified LDL particles are potent chemotactic molecules that induce expression of vascular cell adhesion molecule and intercellular adhesion molecule at the endothelial surface, and promote monocyte adhesion and migration to the subendothelial space, where they differentiate to macrophages, able to function as proinflammatory agents. The final result of this process is theformation of the first typical atherosclerotic lesion. In the subendothelial space, the cross-talk between monocytes, macrophages, foam cells, and T-cells results in cellular and humoral immune responses, and ultimately in a chronic inflammatory state with the production of several proinflammatory molecules [124, 125]. This process continues with the migration of SMC from the medial layer of the artery into the intima, resulting in the transition from a fatty streak to a more complex lesion, the fibrous plaque. There are two types of plaque, defined on the balance between formation and degradation of fibrous cap, i.e. stable and unstable or vulnerable. Stable plaques have an intact, thick fibrous cap composed of smooth muscle cells in a matrix rich in type I and III collagen and the protrusion of this type of plaque into the lumen of the artery produces flow-limiting stenosis, leading to tissue ischemia and usually stable angina [126]. Vulnerable plaques have a thin fibrous cap made mostly of type I collagen and few or no SMCs, but abundant macrophages and proinflammatory and prothrombotic molecules [123, 127]. These plaques are prone to erosion or rupture, exposing the core of the plaque to circulating coagulation proteins, causing thrombosis, sudden occlusion of the artery lumen, and usually an acute coronary syndrome [123, 127].

From a clinical point of view, because the manifestations and symptoms of CAD are varied and overlap with many other conditions, diagnosis based on history and physical examination alone is challenging [128]. Cardiac stress testing, which is designed to identify areas of regional myocardial hypoperfusion or ischemia, is currently the most widely used non-invasive diagnostic method. A large number of protocols for stress testing have been developed and validated. Generally they combine a stressor, usually exercise or a pharmacologic alternative, and an ischemia readout, which may range from simple

electrocardiography (ECG) to imaging modalities such as cardiac ultrasonography (echocardiography), nuclear myocardial perfusion imaging or advanced imaging modalities such as cardiac magnetic resonance imaging (MRI) and computed tomography (CT).

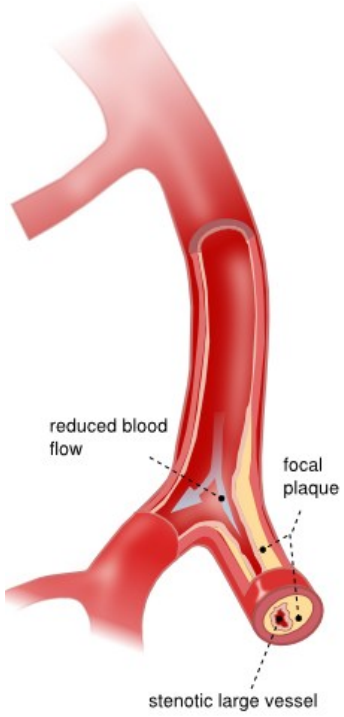


Figure 6: Representation of coronary artery disease.

2.6.2. Microvascular angina

The term 'microvascular angina' (MVA) was introduced in 1985 in order to define patients with coronary microcirculation highly sensitive to vasoconstrictor stimuli, but with a limited microvascular vasodilator capacity. This type of patients showed the typical chest pain syndrome indicative of CAD even if their coronary arteries appeared normal at the angiographic analysis.

MVA is characterized by two major abnormalities, which may combine variously to determine the individual clinical picture: coronary microvascular dysfunction and abnormal cardiac pain sensitivity. Since its first description, it was suggested that in this clinical setting angina is caused by myocardial ischaemia determined by a dysfunction of small resistance coronary artery vessels (<500 μm), not visible at coronary angiography,[129] and indicated by transient ST segment depression and reversible perfusion defects on nuclear imaging during anginal pain, either spontaneous or induced by exercise or

pharmacological stress tests [130]. Furthermore, metabolic evidence of stress induced myocardial ischaemia, including transmyocardial lactate production, coronary sinus oxygen desaturation, and pH reduction, as well as myocardial high energy phosphate depletion on nuclear magnetic resonance,[131] have been documented in about 20% of patients with angina and normal coronary arteries. Support for the notion that microvascular dysfunction is the cause of MVA derives from studies assessing coronary flow response to vasoactive stimuli. These studies have indeed shown an impairment of both endothelium dependent (for example, in response to acetylcholine) and endothelium independent (for example, in response to dipyridamole, papaverine) coronary vasodilation, using different techniques (for example, thermodilution, intracoronary Doppler recording, positron emission tomography, magnetic resonance, etc) for the measurement of coronary blood flow [132].

The causes of microvascular dysfunction in this clinical setting have not been fully clarified as yet. Structural abnormalities, mainly consisting of medial hypertrophy and/or fibrosis of arteriolar vessels, frequently associated with systemic hypertension, have been described in some of these patients. This alteration might be involved in several other typical features of MVA, including increased insulin resistance (associated with endothelial dysfunction), altered adrenergic activity, which may further favour microvascular dysfunction, and also enhanced pain perception. More recently, a role for intracellular rho-kinase, which may enhance vasoconstriction in vascular smooth muscle cells by facilitating calcium overload, has also been suggested in patients with angina and normal coronary arteries [133]. Recent data suggest that low grade inflammation might also play a pathogenetic role in the microvascular dysfunction. Indeed, Cosin-Sales and colleagues have found a correlation between C reactive protein values and clinical and electrocardiographic indices of disease activity in patients with angina and normal coronary arteries. A frequently reported abnormality is ED, as a consequence of an impaired NO release and/or activity, suggested by lower nitrate/nitrite systemic concentrations, and of an increased synthesis of ADMA, which is known to reduce the bioavailability of Arg in endothelial cells [134]. Several findings, however, suggest that ED might cause not only impaired vasodilation, but also an altered vasoconstriction. Indeed, increased plasma concentrations of endothelin-1 have been reported in peripheral blood of MVA patients and have been shown to correlate with coronary microvascular dysfunction [135]. Other studies, however, have also shown impairment of coronary microvascular dilation in response to endothelium independent stimuli, such as adenosine, dipyridamole, and papaverine, suggesting a possible primary SMC abnormality [132]. Also an enhanced

sodium–hydrogen exchanger activity in cell membranes [136] represents a potential cause of microvascular dysfunction as it may induce cellular alkalinisation, thus resulting in increased susceptibility of smooth muscle cells to constrictor stimuli [137].

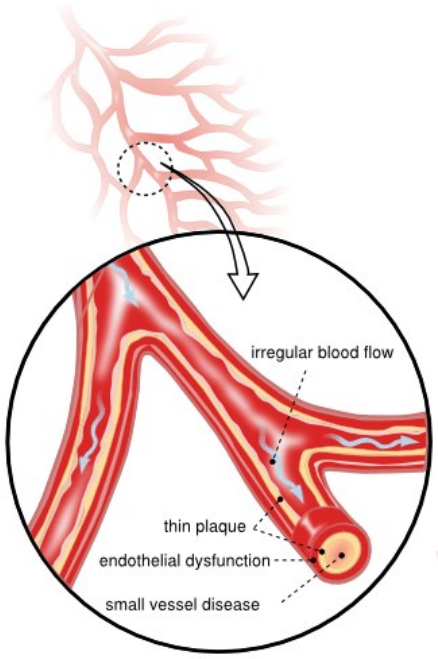


Figure 7: Representation of microvascular angina.

2.7. Alteration of L-arginine/NO pathway in cardiovascular diseases and major risk factors

The biological activity of NO is reduced in several clinical conditions associated with an increased risk for cardiovascular disease, such as hypercholesterolemia, smoking, diabetes mellitus, hypertension, and hyperhomocysteinemia [138]. Clinically, reduced availability of functionally intact NO becomes visible by impaired endothelium-dependent vasodilatation [139], increased platelet aggregation [140], and enhanced monocyte adhesiveness to the endothelium [141]. These pathophysiological events are the earliest visible changes initiating the atherogenic process [142, 143]. A decreased NO bioavailability is well documented also in ischemia-reperfusion injury [144].

As regards to microvascular angina, patients with angina pectoris and angiographically normal coronary arteries showed reduced NO and endothelin-1 responsiveness to

intravenously infused insulin in the presence of high basal endothelin-1 levels and normal basal NO levels [145], which suggests a defect in NO synthesis [146]. In agreement with this hypothesis, Egashira et al also showed that an acute intracoronary infusion of Arg was able to normalize NO-dependent intracoronary vasodilation in these subjects [147], whereas Bottcher et al were unable to confirm these results [148]. An elevation of ADMA levels in plasma might be another possible mechanism to explain the defect in vasodilation in these patients [149]. In fact, previous studies documented that ADMA negatively influences NO-mediated vasorelaxation [73, 150] and is correlated with mononuclear cell adherence to the endothelium [151].

Another potential mechanism contributing to the reduced NO bioavailability, as mentioned above, is an increased oxidative stress. This latter phenomenon can result from peroxynitrite-mediated oxidization of the essential NOS cofactor BH_4 and/or inadequate Arg availability. Based on the hypothesis of the pivotal role of RBCs in vascular homeostasis, several studies have analysed the effects of oxidative stress and of the presence of major cardiovascular risk factors on Arg/NO pathway in RBC compartment [152-154]. One of these studies investigated the effects of a high-cholesterol diet on oxidant/antioxidant status and NOS activity in erythrocytes from rats [152]. They found that cholesterol supplementation causes an increase in xanthine oxidase activity and a decrease in NOS activity, making RBC membranes more sensitive to oxidant stress.

During the same year a paper published by Schnorr and colleagues suggested a dietary intervention with flavanol-rich cocoa in order to diminish arginase activity in RBCs from healthy humans [153]. It has been demonstrated in fact that the ingestion of a cocoa drink, high in flavanols, is able to increase the circulating NO pool in parallel to a L-NMMA-sensitive increase in brachial artery dilation [155]. A mechanistic interpretation of this data is that circulating flavanols (and/or their metabolites) may increase NOS-dependent NO production, by modulation of either NOS activity and Arg availability or enhance NO levels via inhibition of NADPH oxidase [156]. These results suggest a potential utility of therapeutic interventions aimed at improving the balance between NOS and arginase activities. Some years later the involvement of NO produced by RBCs in the pathogenesis of type 2 Diabetes Mellitus (DM) has been analysed [157]. The main finding of this study is the capacity of RBCs to regulate serum levels of molecules involved in Arg/NO pathway through ‘‘buffering’’ their concentrations. In patients with DM this ability was altered, as RBCs showed higher levels of nitrites, Orn, Cit, malondialdehyde, and urea and lower concentrations of Arg. These data would suggest that both NOS and arginase affinities for

the common substrate are significantly increased in RBC of DM patients, demonstrating an enhanced Arg catabolism.

