Oxidative Medicine and Cellular Longevity

Endothelium and Platelets as a Common Ground for Oxidative Stress Induced Alterations in Thrombosis, Haemostasis, and Inflammation in Acute and Chronical Diseases

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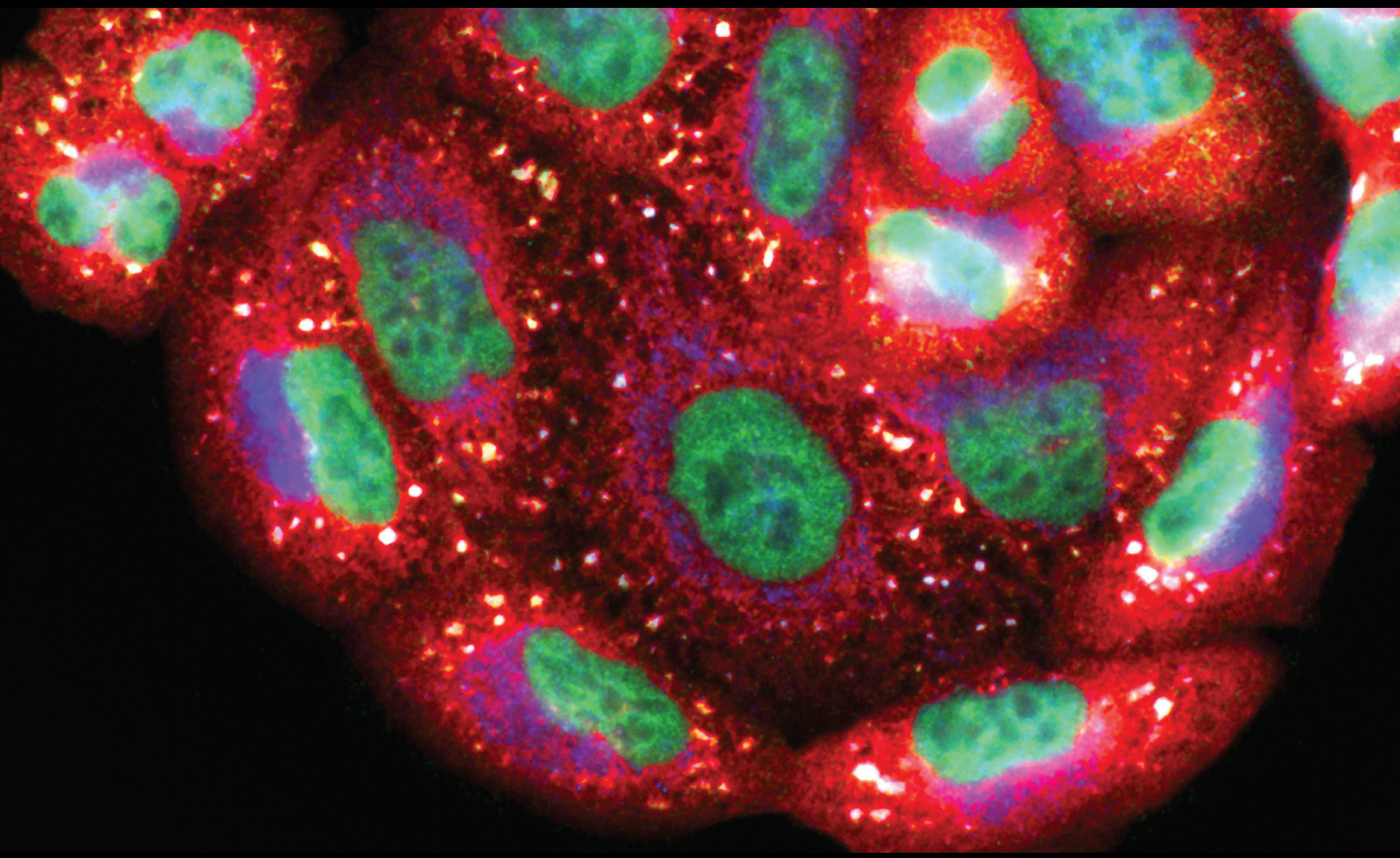

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Erratum

Erratum to "The Inhibition of P-Selectin Reduced Severe Acute Lung Injury in Immunocompromised Mice"

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In the article titled "The Inhibition of P-Selectin Reduced Severe Acute Lung Injury in Immunocompromised Mice" [1], the incorrect file for Figures 6(a) and 6(g) was used during the production process and the figure should be corrected as follows:

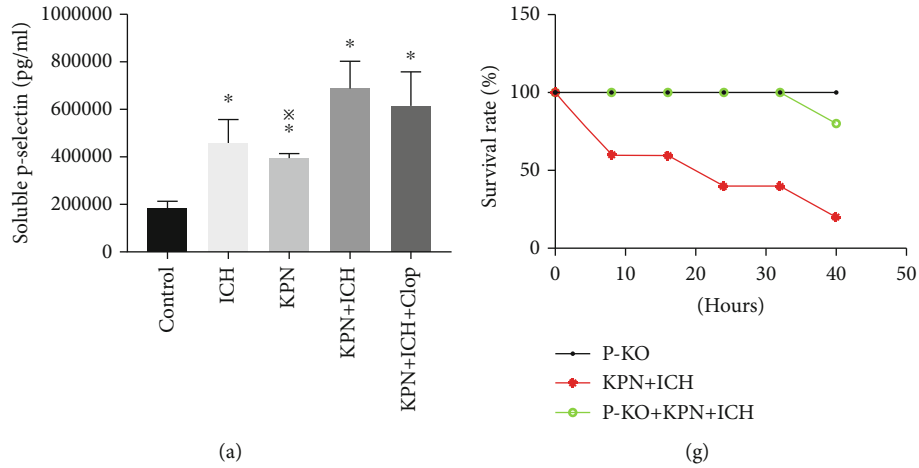

FIGURE 6: Knockout of p-selectin alleviate acute lung injury in ICH mice. (a) The expression of serum P-selectin in mice and (g) the survival curve of mice.

References

[1] Y. Liu, D. Xiang, F. Gao, H. Yao, Q. Ye, and Y. Wang, "The Inhibition of P-Selectin Reduced Severe Acute Lung Injury in Immunocompromised Mice," *Oxidative Medicine and Cellular Longevity*, vol. 2020, Article ID 8430465, 13 pages, 2020.

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Review Article

Effects of REDOX in Regulating and Treatment of Metabolic and Inflammatory Cardiovascular Diseases

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Reduction oxidation (REDOX) reaction is crucial in life activities, and its dynamic balance is regulated by ROS. Reactive oxygen species (ROS) is associated with a variety of metabolic diseases involving in multiple cellular signalling in pathologic and physiological signal transduction. ROS are the by-products of numerous enzymatic reactions in various cell compartments, including the cytoplasm, cell membrane, endoplasmic reticulum (ER), mitochondria, and peroxisome. ROS signalling is not only involved in normal physiological processes but also causes metabolic dysfunction and maladaptive responses to inflammatory signals, which depends on the cell type or tissue environment. Excess oxidants are able to alter the normal structure and function of DNA, lipids, and proteins, leading to mutations or oxidative damage. Therefore, excessive oxidative stress is usually regarded as the cause of various pathological conditions, such as cancer, neurodegeneration, cardiovascular diseases (CVDs), diabetes, and kidney diseases. Currently, it has been possible to detect diabetes and other cardiac diseases by detecting derivatives accompanied by oxidative stress in vivo as biomarkers, but there is no effective method to treat these diseases. In consequence, it is essential for us to seek new therapy targeting these diseases through understanding the role of ROS signalling in regulating metabolic activity, inflammatory activation, and cardiac diseases related to metabolic dysfunction. In this review, we summarize the current literature on REDOX and its role in the regulation of cardiac metabolism and inflammation, focusing on ROS, local REDOX signalling pathways, and other mechanisms.

1. Introduction

Oxidative stress can be defined as active oxygen/nitrogen excessive production of ROS, such as oxidant, and lack of antioxidant enzymes. The detoxification of compounds in the cells is usually normal, but when the oxidant emissions are excessive, the cell produces excessive oxidation material to change DNA lipid and protein structure, leading to cell mutation and oxidative damage. Excessive oxidative stress, therefore, is considered as the causes and consequences of a variety of pathological processes, including cancer, neural degeneration, CVDs, diabetes, and kidney diseases [1, 2]. Some studies have found that a balance of oxidative stress is associated with aging [3]. Most kinds of natural or synthetic antioxidants have been evaluated against the oxidative stress-related pathological changes [1, 4, 5]. Besides, ROS is a by-product of many cell compartment enzymatic reactions occurring on the cytoplasm membrane endoplasmic reticulum (ER), or mitochondria, which can control intracellular environment balance and work as the main regulatory factor of cell dysfunction in the pathophysiology. In different cell types or organizational environment, ROS signals may participate in increased inflammatory of incommensurate reaction or lead to metabolic dysfunction-related diseases, such as atherosclerosis, diabetes, and heart stroke [6]. In addition, emerging studies have revealed that a healthy diet plays a critical role in the prevention of CVDs by modulating the oxidative balance [7, 8]. For example, a healthy diet can prevent atherosclerosis by inhibiting the oxidation of low-density lipoprotein (LDL) and reducing the production of ROS [9]; the results of the PREDIMED study [10] show that highly unsaturated fat and antioxidant-rich dietary patterns are useful for reducing the risk of CVDs. Therefore, understanding of ROS signals in regulating metabolic activity and

inflammation will promote the discovery of new therapies treating CVDs.

2. ROS Generation

The generation of ROS is involved in a series of complex biochemical reactions [11]. The cascade of ROS generation consists of the following five main pathways (Figure 1):

(a) *O_{2^-} formation*: O_2- is produced by the coupling of O_2 with electrons (e^-) from donors, which is usually considered to be the first ROS cascade reaction. In mammalian cells, e^- donors are usually reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH). O_2- can be converted into other kinds of ROS by an oxidation reaction.

(b) *RNS formation*: RNS is a derivative of NO•, and NO• is produced by L-arginine (L-Arg) and catalyzed by NOS. NO• can react quickly with O_2•− to form $ONOO^-$. The second-order rate constant between NO• and O_2•− is nearly 10 times faster than that of O_2•− catalyzed by superoxide dismutase [12, 13]. However, due to the high intracellular SOD content under physiological condition, O_2- was removed before encountering NO•.

(c) *H_2O_2 formation*: H_2O_2 is produced by O_2- mutation catalyzed by superoxide dismutase (SOD). At low pH, a small amount of O_2- mutation occurs spontaneously, and some of which can react with reductive transition metals, such as $[4Fe\text{-}4S]^{2+}$. Some oxidases (e.g., NOX4 and DUOX1/2) have dismutase activity and can directly convert O_2 into H_2O_2 instead of O_2-.

(d) *OH• formation*: OH• can be generated from homolysis fission of ONOOH, and most of OH• is formed by metal ions (iron or copper) catalyzed by H_2O_2 and O_2- through the Habor-Weiss reaction. In diseases with iron accumulation (e.g., atherosclerotic lesions [14] or sickle cell patients [11]), OH• mediated oxidative stress might be the most pivotal mechanism. OH• has strong oxidation ability and short half-life, which is the major cause of biological macromolecule damage by ROS.

(e) *L•/LOO• formation*: The highly active OH• or $ONOO^-$ can react with the polyunsaturated fatty acid (PUFA) of the biofilm for lipid peroxidation, in which OH• can react directly with lipids to capture a hydrogen atom to form a carbon-centered lipid free radical (L•). L• initiates lipid peroxidation under an aerobic condition and generates lipid peroxide group (LOO•), which is a medium oxidant that can extract H from nearby lipids to produce lipid hydrogen peroxide (LOOH). Moreover, L•/LOO• can exist in the reaction process of the lipoxygenase-catalyzed polyunsaturated fatty acid formation of molecular oxygen to form hydroperoxide [15, 16].