As NO derived from NOS is able to regulate coronary blood flow, improve myocardial relaxation, optimize cardiac performance [158], it also participates in the regulation of myocardial metabolism [159] and blood pressure [160]. The use of cross-transplanted chimeric mice genetically competent or deficient for eNOS in circulating blood cells demonstrated in fact a role of RBC NOS in blood pressure regulation and in vivo nitrite homeostasis [160].

The demonstration of the importance of NO derived from RBC arises from the data that eNOS is unable to compensate the depletion of circulating NOS alone in terms of reduction of tissue damage and preservation of left ventricular function The importance of RBC NOS has been recently evidenced also in regulating the severity of myocardial infarction and of left ventricular dysfunction using an animal model [161].

3. Objective

NO is an important signaling molecule involved in the maintenance of vascular function. It promotes several beneficial effects in the vasculature by inducing vasorelaxation, inhibition of leukocyte-endothelium adhesion, smooth muscle cells migration and proliferation, and platelet aggregation. Endothelial cells are considered the major source of NO in the vasculature, however it has been recently shown that also red blood cells (RBCs) may contribute to NO synthesis. RBCs express a functional NOS, similar to the enzyme of endothelial cells, which serves as an intraluminal NO source.

A decreased NO bioavailability, in most cases in association with increased oxidative stress levels, is well documented in several cardiovascular diseases and, between them, in coronary artery disease. Also microvascular angina, a pathological condition characterized by the typical anginal pain, electrocardiographic abnormalities at rest, in the presence of non-obstructed epicardial coronary arteries, has been proposed to be induced by an impaired endothelium-dependent vasodilatation of the coronary microvasculature.

As it has been shown that NOS expression in endothelial cells decreases in the microvasculature, the analysis of NO synthetic pathway in RBCs could open new fields of study in different cardiovascular diseases.

The aim of this study was to investigate the L-Arg/NO metabolic pathway in RBCs from patients with microvascular angina, analysing differences and similarities with coronary artery disease patients, in comparison to healthy subjects.

In addition, the analytes in this cellular compartment were compared to the levels measured in plasma in order to evaluate a potential impairment of Arg/NO pathway in these clinical settings.

These findings could be of particular relevance because RBCs have a systemic impact in terms of NO production, and may represent an important compartment whose alteration participates to the reduction in the overall NO production.

4. Materials and Methods

4.1. Ethical approval

This observational study was conducted with the approval of the local ethics research committee of Centro Cardiologico Monzino (n° S1687/610) and written informed consent to participate was obtained from all subjects. The investigation conformed to the principles outlined in the Declaration of Helsinki.

4.2. Study Population

In this study were enrolled 45 patients and 22 healthy subjects.

Twenty-five patients affected by microvascular angina, characterized by stable effort angina or inducible ischaemia and reduction of the coronary flow reserve, documented by a positive stress test (at least 2.0 mm horizontal or down-sloping ST-segment depression) or by a positive SPECT, despite the absence of coronary disease angiographically documented, were recruited. These patients were compared with 22 angiographically documented CAD patients, whose eligibility was based on the presence of stable exertional angina and positive stress test, as judged by at least 1.5 mm horizontal or down-sloping ST-segment depression. Key angiographic inclusion criteria was the evidence of >75% narrowing in at least one major coronary vessel, with normal left ventricular ejection fraction (>50%) assessed by two-dimensional echocardiography.

For the two patient populations were considered the following exclusion criteria: a history of congestive heart failure, significant valvular diseases, hypertrophic cardiomyopathy, vasospastic angina, recent (<6 months) acute coronary syndrome, surgical or percutaneous revascularization, pacemaker dependency and atrial fibrillation. Patients with renal insufficiency (serum creatinine concentration >1.4 mg/dL), hepatic disease, recent infection, recent major surgical interventions, immunological disorders, chronic inflammatory or neoplastic diseases, were also excluded.

Twenty healthy subjects without cardiovascular risk factors and evidence of CAD were enrolled as control group (Ctrl) from those attending the clinic for global control of cardiovascular risk at Centro Cardiologico Monzino IRCCS. Exclusion criteria were considered: recent infection and major surgical interventions.

4.3. Blood collection

Peripheral blood was drawn from the antecubital vein of patients and controls while fasting, into tubes containing EDTA (9.3 mM; Vacutainer Systems, Becton Dickinson, Franklin Lakes, NJ, USA) to obtain whole blood, plasma and erythrocyte samples. EDTA-anticoagulated blood was centrifuged at 1200 x g for 10 min at 4°C. Plasma was separated and aliquots were stored at -80°C until analyses. Aliquots of packed red cells were lysed by cold deionized water to obtain lysed RBCs and stored at -80°C until analyses.

4.4. Biochemical determinations

4.4.1. L-arginine/NO metabolome

Arg/NO pathway was determined both in lysed RBCs and in plasma. Arg, ADMA, SDMA, Cit and Orn were simultaneously measured by a liquid chromatography – tandem mass spectrometry (LC-MS/MS) method [162]. Prior to analysis, 100 µL of lysed RBCs were mixed with 350 µL of acetonitrile:methanol 50:50 (v/v) after adding internal standards, i.e. $^{13}C_6$-arginine, D_6-ornitine and $^{13}C_1D_4$-citrulline (2 µmol/L) and of D_7-ADMA (0.05 µmol/L) (Cambridge Isotope Laboratories, Inc.; Andover, MA, USA). Precipitated proteins were separated by centrifugation at 10000 x g for 15 min at 4°C.

Liquid chromatography was performed using an Accela pump and autosampler (Thermo Fisher Scientific, San Jose, CA, USA). Separation of analytes was conducted on Luna HILIC analytical column (50x2.0 mm, 3 µm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of aqueous ammonium formate 1.5 mmol/L, pH 3.2 (A) and ammonium formate 1.5 mmol/L, pH 3.2 in acetonitrile/MeOH (95.5:0.5 v/v) (B) at flow rate of 250 µL/min. The mobile phase gradient ran from 10% A to 70% A over 7 min, ran from 70 A to 94.5% A over 2 min, and was held at 94.5% A for 5 min, returning to 10% A over 2 min and held at 10% A for re-equilibration. The sample injection volume was 10 µL, the column temperature was set at 30°C and the sample injector was maintained at 10°C. Total run time per sample was 25 min. Mass spectrometric analysis was performed using a TSQ Quantum Access (Thermo Fisher Scientific, San Jose, CA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface operated in positive mode. The analytes were detected by MS/MS using multiple reaction

monitoring (MRM). The specific collision energy of each analyte were optimized to maximize the ion currents of the selected precursor and product ions. The optimization of the precursor to product ion dissociation was performed by infusion into the MS of separate standard solutions (1 ng/μL) of each analyte (Sigma-Aldrich, St.Louis, MO, USA). The operating conditions for MS analysis were as follows: spray voltage, 2500 V; capillary temperature and voltage, 260°C and 35 V, respectively; sheat gas and auxiliary gas flow, 25 and 20 arbitrary units, respectively. The mass spectrometer was employed in MS/MS mode using argon as collision gas. Instrument control, data collection, and analysis were performed with Xcalibur® software, version 2.0 (Thermo Fisher).

Validation of this method was performed by adding different concentrations of each analyte to mobile phase B or to pooled lysed RBCs. Calibration curves were daily prepared at concentration ranges of 0–20 μmol/L Arg and Orn, 0–10 μmol/L Cit, and 0–200 nmol/L ADMA, SDMA and MMA.

The ratio Arg/(Orn+Cit) as index of global Arg availability [138, 163], and the ratio Orn/Cit as indicator of the relative activity of arginase and NOS [144] were computed.

4.4.2. Glutathione: method development and validation

Reduced glutathione (GSH) and disulphide glutathione (GSSG) were measured on whole blood added with 10% trichloroacetic acid (TCA) in 1 mM EDTA solution to precipitate proteins and stored at -80°C until analysis. Levels of GSH and GSSG were assessed by LC-MS/MS method using a TSQ Quantum Access (Thermo Fisher Scientific, San Jose, CA, USA) triple quadrupole mass spectrometer coupled with ESI operated in MRM in positive mode. Separation of analytes was conducted on a Luna PFP analytical column (100x2.0 mm, 3 μm, Phenomenex, Torrance, CA, USA). The LC mobile phases were (A) ammonium formate 0.75 mM adjusted to pH 3.5 with formic acid and (B) methanol. Separation was performed under isocratic conditions with 99% mobile phase A at flow rate of 200 μL/min and a column temperature of 35°C. The MRM for GSH (m/z 308.1→m/z 76.2 + 84.2 + 161.9) and GSSG (m/z 613.2→m/z 230.5 + 234.6 + 354.8) were performed with collision energy optimized for each compound.

Levels of GSH and GSSG were expressed as μmol/g Hb.

4.4.2.1. Calibration standards and quality controls

Stock solutions of GSH and GSSG were prepared at 1 mM in 10% TCA solution and stored at -80°C. Calibrators containing both GSH and GSSG were prepared daily by diluting the stock solutions with 0,1% formic acid. In house quality control (QC) samples of 3 different concentrations levels (GSH: 8, 2, 0.5 µM and GSSG: 1, 0.25, 0.625 µM), were daily prepared. A pooled whole blood supernatant (PWBS) was aliquoted and stored at -80°C and used in the validation procedures.

4.4.2.2. Linearity and calibration and matrix effects evaluation

Linearity of the assays was assessed by repeat (n=3) analysis of calibrators with concentrations ranging from 0 to 100 µM. Calibration curves were prepared in the concentration ranges 0.5-8 µM and 0.0625-1 µM for GSH and GSSG respectively. The curves were constructed by plotting the peak area *vs* the analyte concentration. Linear regression analysis was used to determine the slope, intercept, and correlation coefficient (r^2). Calibration curves were prepared both in 0.1% formic acid and in 400-fold diluted PWBS to evaluate potential matrix effects by comparing the slope of the standard calibration curve with the slope of matrix-matched standard curve.

4.4.2.3. Precision, accuracy and recovery

Intra-day precision was evaluated by replicate preparation and analysis of QCs (n=6) and of PWBS (n=6) performed on the same day. To assess the inter-day precision, the same analyses were repeated on 3 different days. Intra-day and inter-day precision was expressed as coefficient of variation (CV).

Accuracy was evaluated in "standard addition" experiments. The same GSH and GSSG amounts used for QCs were spiked into both PWBS (n=6) and 0.1% formic acid, and then quantified. Accuracy was evaluated by comparing the estimated (measured) with the added (true) concentrations. Accuracy (bias, %) was expressed as:

((Measured value – True value)/ True value)x100

Recovery (%) was evaluated adding the same GSH and GSSG amounts, used for QCs, into fresh collected whole blood samples (n=6) kept on ice, before the TCA precipitation.

4.4.2.4. Lower limit of detection (LOD) and quantification (LLOQ)

The LOD of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected, but not necessarily quantified, as an exact value.
It was assessed as the smallest detectable signal in diluted whole blood supernatant, above baseline noise (signal to noise ratio, S/N =3).
The LLOQ is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy and was the concentration of GSH and GSSG when their signal was 10 times that of the blank (S/N=10).

4.4.3. RBC-NOS and arginase expression

The evaluation of NOS and arginase expression in RBCs was conducted by immunofluorescence and confocal microscopy and was performed in a subgroup of subjects (n=10 per group matched for age and sex, randomly chosen and representative of the enrolled population). After plasma separation, an aliquot of RBCs was fixed in 2% paraformaldehyde at room temperature (RT) for 30 min, stroked on glass and heat fixed. Non specific reactive sites were blocked with 5% bovine serum albumin solution containing 0.1% saponin for 30 min at RT. RBCs were incubated overnight at 4°C with a monoclonal anti eNOS (2.5 µg/ml; BD Biosciences, Milano, Italy) or polyclonal anti arginase I or monoclonal anti arginase II (4 µg/mL, for both) (Santa Cruz Biotechnology, D.B.A. Italia s.r.l., Milano, Italy) antibodies. After three washings, an anti-mouse or anti-rabbit AlexaFluor488 conjugated secondary antibody (Invitrogen, Life Technologies Italia, Monza, Italy) was added and the immune complexes were visualized by laser scanning confocal microscope (LSM710, Carl Zeiss, Milano, Italy) using a 63X/1.3 oil immersion objective lens. Images were captured and the fluorescence intensity (densitometric sum of grey) was quantified. Data are expressed as the mean level of fluorescence intensity, subtracted of negative control value obtained on the same slide in the absence of primary antibody. Multiple fields of view (at least three randomly selected area) were captured for each slide.

4.4.4. In vitro RBC-NOS activity

NOS activity in RBCs was measured in a subgroup of age and sex matched subjects (n=8 per group, randomly chosen and representative of the enrolled population). The enzymatic activity was measured *in vitro* by the conversion of L-[$^{15}N_2$]arginine to L-[^{15}N]citrulline in

the presence of the arginase inhibitor N(omega)-hydroxy-nor-l-arginine (nor-NOHA). Washed RBCs (10^6cells/µl) were lysed on ice by cold deionized water (1:1, v/v) in the presence of protease inhibitors (phenylmethanesulfonylfluoride, 2 mM; leupeptin, 4 µM; aprotinin, 4 µM). Samples were incubated at 37°C for 2 hr with L-[$^{15}N_2$]arginine (75 µM) and NOS cofactors in the absence or in the presence of nor-NOHA (50 µM). The composition of reaction buffer (in µM) was as follows: Tris-HCl, 250, pH 7.4; $CaCl_2$, 500; BH_4, 0.3; flavin adenine dinucleotide (FAD), 0.1; flavin mononucleotide (FMN), 0.1; nicotinamide adenine dinucleotide phosphate (NADPH), 100. The reaction was stopped by the addition of 5 volumes of acetonitrile/methanol (50:50, v/v). Precipitated proteins were separated by centrifugation at 10000 x g for 15 min at 4°C and stored at -80°C until analysis. The analytes were measured by LC-MS/MS and the activity was quantified as the ratio between the L-[^{15}N]citrulline (µmol/10^6 cells) and residual L-[$^{15}N_2$]arginine (mmol/10^6 cells) [144]. As a positive control, human aortic endothelial cells (HAECs) (500000 cells/well) were used.

4.5. Statistical analysis and scores development

Numerical variables were summarized as mean and standard deviation (SD), unless otherwise stated, and categorical variables were summarized as frequencies and percentages. A sample size of 20 subjects per group allowed a statistical power of 90% to deem as significant a between-group difference in any analyte approximately equal to one standard deviation, with an alpha error of 0.05. Variables were compared between MVA and CAD or Ctrl by T-test or by covariance analysis, adjusting for age and sex. Variables with skewed distribution were log-transformed before analysis. Immunofluorescence intensity was compared between groups by repeated measures covariance analysis, taking into account replicate measures for each subject. All analyses were performed by SAS v. 9.2 (SAS Institute Inc., Cary, NC, USA).

In order to provide a global indicator of all the variables related to NO pathway and to contain inflation of alpha error due to multiple testing, we developed a score similar to the OXY-SCORE, devised by our group few years ago [164]. First, to account for different measurement ranges and units, all the variables were standardized, i.e. the mean was subtracted from individual values and the result was divided by the standard deviation. Second, the standardized values of the variables generally accepted as positively associated to endothelial function (Arg and Cit) were added, whereas standardized values of the

variables negatively associated to endothelial function (ADMA, SDMA, MMA and Orn) were subtracted. It is important to note that these associations were intended as ‘a priori’ and were not inferred from the present study. We created a first score using variables measured in plasma (NO plasma score) and another score using variables measured in the RBCs (NO RBC score). Similarly, we created oxidative stress score, a simplified version of the OXY-SCORE including GSSG (with a plus sign) and GSH (with a minus sign).

5. Results

5.1. Set up of glutathione measurement

5.1.1. Sample preparation and analytical method conditions

Sample preparation is a very critical phase in GSH measurement, due to its instability in aqueous solution and potential thiol oxidation by transition metals. For this reason EDTA, a chelating agent, was used for blood treatment and a deproteinization step, using TCA, was immediately performed after sample collection. This acid was reported to be the best precipitating reagent by which GSH oxidation with time is minimal [165-167]; in this method GSH decrease is thus prevented by keeping whole blood samples on ice and by storage at -80°C after the rapid treatment with TCA.

The stability of GSH and GSSG was tested by analysing, after 1 day and after 7 days, standard samples (GSH and GSSG 2 µM) and PWBS aliquots kept at different temperatures (-80, -20, +4 and +21°C). A GSH loss of 10% with a concomitant increase of GSSG was observed after one week at room temperature (+21°C). GSH concentration was stable, in both standard solution or PWBS, for at least 7 days at -80°C, -20°C and +4°C in agreement with Steghens et al [168] (Table 1).

		GSH %		GSSG %	
		24 h	**7 d**	**24 h**	**7 d**
Std	-80°C	99.6	99.2	104.8	93.2
	-20°C	99.7	99.5	95.7	90.3
	+4°C	94.4	96.9	101.1	91.1
	RT	86.9	86.9	94.3	73.9
PWBS	-80°C	97.4	98.9	92.4	83.1
	-20°C	80.6	93.4	106.1	89.9
	+4°C	95.2	94.3	84.8	61.5
	RT	94.5	80.0	90.0	78.4

Table 1: Stability of GSH and GSSG. Pure standard (2 µM) and PWBS were stored at 4 different temperatures (-80°C, -20°C, +4°C and RT) for 24 hours or for 7 days. The displayed values are percent related to sample analyzed immediately after the collection.

GSH and GSSG are very hydrophilic so that they are not easily retained on regular reversed phase columns. Good retention and separation of these two compounds were achieved using a pentafluorophenyl propyl ligand bonded to silica. Total run time was 10 min and retention times were 3.12±0.05 (mean±SD) and 4.72±0.02 min for GSH and

GSSG, respectively (Fig 8). Chromatograms of both analytes highlighted the specificity of the assay with no observed interferences in the regions of elution.

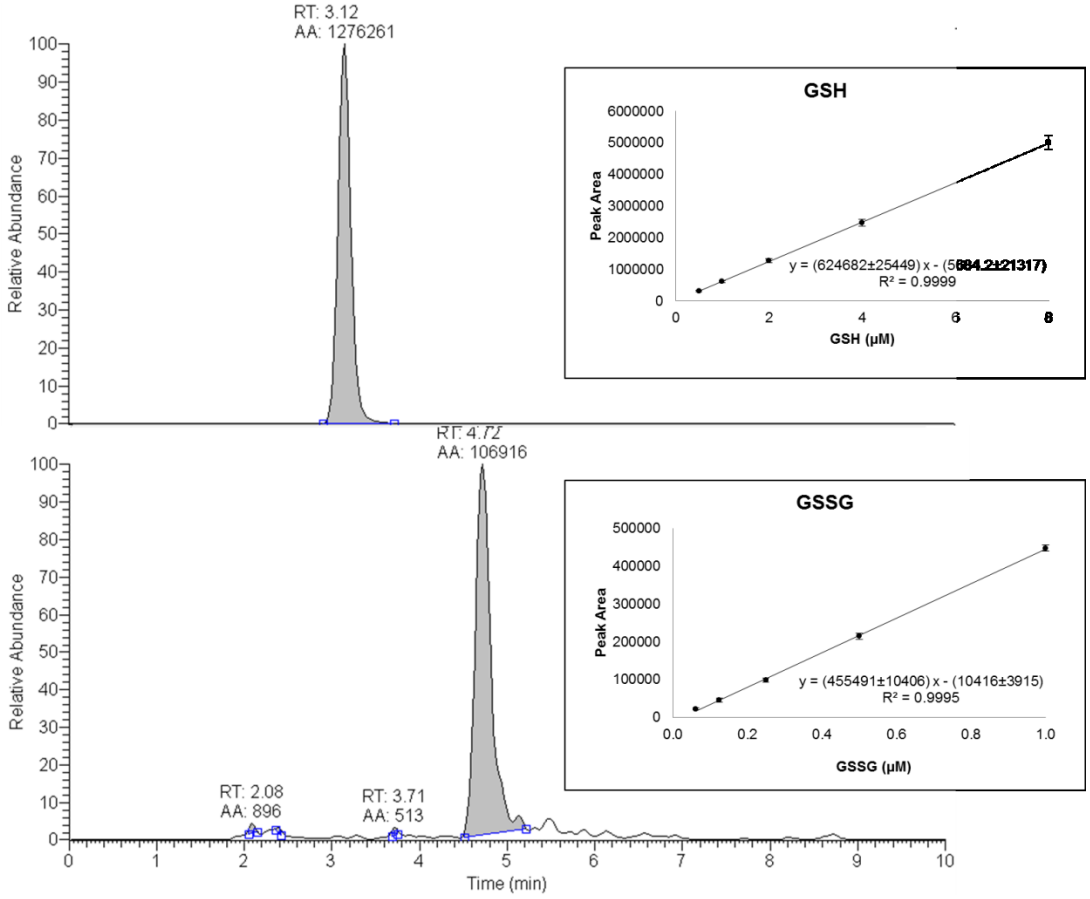


Figure 8: Typical chromatograms produced by the LC-MS/MS method of a standard solution of 2 µM GSH and 0.25 µM GSSG. In the inset the corresponding calibration curves. Each data point represents the mean area ± SD from three different calibration curves plotted on three different days.