3. Dynamics of ROS

In order to maintain the stability of ROS, there are five active oxygen scavenging pathways:

(a) O_2- mutated to H_2O_2 by superoxide dismutase (SOD)

(b) Catalase (CAT) decomposes H_2O_2 to produce H_2O and O_2

(c) *Glutathione redox cycle*: using glutathione as an electron donor, H_2O_2 and LOOH are decomposed by glutathione peroxidase (GPX).

(d) *Thioredoxin reduction cycle*: using reduced thioredoxin (TrxR) as electron donor, H_2O_2 was reduced by redox protein (PRDX) 1-5 to produce H_2O.

(e) Exogenous detoxification of glutathione transferase (GST)

4. Oxidative Stress and CVDs

CVD is the leading cause of death worldwide [17], which is a complex pathophysiological disease involved in many factors. The dysdynamics of ROS has been regarded as one of the potential pathogenic factors [18–20]. Increased ROS level is able to lead to decreased availability of nitric oxide and vasoconstriction, which subsequently promotes arterial hypertension [21]. ROS also has negative effects on myocardial calcium treatment, inducing arrhythmias and cardiac remodeling by facilitating hypertrophic signal transduction and apoptosis [22, 23]. In addition, it also promotes the formation of atherosclerotic plaques [24].

4.1. Arterial Hypertension. It is estimated that the global prevalence of hypertension was 1.13 billion in 2015, the risk of which becomes higher with age [25]. A large number of studies have shown that ROS plays an important role in the pathogenesis of hypertension [26–28].

In the vascular system, ROS is mainly produced by vascular endothelial cells, adventitia cells, and smooth muscle cells, primarily induced by NADPH oxidase which produces O_2- upon being stimulated by Angiotensin II (Ang-II), Endothelin-1 (ET-1), or urotensin II (U-II). On the other side, increased mechanical forces caused by elevated blood pressure, such as unidirectional laminar flow and oscillatory shear stress, can help to increase the accumulation of ROS. Ca^{2+} is involved in the regulation of cell contraction, secretion, metabolism, gene expression, and cell survival [29]. The interaction of ROS and Ca^{2+} plays an important role in the occurrence and development of CVDs [30–32]. Store-operated Ca^{2+} channel (SOCC) is a ubiquitous Ca^{2+} influx pathway and is the dominant Ca^{2+} channel in unexcited cells [33, 34]. ORAI/STIM is a highly selective calcium channel and an important component of SOCC [35, 36]. ORAI/STIM channel is involved in a variety of cardiovascular physiological processes [37]. Oxidative stress can regulate the activity of the ORAI/STIM channel by uncoupling ORAI/STIM complex, regulating the gene expression of ORAI or STIM

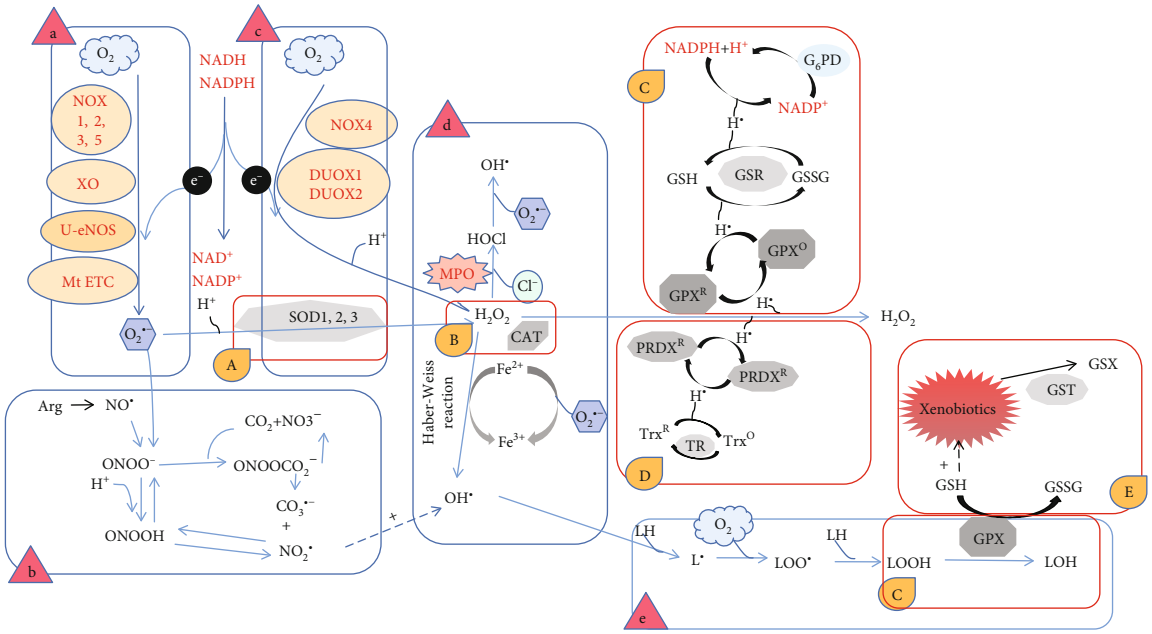

FIGURE 1: ROS generation and clearance. I. ROS generation: (a) superoxide formation; (b) reactive nitrogen species (RNS) formation; (c) hydrogen peroxide formation; (d) hydroxyl radical formation; (e) lipid radical formation. II. ROS clearance: (A) superoxide dismutation; (B) hydrogen peroxide decomposition; (C) glutathione redox cycle; (D) thioredoxin redox cycle; (E) glutathione-*S*-transferase detoxification.

protein, and oxidizing ORAI or STIM protein [35, 38]. Studies have shown that ROS regulates the ORAI/STIM channel by directly targeting the conserved cysteine residues in ORAI and STIM molecules [39, 40]. ROS can also act as the second messenger in cells to promote the increase of intracellular Ca^{2+} concentration and lead to vasoconstriction, thus assisting the pathogenesis of hypertension [41]. Ang-II-induced hypertension involves redox-dependent signal cascade activation and NADPH oxidase-induced ROS production [42]. Some common antihypertensive medications, such as Ang-I receptor blockers and angiotensin-converting enzyme (ACE) inhibitors, have been shown to reduce blood pressure partly by inhibiting NADPH oxidase and reducing the ROS production [43].