5.1.2. Method validation

The method has been validated following the FDA international guidelines and the tested parameters are summarised in Table 2.

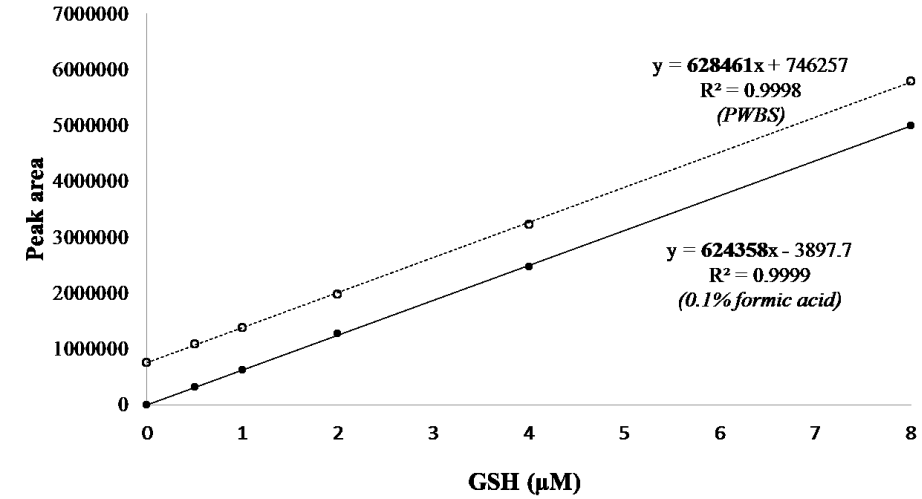
	GSH	GSSG
LOD (µM)	0.003	0.004
LLOQ (µM)	0.5	0.0625
Intra-day precision (%CV)		
QC level 1	0.9	0.7
QC level 2	2.3	5.5
QC level 3	5	9.9
PWBS	3.2	4
Inter-day precision (%CV)		
QC level 1	4.9	2.3
QC level 2	6.2	5.5
QC level 3	9.6	11.6
PWBS	4	6.1
Accuracy (bias, %)		
QC level 1	0.7	3
QC level 2	-2.1	7.9
QC level 3	2.8	7.1

Table 2: Methods validation data. Concentration levels of QCs used for the validation procedure were: Level 1= 8 µM GSH and 1 µM GSSG; Level 2= 2 µM GSH and 0.25 µM GSSG; Level 3= 0.5 µM GSH and 0.0625 µM GSSG. The pooled whole blood acidic supernatant (PWBS) was diluted, with formic acid 0.1%, 400-fold.

This LC-MS/MS method showed high sensitivity, in particular as regards GSSG quantification, as highlighted by LOD and LLOQ values. On the basis of these results calibration curves ranged between 0.5-8 µM for GSH and 0.025-1 µM for GSSG, although linear ranges were found to be 0.01-50 µM for both GSH and GSSG.

The intra- and inter-day precision for QCs and for PWBS, showed in table 2, were within the acceptable ranges required for validation of the assay.

As regards matrix effect evaluation, in this LC-MS/MS assay the slopes of the standard and of the matrix-matched calibration curves, are virtually the same, indicating no matrix effect (Fig 9).



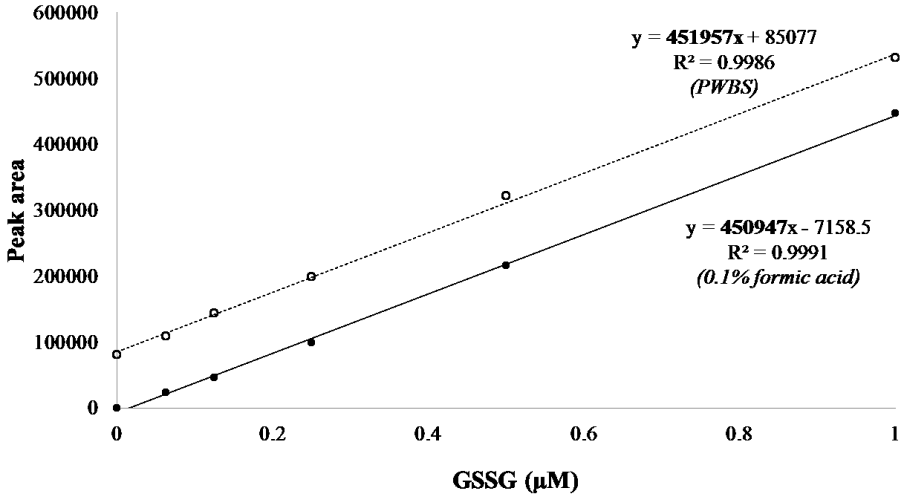


Figure 9: Evaluation of matrix effects. Calibration curves were prepared both in 0.1% formic acid and in 400-fold diluted PWBS (matrix) to evaluate potential matrix effects by comparing the slope of the standard calibration curve with the slope of matrix-matched standard curve. The curves were constructed by plotting the peak area versus the analyte concentration. Linear regression analysis was used to determine the slope, intercept, and correlation coefficient (r^2).

As regards recovery, it was calculated from whole blood samples spiked with 3 different concentration levels and showed a mean of 102.4% (range 98.8-108.7) and 108.6 (range 102.5-115.1), for GSH and GSSG, respectively.

5.2. Study Population

The principal demographic and clinical characteristics of the two patient groups and of healthy subjects analysed in this study are depicted in Table 3.

Variable	MVA (n=25)	CAD (n=22)	Ctrl (n=20)	p value ANOVA	p value MVA *vs* CAD
Age (years)	56.5±10.3	66.1±8.6	55.5±10.2	*0.001*	*0.0012*
Male gender n(%)	14 (56.0)	17(77.3)	14(70)	0.2858	0.1475
BMI	25.8±3.3	27.3±3.11	24.36±2.35	*0.0223*	0.1128
Total cholesterol (mg/dL)	226.9±59.2	208.3±29.2	208.1±26.8	0.2678	0.1963
HDL-cholesterol (mg/dL)	54.9±17.3	46.9±17.2	54.5±17.3	0.2576	0.1282
LDL-cholesterol (mg/dL)	150.0±52.4	133.8±36.8	132.3±22.1	0.3146	0.2419
Triglycerides (mg/dL)	105.5±66.9	131.5±69.4	95.5±32.5	0.1844	0.2031
Systolic Blood pressure (mmHg)	130.0±13.2	138.9±18.3	130.0±14.0		
Diastolic Blood pressure (mmHg)	78.0±8.3	80.0±9.1	78.0±6.0		
Creatinine (mg/dL)	0.85±0.22	0.86±0.22	0.81±0.14	0.7763	0.8049
Current Smoker	3(12.0)	3(13.64)	0(0)	0.3664	
HyperCholesterolemia	14 (56.0)	14(63.6)	2(12.5)	*0.0035*	
IperTrygliceridemia	1 (4.0)	2(9.1)	1(6.25)	0.8227	
Hypertension	11 (44.0)	14(63.6)	2(12.5)	*0.007*	
Pharmacological treatments					
Converting enzyme inhibitors	2 (8.0)	6(27.3)	0(0)	*0.0301*	
Antithrombotics	23 (85.1)	17(77.3)	0(0)	*0.00012*	
Beta-Blockers	10 (40.0)	5(23.8)	1(5.88)	*0.0394*	
Calcium channel blockers	1 (4.0)	4(18.2)	1(5.88)	0.2649	
Diuretics	2 (8.0)	2(9.1)	0(0)	0.5512	
Statins	4 (16.0)	4(18.2)	2(11.8)	0.9125	
Hypoglycemics	0 (0)	0(0)	0(0)	-	
Angiotensin receptor blockers	4 (16.0)	6(27.3)	0(0)	0.1035	

Table 3: Demographic and clinical characteristics of the three groups of subjects. Quantitative variables were expressed as mean±SD and categorical variables as n(%).

As regard to demographic characteristics, no significant differences were found among groups except for age and BMI, that were considered as confounders for group comparisons. From a clinical point of view, there were more hypercholesterolemic and hypertensive subjects among CAD patients, but both LDL cholesterol and systolic/diastolic

pressure values were similar between patients and Ctrl, as the result of pharmacological treatments.

5.3. Metabolites involved in L-arginine/NO Pathway

5.3.1. Red Blood Cell compartment

In order to evaluate the potential impairment of Arg/NO pathway in MVA patients, we simultaneously measured the principal metabolites involved in this pathway in RBC compartment. In these cells, the concentrations of the enzymatic substrate Arg and of the two enzymatic products Cit and Orn didn't significantly differ in the three studied groups. The ratio between Orn and Cit, an indirect index of the activity of Arg metabolic enzymes arginase and NOS, was lower in MVA than CAD patients but similar to Ctrl (Fig 10).

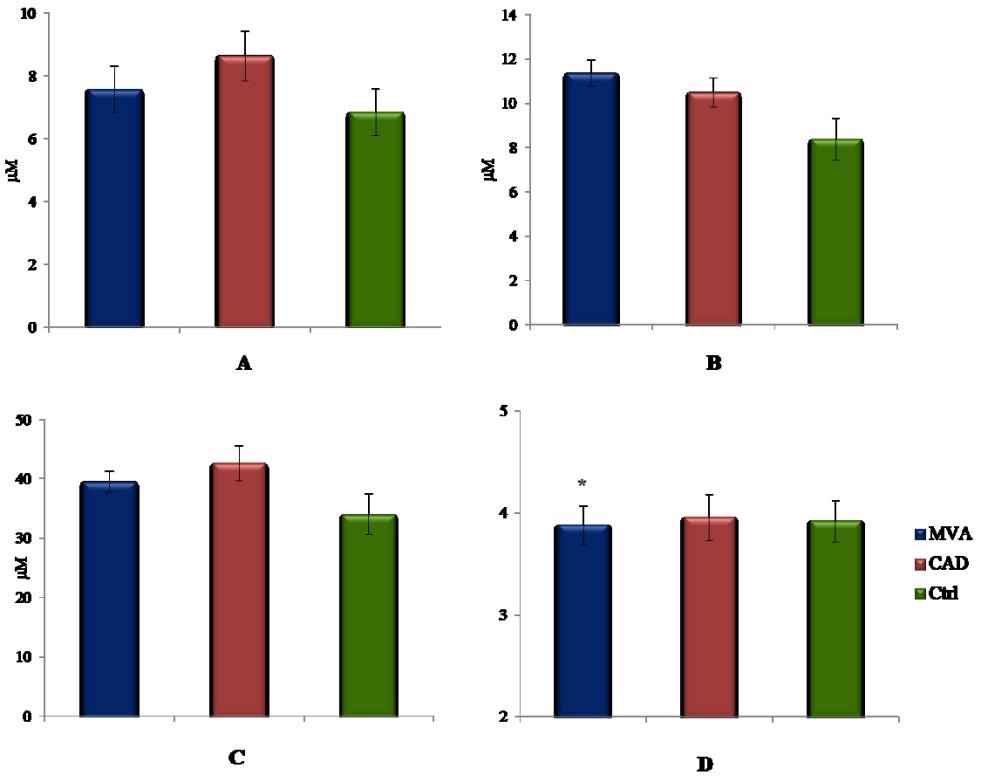


Figure 10: Concentrations of Arg (a), Cit (b), Orn (c) and Orn/Cit ratio (d) measured in RBCs isolated from patients with microvascular angina (MVA, n = 25) or coronary artery disease (CAD, n = 22) or healthy subjects (Ctrl, n = 20). The results are expressed as median±ES. * $p<0.05$ *vs* CAD, adjusted for age and gender after log-transformation of the data.

The levels of NO inhibitors ADMA and SDMA were higher in MVA and CAD patients than in Ctrl, and MMA levels were the highest in MVA. However the Arg bioavailability, defined as the ratio between Arg and the sum of Cit and Orn, was similar in the three studied groups (Fig 11).

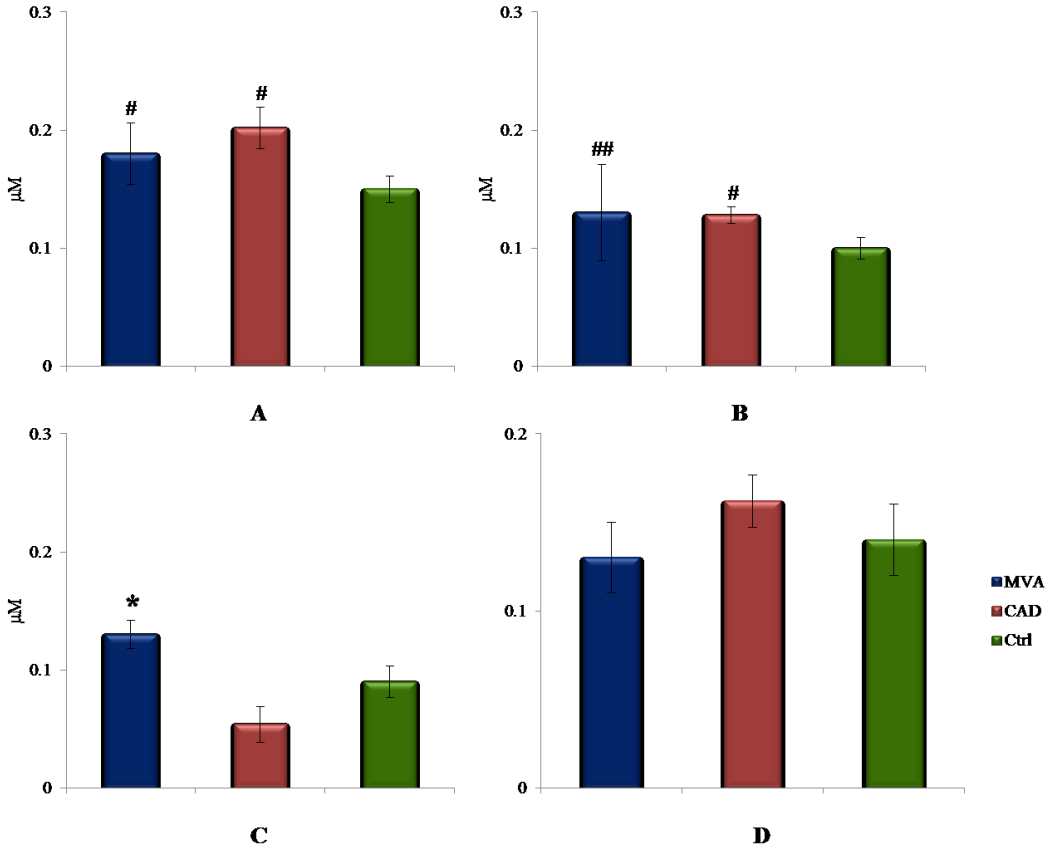


Figure 11: Concentrations of ADMA (a), SDMA (b), MMA (c) and Arg bioavailability (d) measured in RBCs isolated from patients with microvascular angina (MVA, n = 25) or coronary artery disease (CAD, n = 22) or healthy subjects (Ctrl, n = 20). The results are expressed as median±ES. * $p<0.05$ *vs* CAD; # $p<0.05$, ## $p<0.01$ *vs* Ctrl, adjusted for age and gender after log-transformation of the data.

5.3.2. Plasma compartment

We further investigate the levels of the same analytes in plasma, in order to compare them to the cellular compartment. In plasma compartment, MVA patients showed Arg and Cit levels similar to those of CAD patients and Ctrl. As regards Orn, CAD had higher concentrations with respect to Ctrl. As a consequence, the Orn/Cit ratio in plasma of CAD

was higher than in Ctrl, while in MVA this ratio showed levels intermediate between those of CAD and Ctrl.

ADMA levels, instead, were higher in both MVA and CAD patients compared to Ctrl. SDMA and MMA levels didn't differ among the three groups studied. In accordance to these findings, the Arg bioavailability was lower in MVA and CAD than in Ctrl (Table 4).

	Plasma			*RBC*		
	MVA (n=25)	CAD (n=22)	Ctrl (n=20)	MVA (n=25)	CAD (n=22)	Ctrl (n=20)
Arg	74.22 [69.15-87.08]	82.88 [64.81-95.74]	84.80 [73.13-98.01]	7.57 [5.00-9.53]	8.64 [5.31-10.48]	6.84 [3.86-7.80]
Cit	27.45 [24.21-31.16]	27.61 [20.41-33.83]	26.55 [24.13-30.68]	11.36 [9.58-12.35]	10.48 [8.36-12.11]	8.37 [7.04-10.78]
Orn	47.66 [41.19-54.11]	51.82$^{\#}$ [48.72-61.68]	40.84 [34.09-46.08]	39.48 [29.52-49.06]	42.59 [36.74-44.90]	33.93 [23.77-43.07]
Orn/Cit ratio	1.74 [1.42-2.21]	1.97$^{\#\#\#}$ [1.69-2.40]	1.48 [1.36-1.71]	3.88* [3.10-4.41]	3.96 [3.51-5.03]	3.92 [3.20-4.32]
ADMA	0.51$^{\#}$ [0.43-0.60]	0.49$^{\#}$ [0.45-0.59]	0.41 [0.35-0.47]	0.18$^{\#}$ [0.14-0.27]	0.20$^{\#}$ [0.15-0.25]	0.15 [0.12-0.20]
SDMA	0.47 [0.42-0.60]	0.54 [0.45-0.63]	0.42 [0.36-0.48]	0.13$^{\#\#}$ [0.09-0.16]	0.13$^{\#}$ [0.12-0.15]	0.10 [0.06-0.11]
MMA	0.12 [0.09-0.13]	0.11 [0.08-0.13]	0.12 [0.10-0.14]	0.13* [0.07-0.18]	0.05 [0.04-0.09]	0.09 [0.05-0.12]
Arg bioavailability	1.06$^{\#}$ [0.90-1.26]	1.01$^{\#}$ [0.87-1.22]	1.25 [1.04-1.41]	0.13 [0.11-0.19]	0.16 [0.12-0.19]	0.14 [0.09-0.20]

Table 4: Biochemical determinations in plasma and RBCs. Quantitative variables are expressed as median [interquartile interval]. * p<0.05 *vs* CAD; # p<0.05, ##p<0.01 and ### p<0.001 *vs* Ctrl, adjusted for age and gender after log-transformation of the data.

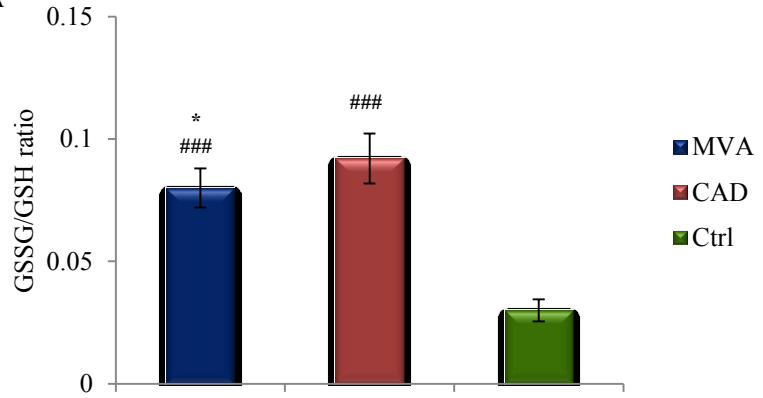
5.4. Oxidative stress status

In order to evaluate the oxidative stress status in the three study groups, we measured in whole blood the levels of the oxidised and the reduced form of glutathione, whose ratio is a recognised index od oxidative stress.

Patients with MVA had higher GSSG/GSH ratio with respect to Ctrl, but lesser than those determined in CAD patients (Fig 12, panel A). Specifically, both groups of patients showed lower levels of GSH and higher levels of GSSG with respect to Ctrl (Fig 12, panel B).

Interestingly, a positive correlation between GSSG/GSH and Orn/Cit ratio (r= 0.56, P=0.007) was found only in CAD patients.