4.2. Atherosclerosis. Atherosclerosis is one of the main causes of cardiovascular death in developed countries [17]. More and more evidence shows that oxidative stress plays a key role in the formation of atherosclerosis [44, 45]. The activation of proinflammatory signal pathway, the expression of cytokines/chemokines, and the increase of oxidative stress are some of the mechanisms underlying atherosclerosis [20].

ROS is an autophagy trigger factor. Excessive ROS in cells is able to cause oxidative stress, which will further activate autophagy [46, 47]. Autophagy is closely related to the development of atherosclerosis [48, 49]. Excessive autophagy can lead to autophagic cell death [50]. Autophagic death of endothelial cells can damage plaque, form thrombus, and cause atherosclerosis [51]. Therefore, elucidating the specific mechanism of ROS-regulating autophagy may be a feasible way to treat atherosclerosis.

Oxidative stress reduces the expression of prethrombotic antioxidant P-oxidase-2 (PON2) in human atherosclerotic plaques, especially in endothelial cells [52]. Ebert et al. revealed the redox-dependent mechanism of PON2, which involves tissue factor (TF) activity in endothelial cells and prevents systemic coagulation activation and inflammation (Figure 2) [52].

NADPH oxidase is the main source of ROS in atherogenesis, enhancing the production of superoxide and aggravating oxidative stress, leading to the occurrence and development of arterial disease [53, 54]. Gray et al. [55] knocked out the NOX1 and NOX4 genes in streptozotocin (STZ-) induced diabetic $ApoE^{-/-}$ mice and found that loss of NOX1 had a significant antiatherosclerotic effect, which was related to the decreased production of ROS. GPX4 is one of the glutathione peroxidases, which can effectively interact with lipid hydrogen peroxide and catalyze the degradation of peroxides [56]. Mitochondrial GPX4 can avoid ROS damage and maintain intravascular homeostasis by clearing ROS. Overexpression of mitochondrial GPX4 can alleviate myocardial ischemia/reperfusion injury [57]. GPX4 can inhibit ferroptosis by scavenging lipid peroxides and improve the function of the heart [58–60]. GPX4 overexpression inhibits atherosclerosis in $ApoE^{-/-}$ mice [61, 62]. Hyperglycemia can increase the production of ROS, such as O_2- and peroxide, through the mitochondrial electron transport chain, and then form a positive feedback effect [63, 64]. For example, PKC can be activated by O_2-. Then, activated PKC can promote the production of NADPH oxidase-dependent ROS [65, 66]. O_2- in mitochondria can increase the production of intracellular advanced glycation end products (AGEs) in cells [67, 68]. AGEs can add oxygen radical, and the activation of AGEs receptor can cause intracellular oxidative stress, which in turn causes inflammation in endothelial cells [69–71]. Therefore, AGEs eventually lead to atherosclerosis by modifying the extracellular matrix and circulating lipoproteins and activating AGEs receptors [66]. In addition, Zhu et al. showed that AGEs could accelerate

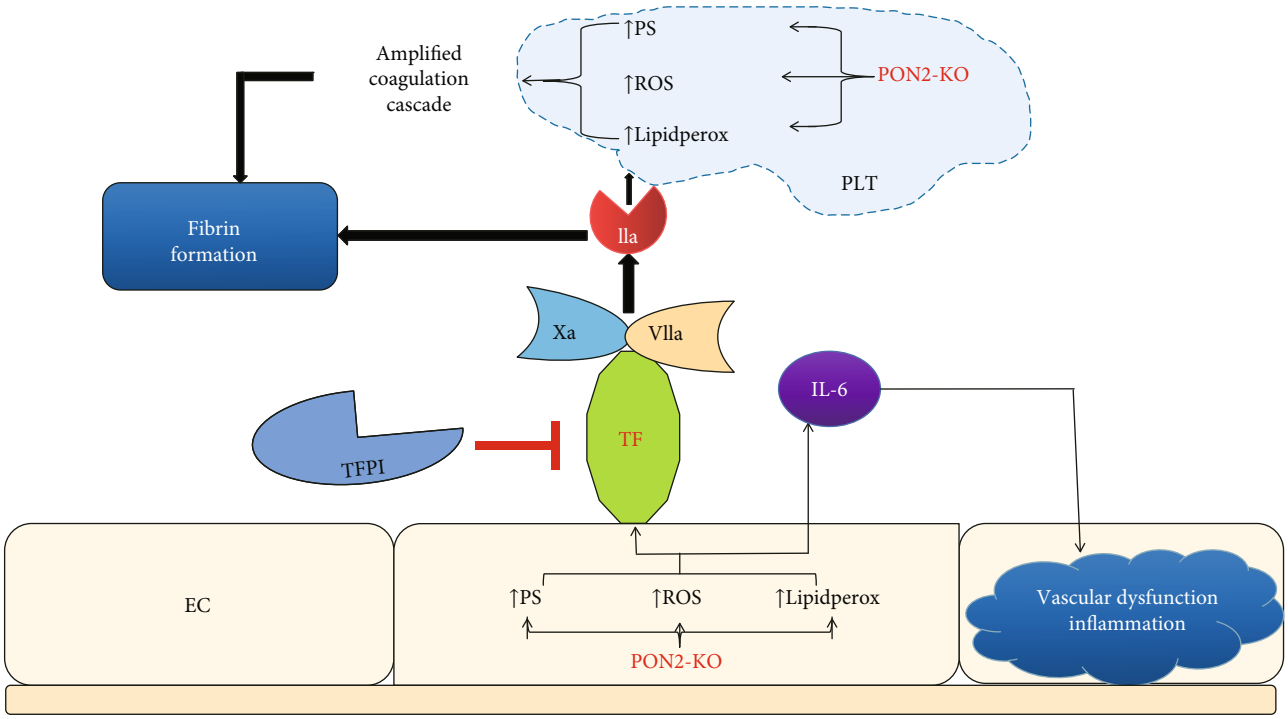

Figure 2: Schematic of EC and platelet-mediated procoagulant and vascular inflammatory processes in pon2$^{-/-}$ environment. EC-mediated systemic inflammation is established by elevated levels of interleukin-6 (IL-6), which may promote vascular inflammation and dysfunction. Knockout of PON2 can lead to the accumulation of phosphatidylserine (PS), ROS, and lipidperox in ECs; result in the production of fibrin through cascade reaction; and ultimately consolidate the function of blood coagulation.

vascular calcification through the pathway of hypoxia-inducer/pyruvate dehydrogenase kinase 4 [72].