A



B

	MVA (n=25)	CAD (n=22)	Ctrl (n=20)
GSH (µmol/g Hb)	6.40 # [4.90-7.20]	6.10 # [5.30-6.70]	7.60 [6.80-8.60]
GSSG (µmol/g Hb)	0.50 # [0.35-0.59]	0.62 # [0.50-0.72]	0.26 [0.21-0.32]

Figure 12: GSSG/GSH ratio in whole blood. The results are expressed as median ± SE for GSSG/GSH ratio (a) or as median [interquartile range] (b) for GSH and GSSG in whole blood from patients with microvascular angina (MVA n = 25) or coronary artery disease (CAD n = 22) or healthy subjects (Ctrl n = 20). * p<0.05 *vs* CAD, ### p< 0.001 *vs* Ctrl.

In Figure 13 was shown the distribution of the analytes measured in plasma or RBCs of MVA and CAD patients expressed as fold change over Ctrl. In general, the analytes of the NO pathway behaved similarly in MVA and CAD and they were moderately elevated with respect to Ctrl, both in plasma and in RBCs. A special case is represented by MMA in RBCs, whose levels were higher in MVA with respect to Ctrl and CAD patients. As expected, the oxidative stress, in particular the oxidized form of glutathione, was higher in both MVA and CAD patients with respect to Ctrl.

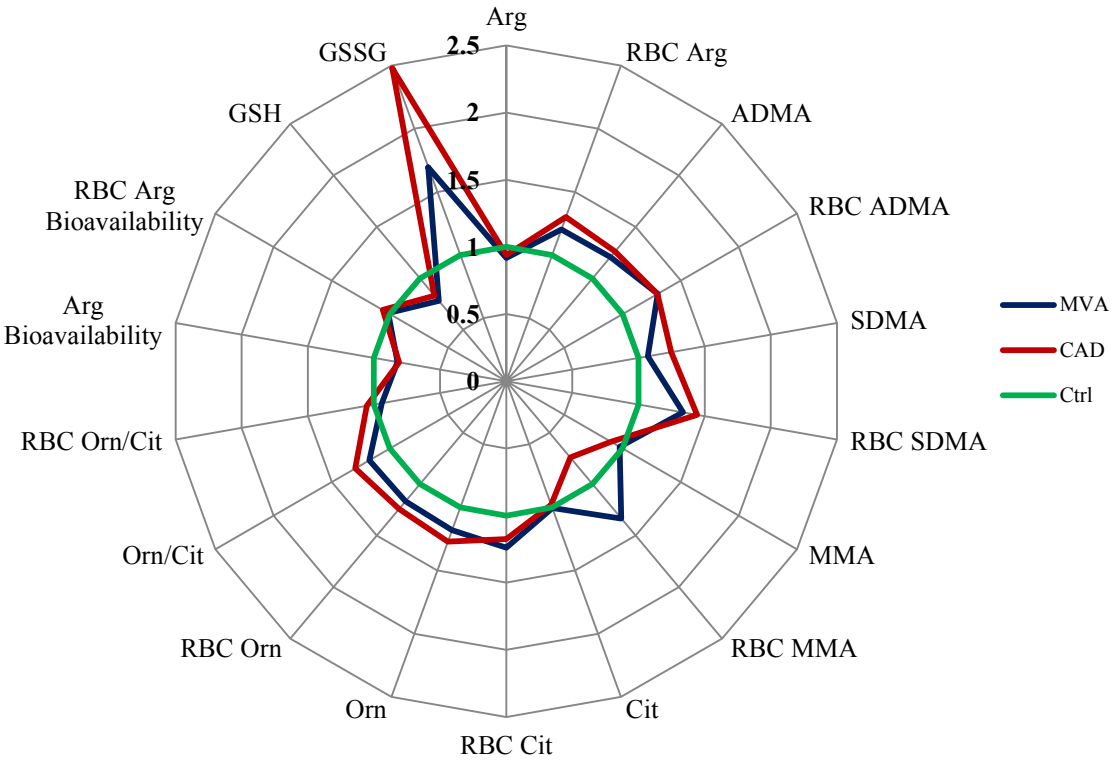


Figure 13: Levels of analytes involved in Arg/NO pathway measured in plasma and in RBCs isolated from patients with microvascular angina (MVA *n* = 25) or coronary artery disease (CAD *n* = 22) or healthy subjects (Ctrl *n* = 20). The results are expressed as fold change over Ctrl.

5.5. RBC-NOS and arginase expression

Confocal microscopy analysis of NOS expression in RBCs revealed a distinct ring of immunofluorescence staining surrounding the cytoplasm and, to a lesser extent, punctuate immunofluorescence structure through the entire cytoplasm.

The analysis of fluorescence intensity revealed strong quantitative differences between both patient groups and Ctrl. Specifically, RBCs of MVA and CAD patients had significantly lower RBC-NOS fluorescence, localized in the membrane and into the cytosol, with respect to Ctrl (Fig 14).

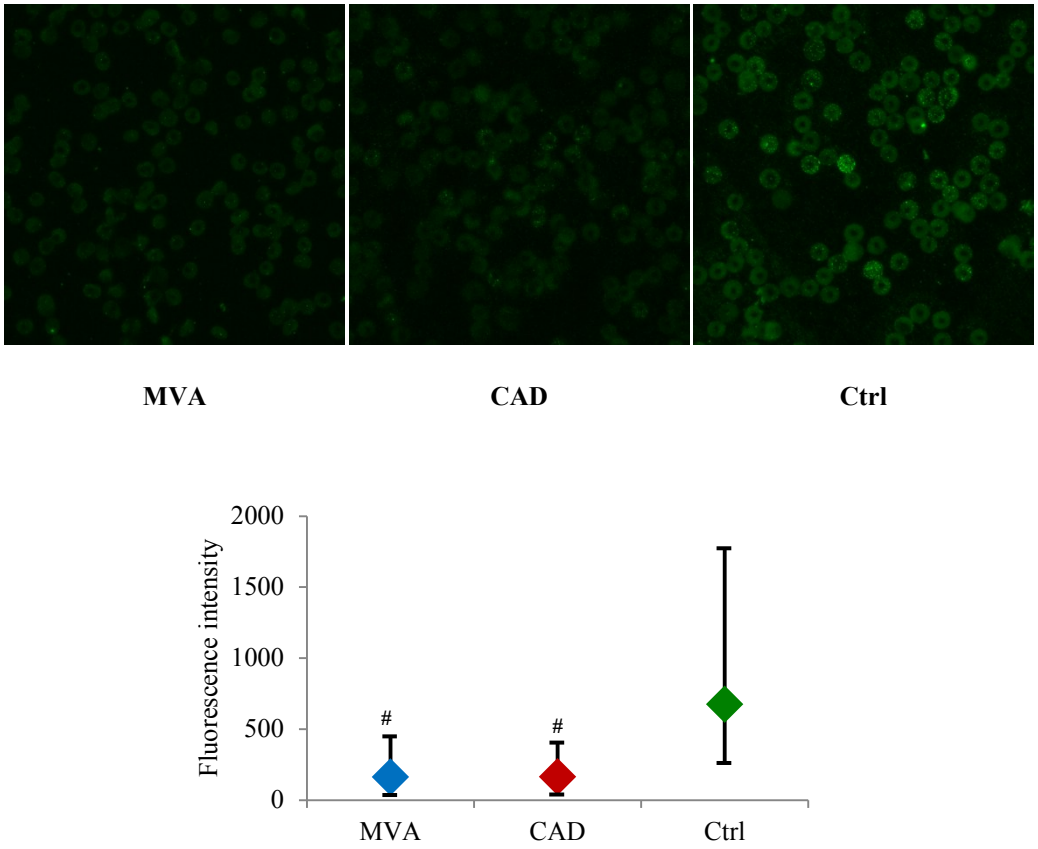


Fig 14: NOS expression in human RBCs. Representative immunofluorescent images (630 x magnification) of RBCs isolated from patients with microvascular angina (MVA) or coronary artery disease (CAD) or healthy subjects (Ctrl). Data are expressed as the mean of fluorescent intensity ± SD subtracted of the negative control value (at least three fields were analyzed, n = 10 subjects for each group). # $p<0.05$ *vs* Ctrl.

We also evaluated the expression of both isoforms of arginase, the other enzyme able to metabolize arginine in erythrocytes. As regard to the isoform II, it was not detectable in RBCs of Ctrl and of patients (data not shown). In contrast arginase I, is less expressed in RBCs of MVA patients and in Ctrl than in CAD ones (Fig 15).

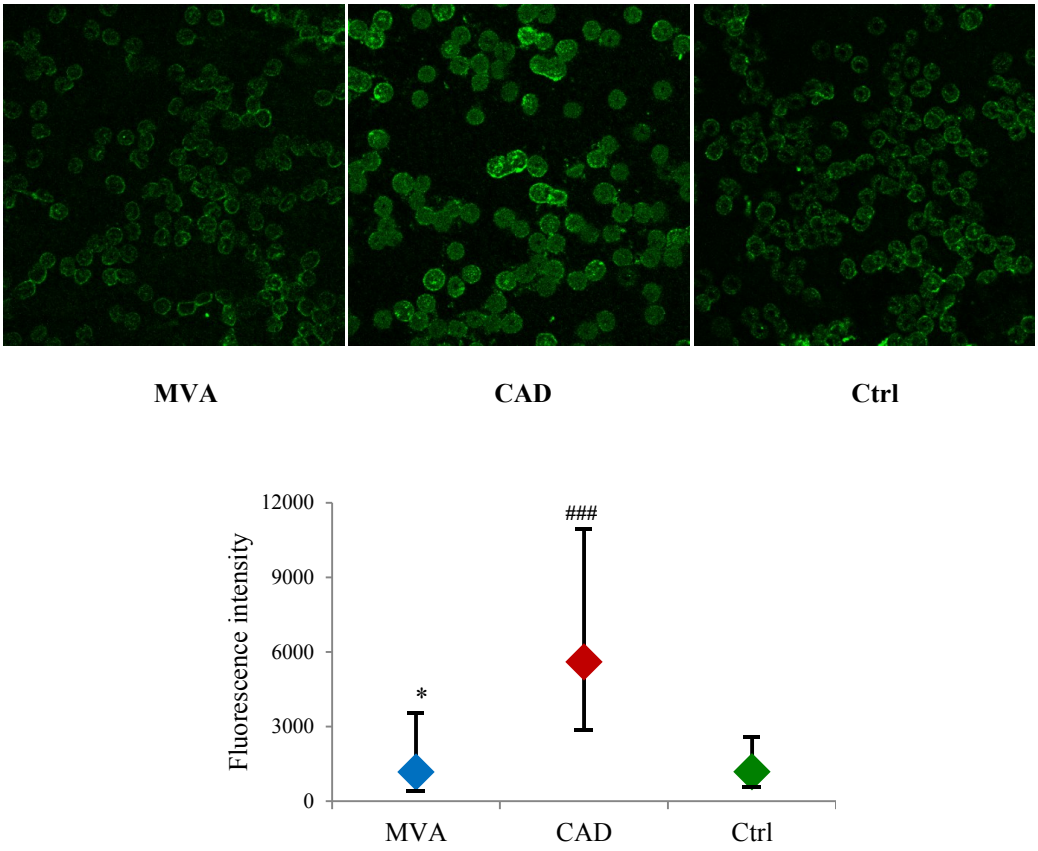


Figure 15: Arginase I expression in human RBCs. Representative immunofluorescent images (630 x magnification) of RBCs isolated from patients with microvascular angina (MVA) or coronary artery disease (CAD) or healthy subjects (Ctrl). Data are expressed as the mean of fluorescent intensity ± SD subtracted of the negative control value (at least three fields were analysed, n = 10 subjects for each group). * $p<0.05$ *vs* CAD; ### $p<0.001$ *vs* Ctrl.