It is worth mentioning that a recent research found that colchicine, which is a drug widely used in the treatment of nonspecific inflammation, could combine with cholesterol crystal (CC), an important pathological marker for the vulnerability of atherosclerotic plaques [73]. This combination can reduce the intake of cholesterol crystals by endothelial cells, thus attenuating the cellular oxidative stress and endothelial cell prolapse by regulating the AMPK/SIRT1 signaling pathway [74].

4.3. Diabetic Cardiomyopathy. The complications of diabetes mainly include nephropathy, neuropathy, retinopathy, and heart diseases, which are linked with the activation of a series of oxidative stress in the body [75]. ROS can interact with a variety of biological macromolecules, such as DNA, proteins, and lipids [76, 77]. In the case of DNA damage, ROS induces DNA strand breaks and the formation of 8-hydroxydeoxyguanosine, which is a prominent feature of the diabetic heart [78]. The passive stiffness of myocardium is redox dependent, which leads to the increase of cardiac stiffness through actin oxidation and disulfide bond formation [79, 80]. In patients with diabetes, oxidative stress leads to decreased actin phosphorylation by damaging the NO/cGMP/PKG signaling [81], increased cardiomyocyte stiffness, and collagen and AGE deposition [82]. Polyunsaturated fatty acids rich in membrane lipids are easily oxidized by ROS, which is also involved in the formation of atherosclerotic plaques [83]. Lipid oxidation can lead to excessive formation of carbonyl compounds, such as aldehydes, which can accelerate a variety of pathologies [84]. NADPH oxidase is the main source of cardiac ROS, in which NOX2 and NOX4 are the two main subtypes expressed in the heart. It has been found that ROS produced by NOX is a common downstream mediator of various hemodynamic and metabolic pathways. ROS is involved in the occurrence of endothelial dysfunction and the development of diabetic vascular complications during hyperglycemia [85]. Glucose autoxidation, PKC activation, GAPDH inhibition, AGE formation, and polyol pathway activation can in turn exacerbate oxidative stress [86–88]. For example, the activation of the PKC pathway can lead to an increased expression of nuclear factor κB(NF-κB) [65, 89]. NF-κB can increase the expression of inducible nitric oxide synthase and increase the production of nitric oxide. Excessive nitric oxide reacts with peroxynitrates to produce peroxynitrates. Peroxynitrates can induce the formation of mitochondrial permeability transition holes, resulting in the increase of ROS production and the loss of cytochrome C, which exacerbates the development of diabetic cardiomyopathy [65]. Related studies found that in the aorta of STZ-induced diabetic ApoE$^{-/-}$ mice, the levels of NOX2 and NOX4 increased; in db/db mice (type II diabetes model), the expression of NOX1 and NOX4 was upregulated, and their activation resulted in the oxidation of ROS downstream molecules (e.g., tetrahydrobiopterin) and increased inflammatory response, indicating that NOX1, NOX2, and NOX4 are all involved in the pathological process of diabetic cardiomyopathy [90].

Of course, hyperglycemia is not the only pathogenic factor of diabetic cardiomyopathy, and the excessive oxidation

of free fatty acids is also not ignored, which will lead to the activation of oxidative stress mitochondria and endoplasmic reticulum stress proinflammatory signals [91–93]. What can be seen is that a large number of changes in the diabetic heart include the overexpression of ROS and abnormal redox status. It is believed that the genes or drugs that target to block the ROS pathway in the future will bring the dawn of cure to patients with diabetic cardiomyopathy.

ROS also participates in the cardiac hypertrophy signaling transduction. In insulin-induced cardiac hypertrophy, the ROS level was upregulated and the levels of catalase were decreased [94]. Hypertrophy agonist Ang-II can increase the ROS levels in cardiomyocytes, and mitochondrial oxidative stress in turn contributes to Ang-II-induced cardiac hypertrophy [95]. Antioxidant administration can inhibit cardiac hypertrophy [96]. Tumor necrosis factor-alpha causes hypertrophy via the generation of ROS in cardiomyocytes [97]. Several hypertrophic stimuli need ROS to trigger cardiac hypertrophy. ROS could be a potential biological target for the novel therapy for maladaptive cardiac hypertrophy.

4.4. Myocardial Infarction (MI). MI is one of the leading causes of disability and death in patients with CVDs in the world [98]. Programmed cardiomyocyte death, that is, apoptosis or autophagy, is considered to be the cause of MI. Cardiomyocyte apoptosis induced by ROS is controlled by a complex network of signal pathways involving noncoding RNAs [99]. For instance, under anaerobic conditions, mitochondrial fission and apoptosis-related circRNA (MFACR) suppresses the uninterrupted expression of miR-652-3p and MTP18 proteins, which leads to the imbalance of ROS, triggers the accumulation of mitochondrial fragments, and then results in apoptotic cell death [100]. ROS is involved in the toll-like receptor 4 (TLR4) and its downstream molecular pathway in mediating sympathetic activity post-MI within the paraventricular nucleus (PVN) [101]. The activation of TLR4 enhances the sympathetic activity after myocardial infarction by activating the microglia NF-κB and ROS in the paraventricular nucleus of the hypothalamus.