5.4. RBC-NOS activity

The activity of RBC-NOS was tested *in vitro* by measuring the hydrolysis of the L-[$^{15}N_2$]arginine to the labeled product L-[^{15}N]citrulline, which is produced in equimolar amounts as NO during the reaction. After incubation of RBCs with L-[$^{15}N_2$]arginine a marked accumulation of Orn was found (mean increment over basal of 27.8±7.8, 26.3±8.3 and of 29.1±9.8 µmol/10^6 cells in MVA, CAD patients and Ctrl, respectively, P=0.001 for each). These data documented and confirmed the predominant activity of arginase *vs* NOS in RBCs. The evaluation of NOS activity could be performed in fact only in the presence of arginase inhibitor Nor-NOHA. In these conditions the RBC-NOS activity, assessed by the ratio between the L-[^{15}N]citrulline and residual L-[$^{15}N_2$]arginine, was similar between the

two groups of patients but significantly reduced in CAD and MVA with respect to Ctrl (P=0.051 and P=0.049 for MVA *vs* Ctrl and for CAD *vs* Ctrl, respectively) (Fig 16).

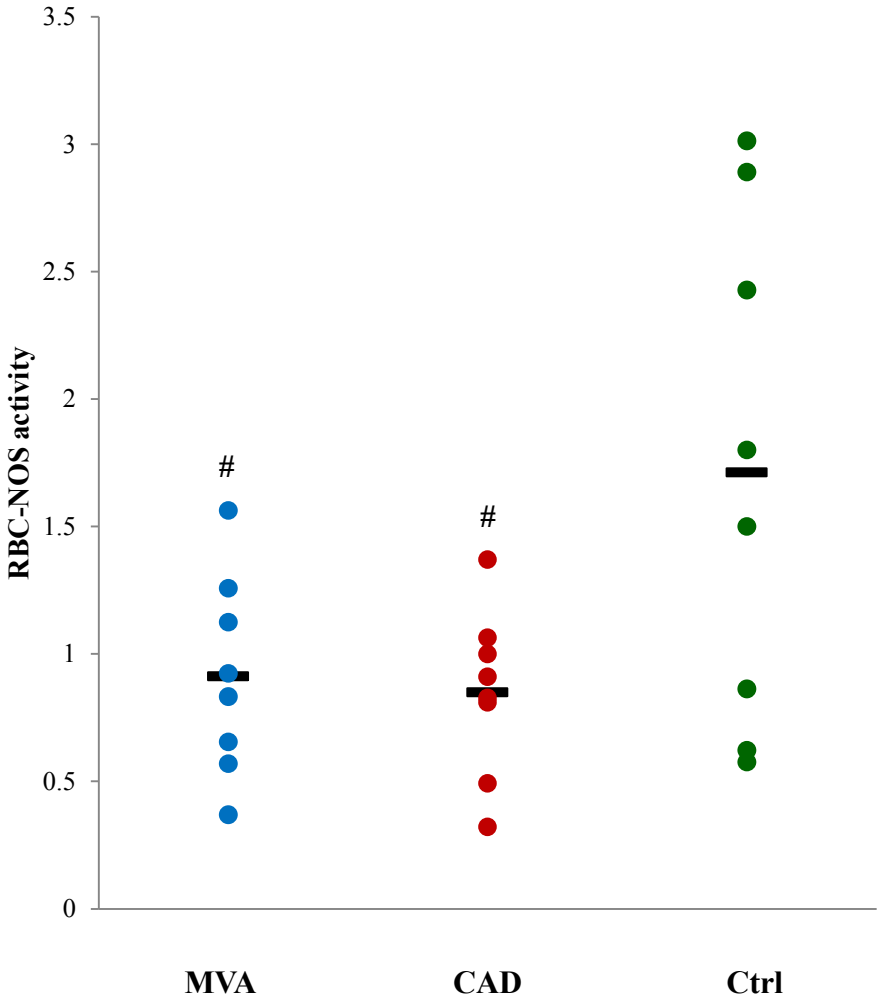


Figure 16: NOS activity in lysed RBCs. RBC-NOS activity was measured after the addition of [$^{15}N_2$] L-arginine in the presence of arginase inhibitor nor-NOHA (50 µM). The levels of [^{15}N] L-citrulline and [$^{15}N_2$] L-arginine were determined by LC-MS/MS. The RBC-NOS activity was evaluated by the ratio between [^{15}N] L-citrulline formed and [$^{15}N_2$] L-arginine residue. Individual data are represented as geometric means for MVA, CAD, and Ctrl (n=8 for each group). # $p<0.05$ *vs* Ctrl.

5.7. Summary scores of NO Pathway and oxidative stress

In order to summarize the Arg/NO pathway in the examined clinical settings, the analytes, i.e. substrate, inhibitors and enzymatic products involved in NO synthesis, were combined into appropriate scores (see methods). In Figure 17, the Cartesian plane was defined by the

NO plasma score (X-axis) and the NO RBC score (Y-axis); the intersection of the axes identifies the midpoint of the entire sample, and the units are expressed in terms of standard deviations. The Ctrl group was placed in the first quadrant (positive values for both scores), whereas the two patient groups were placed in the third quadrant (negative values for both scores). To be noticed that the MVA group was located in a more negative position, along the NO RBC score axis, compared with the CAD group, however the difference did not reach statistical significance.

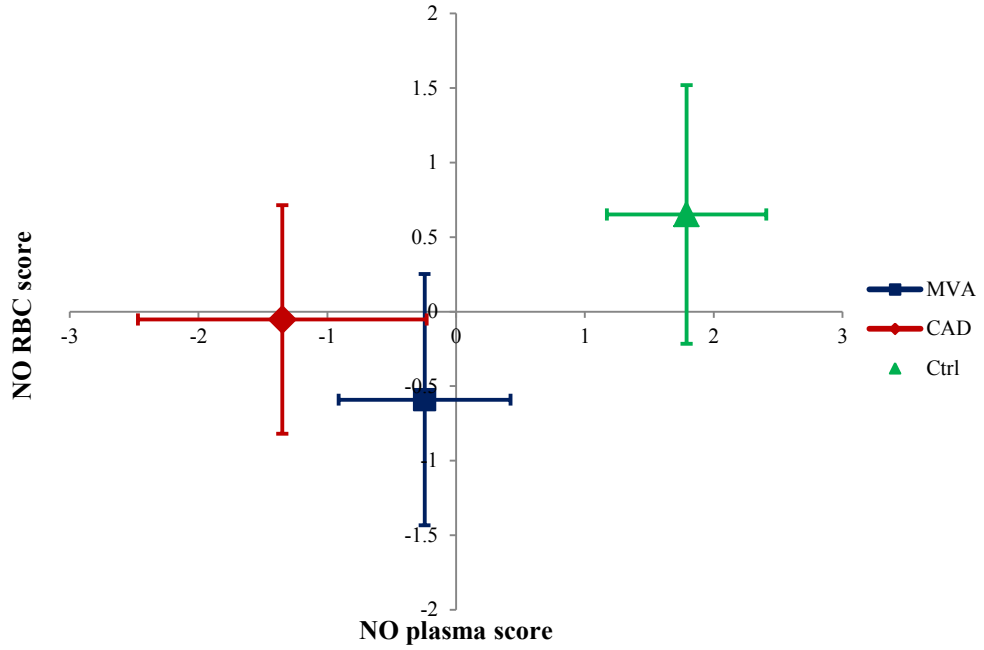


Figure 17: Arg/NO pathway score in plasma and in RBCs. The scores are computed after standardization of the analytes involved in NO synthesis (Arg, Cit, Orn, ADMA, SDMA, MMA). The standardized values of the variables positively associated with endothelial function are added, whereas the values of variables negatively associated with endothelial function are subtracted. The NO score is calculated in plasma and in RBC compartment. NO RBC score: $p< 0.05$ MVA *vs* Ctrl; NO plasma score: $p<0.001$ MVA *vs* Ctrl and CAD *vs* Ctrl.

Figure 18 shows the Cartesian plane defined by the oxidative stress score (X-axis) and the NO plasma score (Y-axis). In this graph, the control group was placed in the quadrant characterized by a negative oxidative score and by a positive NO score. In contrast, both groups of patients were placed in the quadrant relative to a positive oxidative score and a negative NO score, with the CAD group located in a more extreme position with respect to MVA (although this difference did not reach statistical significance: P=0.08 for multivariate ANOVA).

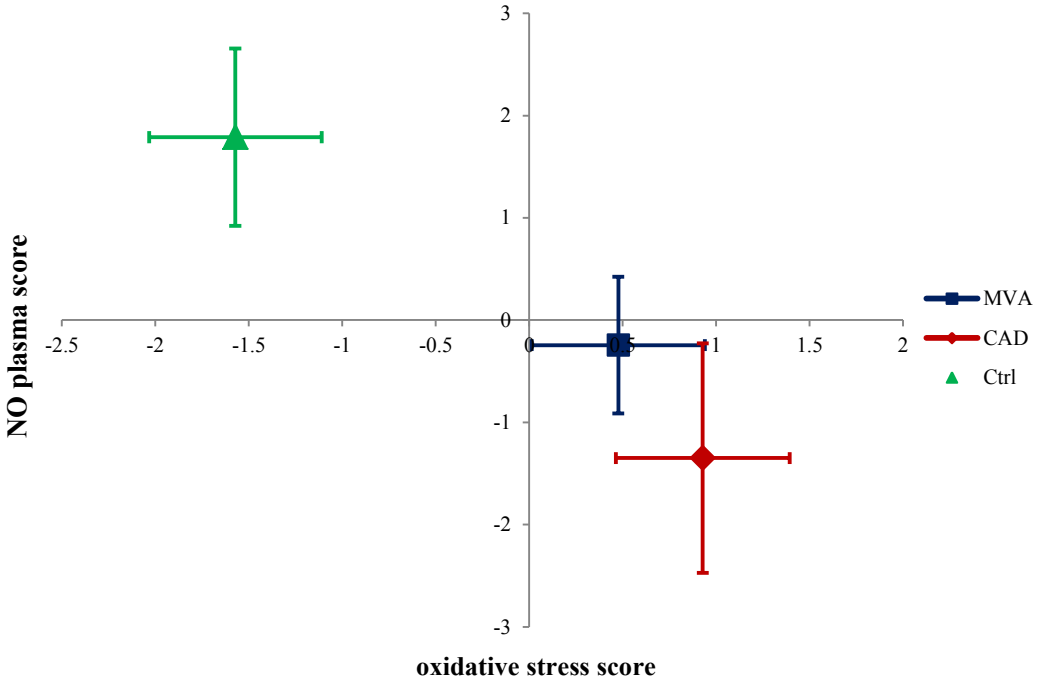


Figure 18: NO plasma and oxidative stress scores. The NO plasma score is computed after standardization of the analytes involved in NO synthesis (Arg, Cit, Orn, ADMA, SDMA, MMA). The standardized values of the variables positively associated with endothelial function are added, whereas the values of variables negatively associated with endothelial function are subtracted. The oxidative stress score is calculated by the values of GSSG (plus sign) and GSH (minus sign). NO plasma score: $p< 0.001$ MVA vs Ctrl and CAD vs Ctrl; oxidative stress score: $p<0.001$ MVA *vs* Ctrl and CAD *vs* Ctrl.

6. Discussion and conclusions

Initially, it was hypothesized that RBCs serve as scavengers or transporters for NO produced by endothelial cells [169] but, recently, an active role of RBCs in NO biosynthesis has been recognized [49]. In parallel to these findings, it has been demonstrated that alterations of this cellular compartment associates with a poor prognosis in patients with coronary disease and plasma Hb is an independent predictor of major adverse cardiovascular events in patients with acute coronary syndromes [170, 171]. The pathophysiology of MVA is not completely understood, as yet, even if the several metabolic, haemodynamic and vasospastic alterations have been linked to this syndrome. Recently it has been reported that red cell distribution width (RDW) values are significantly higher in both MVA and CAD patients compared to healthy subjects [172]. However, as documented by the absence of modifications in RDW values (data not shown), in our study the impairment of NO pathway in RBCs of MVA patients is not associated with changes in the size of circulating RBCs.

In this study we have investigated the overall metabolic pathway involved in NO biosynthesis in RBCs and we compared them to the levels of metabolites involved in this pathway in plasma. We report here unprecedented data that depict an impairment of NO biosynthetic pathway in RBCs of MVA and CAD patients, compared to those of healthy subjects. This impairment might be ascribed to the higher levels of ADMA and SDMA in RBCs from MVA compared to Ctrl, that do not markedly differ however from those found in CAD. Increased levels of these NO biosynthesis inhibitors in plasma of patients with a stable cardiovascular disease have been previously reported by others and by our group [173-175] and in some cases the impaired renal function of CAD has been considered to be the main reason for these findings [176, 177]. Patients in this study, however, had a renal function within the normal range, and, accordingly, plasma levels of SDMA were similar to Ctrl. Instead, RBC levels of SDMA in these patients were significantly greater than in Ctrl.

Concerning the other factors involved in NO biosynthesis, several observations were done. First, Arg bioavailability, e.g. Arg/Orn+Cit, was comparable between patients and Ctrl in RBCs, but not in plasma. This finding points out toward a potential dynamic equilibrium, which is relevant in order to maintain intracellular homeostasis. The higher levels of Orn in plasma of CAD patients reflect the preponderance of the metabolic pathway that transforms Arg into Orn, as also reflected by the higher levels of Orn/Cit ratio.

Finally, Cit levels were similar in patients and in controls, both in plasma and in the cellular compartment analysed. In this context it is of relevance to underline that Cit may

derive not only from the activity of NOS on Arg, but also from that of dimethyl arginine dimethylaminohydrolase (DDAH) on ADMA. It should be noted, however, that these pathways supply only a minor fraction of plasma Cit as its major source is the small intestine [178]. Indeed, in our opinion, the lack of difference in Cit levels between patients and Ctrl is due to a continuous exchange between the cellular and plasma compartments, rather than to a direct effect of the degradation of ADMA, as the presence of DDAH in RBCs is controversial [58, 59]. As a consequence, in a Cartesian plane, defined by NO scores, the MVA group is located in a negative position along the NO RBC score axis with respect to Ctrl, thus suggesting a possible alteration in NO production, more pronounced in MVA with respect to CAD.

The assessment of the NO pathway in RBCs should take into account the expression and activity of the NOS enzyme. In agreement with previous studies, we detected the presence of NOS in RBCs mainly in the membrane compartment, and this compartmentalization might be relevant for the export of NO toward other cells or to plasma, as previously suggested by Cortese-Krott et al [179]. In addition, intracellular NO in RBCs could induce electromechanical modifications of proteins and lipids/lipoproteins present in the membranes, thus preventing NO consumption by RBCs [180]. Although the presence of NOS in RBCs has been confirmed, data concerning the activity of this enzyme are still controversial [49-52, 179]. However variable experimental conditions and methodological shortcomings were reported. For instance, Kang et al [51] carried out the study in homogenized cell fractions and it is possible that cofactors necessary for NOS activity such as FAD and BH_4 are lost during homogenization as suggested by Mehta [181]. Bohmer [52] did not find a functional RBC-NOS but he didn't take into consideration the activity of arginase.

In our study NOS activity in RBCs was measured in cell lysates, in order to preserve the whole metabolic cellular system, by the formation of [^{15}N] L-citrulline *in vitro,* after the inhibition of arginase. Indeed, arginase, by competing with NOS for the substrate Arg, can limit its availability for NO biosynthesis in intact cells. Although the affinity of Arg is much higher for purified NOS (Km approximately 2-20 μM) than for arginase (Km approximately 2-20 mM), the maximum activity of arginase at physiological pH is 1000-fold greater than that of NOS [182]. Indeed, overexpression of the two isoforms of arginase (isoforms 1 and 2) in endothelial cells has been reported to suppress NO generation [183], which suggests that the inhibition of the two arginase isoforms is associated with an increase of NO biosynthesis by endothelial cells [184]. In addition, in coronary arteries of

diabetic rats, an increased arginase activity resulted in a reduced bioavailability of Arg as a substrate for eNOS [185].

The importance of endothelial arginase in different pathological conditions as hypertension, diabetes, ischaemia-reperfusion, cystic fibrosis, sickle cell disease and asthma has already been reported [186]. In RBCs of hypertensive patients, increased arginase activity accompanied by reduced levels of nitrite and nitrate was found [187].

Overall, increased arginase activity in endothelial cells has been proposed to promote a proatherogenic effect due to the reduction of cell NO biosynthesis [188]. Accordingly, our data show that in RBCs the substrate Arg is preferentially metabolized by the arginase enzyme, as documented by the selective increase in Orn levels after the addition to RBCs of [$^{15}N_2$] L-arginine. Arginase I, which is the only arginase so far described in RBCs, accounts for about 98% of total blood arginase activity [189]. In our condition, greater amounts of arginase I in CAD patients, were found. Since it has been reported that erythroid progenitor cells, express both arginase I and arginase II [55], we measured also this enzyme in RBCs. According to the literature we failed to detect measurable amounts of arginase II in Ctrl or in patients.

This predominant activity of arginase might explain the apparent inconsistency of our data with those previously published by others, which reported that NOS enzyme in RBCs is inactive [51, 52].

Interestingly, we found that the lower expression of NOS detected by confocal microscopy in RBCs of MVA and CAD patients went in parallel with a reduced activity of the enzyme. We cannot exclude the effects of pharmacological treatments, ongoing in patients, on NOS activity. Indeed some authors evidenced that an angiotensin converting enzyme inhibitor, lisinopril, monotheraphy or combined with statin therapy, decreases arginase activity in RBCs [187]. In addition statins [190] and aspirin [191] were described to induce NOS activity in RBC *in vivo*. On these bases, these drugs could result in an increase of NO RBC production masking a more conspicuous difference in MVA and CAD *vs* Ctrl.

Arg metabolic pathway may be impaired by the occurrence of oxidative stress, which in turn stimulates arginase activity [186] and conversely inhibits NOS activity [192, 193].

Of relevance is the observation that the ratio between oxidized and reduced forms of glutathione was almost doubled in whole blood of MVA patients with respect to Ctrl, suggesting an increased oxidative stress in this condition. A role of oxidative stress in lowering NO bioavailability has previously highlighted, but the information in MVA is still

scanty [130, 146, 194]. However the decrease of GSH in MVA patients is in accordance with data reported by Dhawan et al. [195], who showed a positive correlation between GSH levels and coronary flow velocity reserve, thus predicting impaired microvascular function.

We attained also a marked increase of GSSG/GSH, based on the increase of oxidized glutathione, that was even more pronounced in CAD patients.

Interestingly, we also found a positive correlation between GSSG/GSH and Orn/Cit only in CAD patients, further supporting the predominant activation of arginase over NOS in the presence of oxidative stress in this clinical setting.

The concomitant assessment of oxidative stress and NO pathway in patients indicates that both MVA and CAD are placed in the Cartesian plane quadrant relative to a positive oxidative score and a negative NO score, with the CAD group located in a more extreme position with respect to MVA.

In conclusion, the study above described shows for the first time that RBCs of patients with MVA contain higher levels of inhibitors of the NO synthesis than Ctrl, and that these levels do not markedly differ from those found in CAD patients. A similar picture is found in plasma, as previously described by others [134, 196]. Finally, NOS expression in RBCs was found markedly reduced in both MVA and CAD patients. The reduced expression of this enzyme evidenced in MVA patients was related to a reduced activity, suggesting the presence of an intermediate phenotype, between CAD and Ctrl, in this clinical setting. The increased oxidative stress found in both patient groups argued the presence of this common eziology. To confirm this data, arginase expression was found increased in both pathological settings.

The finding that RBC-NOS activity and expression was altered in MVA is of particular relevance because these cells may represent an important NO source in a pathological condition characterized by alterations in microcirculatory bed.

In CAD patients therapeutic interventions aimed at reducing intracellular oxidative stress, e.g. restoration of GSH levels, might be effective in improving the balance between NOS and arginase activities.

7. References

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