4.5. Heart Failure (HF). Heart failure (HF) is a progressive disease with an annual mortality rate of about 10%. Although effective treatment has improved the outcome, the prognosis is still poor [102]. Related experiments and clinical studies have shown that the increase of ROS is related to the pathogenesis of HF [103–106]. ROS stimulates myocardial growth, matrix remodeling, and cellular dysfunction by activating various hypertrophic signal kinases and transcription factors. Activation of G protein-coupled receptor (GPCR) can lead to the production of ROS. Some data shows that ROS can directly induce the dissociation and activation of G protein [107–110]. Therefore, ROS may promote the hypertrophic growth signal of neonatal rat ventricular myocytes by directly activating G protein. ROS also stimulates apoptosis signal kinase-1, a redox-sensitive kinase that, when overexpressed, leads to NF-κB-induced hypertrophy [111]. Mitochondrial ROS and mitochondrial matrix calcium ($[Ca^{2+}]m$) also participate in the pathogenesis of obesity-induced heart failure [112, 113], which may attack cardiomyocytes through the mechanism of free radical injury and combined with inflammatory cytokines (such as TNF-α and IL-6), resulting in the apoptosis of some cardiomyocytes, decreased cardiac function, and compensatory proliferation of cardiomyocytes, finally leading to myocardial hypertrophy [114].

In vitro hydrogen peroxide treatment induces oxidative stress in cardiomyocyte and leads to all kinds of cellular physiological or pathological processes, including necrosis and apoptosis. Wang et al. had identified several de novo pathways that underlie these processes. Several noncoding RNAs play functional roles in these pathways. In the programmed necrosis induced by hydrogen peroxide, long noncoding RNA NRF can combine with miR-873 and regulate the RIPK1/RIPK3 expression [115]. E2F1/miR-30b/Cyclophilin D forms a pathway in regulating hydrogen peroxide-induced necrotic cell death [116]. During hydrogen peroxide induced apoptosis, Wang et al. found that both E2F1/miR-421/Pink signal pathway and miR-361/PHB1 function in regulating mitochondria fission and apoptosis [118, 119]. All these results indicate that functional noncoding RNAs also play important role in a series of hydrogen peroxide-induced cellular responses. And these studies suggest that there might be relationships between functional noncoding RNAs and ROS, which await further study to unveil.

4.6. Atrial Fibrillation (AF). Atrial fibrillation (AF) is the most common arrhythmia in clinics, and its risk increases with age [119]. Both human and animal data confirm the role of oxidative stress in the pathogenesis of AF [120, 121]. So far, there are some antioxidants that can positively affect the development of AF [122]. Type 2 ryanodine receptor (RyR2) is the main calcium release channel in atrial myocytes. It is a dysfunction caused by oxidative stress which disturbs the intracellular Ca^{2+} homeostasis that is linked with the pathogenesis of AF [123]. In atrial myocytes, RyR2 is oxidized by mitochondrial-derived ROS, resulting in increased intracellular Ca^{2+} leakage. It is worth noting that studies have shown that reducing the production of ROS can reduce atrial diastolic Ca^{2+} leakage, thus hindering the development of AF [124].

4.7. DNA Methylation and CVDs. DNA methylation, in which methyl is added to the C-5' position in the dinucleotide sequence of cytidine-phosphate-guanosine (CpG) to inhibit gene activity by preventing transcription factors from binding to the promoter or by recruiting chromatin modifying enzymes [125]. DNA methylation is catalyzed by three different DNA methyltransferases (DNMTs): DNMT3a and DNMT3b are mainly responsible for the ab initio methylation of embryonic and postpartum tissues, while DNMT1 subsequently maintains methylation [126].

The latest advances in next-generation sequencing technology have provided de novo understanding of DNA methylation. And more and more studies found that there are significant contributions of noncoding RNA in the pathophysiology of HF [127].

Long noncoding RNAs (lncRNAs) can regulate gene expression at the epigenetic level by directly or indirectly regulating the interaction with other molecules [128]. lncRNAs show epigenetic characteristics similar to those of coding

genes, such as maternal effects, DNA methylation and histone modification, and posttranscriptional regulation [129].

In a series of causes, abnormal gene expression may be related to specific DNA methylation. The specific knockout of DNA methylase DNMT3b gene in the heart can lead to cardiomyocyte interstitial fibrosis and sarcomere disorder and accelerate the deterioration of systolic function and thinning of the ventricular wall during HF [130]. lncRNA-H19 is closely related to genomic imprinting [131]. It can change the methylation level of DNA by regulating the activity of S-adenosyl methionine(SAM), which plays an important role in cardiovascular diseases [129]. lncRNA-Mhrt can directly interact with histone modifiers to regulate chromatin modification, and its upregulated expression can prevent pathological myocardial hypertrophy [128]. lncRNA upperhand can regulate the expression of the hand2 gene related to cardiac development by allele specificity and cis-regulation [132].

The interaction between lncRNA-Chaer and the catalytic subunit of histone modification complex PRC2 interferes with the targeted genomic site of PRC2, thus inhibiting the methylation of histone H3 lysine 27 residues in the promoter region of cardiac hypertrophy related genes [133]. Inhibition of Chaer can significantly reduce myocardial hypertrophy and dysfunction.

The application of the targeted drugs to interfere with epigenetic dynamics is likely to become a new direction of drug research and development for cardiovascular diseases in the future. For example, trichostatin A, a class I and II histone deacetylase (HDACs) inhibitor, can prevent ischemia-induced left ventricular remodeling by inhibiting the TNF-α transcription and promote angiogenesis and cardiomyocyte survival by enhancing the Akt phosphorylation [134]. HDAC inhibitor sodium butyrate can block NF-κB signal transduction and inflammatory factors and improve myocardial infarction and atherosclerosis [135]. In addition, folic acid, histone deacetylase inhibitor apicidin, peroxisome proliferator-activated receptor-gamma agonist, and valproic acid were found to contribute to the restoration of chromatin modification in cardiac metabolism [136].

5. Diet Participates in the Regulation of ROS

More and more evidences have pointed that diet is closely linked to inflammation. And some researchers have found that it is possible to reduce the incidence of coronary heart disease through controlling diet [137]. If eating high-refined starch, sugar, saturated fatty acids, and trans fatty acids is kept for a long time, it will lead to a lack of natural antioxidants, fibers, and omega-3 fatty acids, which produce excessive proinflammatory cytokines.

In order to explore the relationship between diet and inflammation, Cavicchia et al. [138] proposed the inflammatory diet index (DII) for the first time in 2009, which is a dietary tool derived from the literature to evaluate the overall inflammatory potential of individual diet. DII consists of a variety of dietary ingredients, classified according to proinflammatory and anti-inflammatory components (Table 1). In recent years, DII has been widely used in clinical research, for example, cancer and CVDs [139]. DII can provide new ideas for the diagnosis and treatment of diseases, but related research remains unclear. In the study of diet and CVDs, we need to focus on the huge role of gut microbes and their metabolites in CVDs [140]. There are huge microecosystems

Table 1: Components of the inflammatory dietary index.

Name	Proinflammatory or anti-inflammatory	Total inflammatory score
Alcohol (g)	Anti-inflammatory	-0.278
Anthocyanidins (mg)	Anti-inflammatory	-0.131
Beta carotene (μg)	Anti-inflammatory	-0.584
Black/green tea (g)	Anti-inflammatory	-0.536
Caffeine (g)	Anti-inflammatory	-0.110
Carbohydrate (g)	Proinflammatory	0.097
Cholesterol (mg)	Proinflammatory	0.110
Energy (kcal)	Proinflammatory	0.180
Eugenol (mg)	Anti-inflammatory	-0.140
Fiber (g)	Anti-inflammatory	-0.663
Flavan-3-ol (mg)	Anti-inflammatory	-0.415
Flavonols (mg)	Anti-inflammatory	-0.467
Folic acid (μg)	Anti-inflammatory	-0.190
Garlic (g)	Anti-inflammatory	-0.412
Ginger (g)	Anti-inflammatory	-0.453
Iron (mg)	Proinflammatory	0.032
Isoflavones (mg)	Anti-inflammatory	-0.593
Magnesium (mg)	Anti-inflammatory	-0.484
Monounsaturated fatty acids (g)	Anti-inflammatory	-0.009
Niacin (g)	Anti-inflammatory	-0.246
Omega 3 (g)	Anti-inflammatory	-0.436
Omega 6 (g)	Anti-inflammatory	-0.159
Onion (g)	Anti-inflammatory	-0.301
Oregano/thyme (mg)	Anti-inflammatory	-0.102
Pepper (g)	Anti-inflammatory	-0.131
Polyunsaturated fatty acids (g)	Anti-inflammatory	-0.337
Protein (g)	Proinflammatory	0.021
Riboflavin (mg)	Anti-inflammatory	-0.068
Rosemary (mg)	Anti-inflammatory	-0.013
Saturated fat (g)	Proinflammatory	0.373
Selenium (μg)	Anti-inflammatory	-0.191
Thiamine (mg)	Anti-inflammatory	-0.098
Total fat (g)	Proinflammatory	0.298
Trans fat (g)	Proinflammatory	0.229
Turmeric (mg)	Anti-inflammatory	-0.785
Vitamin A (RE)	Anti-inflammatory	-0.401
Vitamin B6 (mg)	Anti-inflammatory	-0.365
Vitamin B12 (μg)	Proinflammatory	0.106
Vitamin C (mg)	Anti-inflammatory	-0.424
Vitamin D (μg)	Anti-inflammatory	-0.446
Vitamin E (mg)	Anti-inflammatory	-0.419
Zinc (mg)	Anti-inflammatory	-0.313

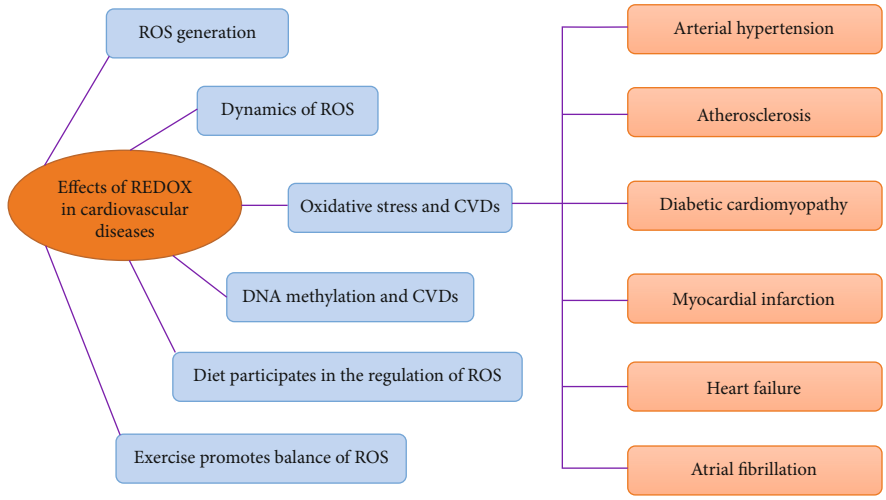

Figure 3: Summary exhibiting effects of REDOX in cardiovascular disease. ROS plays an important role in cardiovascular disease and serious heart disease. The pathogenesis of cardiovascular diseases and metabolic diseases is complex, and understanding the tissue-specific REDOX signal is very important for us to develop new methods to treat diseases. The contribution and influence of reactive oxygen species on metabolic processes is an important area of scientific discovery. Exploring the regulatory effects of a healthy diet and exercise on the improvement of oxidative stress, inflammation, and cardiac dysfunction will also provide new directions for the treatment of CVD.

in the intestinal tract, in which there are a large number of bacteria, fungi, viruses, protozoa, etc. Its metabolites play an important role in host metabolism, neurodevelopment, energy balance, and immune regulation, as well as the occurrence and development of cardiovascular diseases [141]. For example, intestinal microorganisms can promote vascular dysfunction and hypertension induced by Ang-II through vascular immune cell infiltration and inflammation [142, 143]. In patients with heart failure, the decrease of cardiac output and blood redistribution lead to reduced intestinal perfusion and breakdown of the intestinal barrier, as intestinal microbes and endotoxins enter the bloodstream and increase systemic inflammation, which in turn increases heart failure [144]. The evidence suggests that trimethylamine-*N*-oxide (TMAO) and short-chain fatty acids (SCFAs), the main metabolites of intestinal microorganisms, are involved in the pathogenesis of cardiovascular diseases [145]. TMAO can induce endothelial dysfunction and monocyte adhesion by activating NF-κB, protein kinase C, and pyran domain of nucleotide-binding oligomerization domain-like receptor family, and increase the expression of vascular endothelial inflammatory factors [146, 147]. At the same time, TMAO can also upregulate scavenger receptors in macrophages, promote the accumulation of cholesterol and formation of foam cells in macrophages, and further promote the formation of vascular plaques [148] and promote the inflammatory reaction of blood vessels through the MAPK and NF-κB pathways [149]. SCFAs play a key role in maintaining intestinal barrier function and play a positive role in cardiac metabolic health [150]. In addition, some probiotics and their fermented products have been proved to inhibit the production of nitrogen oxides in macrophages, reduce the types of reactive oxygen species, increase dietary calcium absorption, and thus reduce blood pressure [151].

A dietary intervention has been shown to reduce the risk of cardiovascular disease events. High-fat and high-sugar diets can lead to abnormal intestinal flora and increase the risk of cardiovascular disease [152]. Increasing the carbohydrate diet can change the composition of Rosella and rectal true bacilli [153]. A diet rich in dietary fiber can promote the growth of beneficial bacteria and inhibit the growth of the conditional pathogenic bacteria [154]. A high-fiber diet can increase acetate-producing microorganisms, lower blood pressure, and improve ventricular remodelling and fibrosis [155]. Another example in rats after partial nephrectomy indicated that the use of curcumin in ginger can retain the ejection fraction and reduce the lipid peroxidation of the heart muscle [156]. Allicin (40 mg/kg/day, orally), which is a component of garlic extract, could reduce hypertension, lipid, and protein oxidation in the heart, meanwhile accelerate the levels of antioxidant enzymes [157]. Supplementation of 800 IU/day vitamin E as an antioxidant can reduce CVD endpoints and myocardial infarction in haemodialysis patients with CVDs [158].

Aloe is an edible plant in daily life [159], which contains a compound called aloe-emodin (AE) [160, 161]. Yu et al. [162] found that in the H_2O_2-induced apoptosis model of neonatal rat ventricular myocytes, AE can prevent myocardial infarction by upregulating miR-133, inhibiting the ROS production, and inhibiting the caspase-3 apoptosis signal pathway. In addition, AE treatment significantly reversed the H_2O_2-induced upregulation of Bax/Bcl-2 and loss of mitochondrial membrane potential. Chen et al. [163] established a rat cardiac inflammation model induced by hyperlipidaemia, and then administered AE to study the potential role and mechanism of AE regulating cardiac oxidative stress and inflammation induced by hyperlipaemia. They found that compared with the normal diet (ND) group, the expression levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α were significantly upregulated in the hyperlipaemia group, while the expression levels of IL-1, IL-6, and TNF-α were dramatically decreased in the AE treatment group. In addition, AE also

inhibited the expression of vascular cell adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM-1). In vitro, AE decreased the expression of IL-1β, IL-6, and TNF-α in palmitic acid (PA-) treated H9C2 cells in a dose-dependent manner. Further experiments showed that AE inhibited PA-induced cell death and promoted the production of intracellular ROS [163]. This study indicates that AE may reduce cardiac inflammation induced by hyperlipidaemia/-plasminogen activator by inhibiting the TLR4/NF-κB signal pathway, which may be a promising therapeutic strategy for the prevention of myocardial injury.

Intestinal flora and cardiovascular disease is a new research field in the future, but the specific mechanism of the interaction between intestinal flora and body is not clear. Maintaining the homeostasis of intestinal flora and correcting the imbalance of intestinal flora will become a new target for the prevention and treatment of cardiovascular diseases.

6. Exercise Promotes Balance of ROS

Physical activity has long been considered to be beneficial for CVDs. However, the molecular mechanisms by which triggering and sustaining exercise are beneficial for the heart are poorly understood, which is expected for new therapeutic targets. To explore these mechanisms, Moreira et al. identified cardiac gene targets in rat models by using RNA sequencing [164], whose expression could be disrupted in heart failure but was recovered by exercise. Through a series of elaborate validation, they screened 16 targets to assess whether targeted interference with the silencing RNA of these genes can affect the abundance of a CVD biomarker (BNP, B-type natriuretic peptide) in human cardiomyocytes. Among them, the Proline Dehydrogenase (PRODH) expression is reduced in human failing hearts, but rescued by exercise in a rat model of HF. The knockdown of PRODH also resulted in the rise of the BNP expression in human cardiomyocytes.

Compared with the traditional drug treatments, natural methods of improving the collaterals through exercise training seem to be more effective, especially for patients with intermittent claudication. Exercise has a variety of positive effects on the body, but it also has systemic benefits [165, 166]. In general, physical activity has been shown to greatly improve cardiovascular function, and this is partly due to improved bioavailability of NO, increased endogenous antioxidant defence, and decreased expression of the enzyme involved in the ROS production [167].

7. Summary

ROS is not only a natural by-product of metabolic responses in various cell compartments but also a signalling molecule that regulates specific biochemical pathways in normal cell function and survival. However, the dysregulation of ROS signalling or excessive production of nonspecific ROS can affect the pathophysiology of heart diseases. As this review highlights (Figure 3), ROS are particularly important in cellular metabolism and inflammatory signalling. Therefore, it is not surprising that ROS plays an important role in cardiac diseases associated with metabolic disorders and inflammation. The pathogenesis of cardiovascular and metabolic diseases is complex, and understanding tissue-specific REDOX signals is important for us to develop new and novel therapies to treat diseases. Metabolic dysregulation is a major driver of cell dysfunction and disease progression, and exploring the contribution and effect of reactive oxygen species on metabolic processes is an important field of scientific discovery. At the same time, exploring the effects of a healthy diet and exercise on the regulation of oxidative stress, inflammation, and the improvement of cardiac dysfunction will also provide a new direction for the treatment of CVDs.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

Kai Wang and Yanhan Dong contributed equally to this work.

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Research Article

Stimulation of Epithelial Sodium Channels in Endothelial Cells by Bone Morphogenetic Protein-4 Contributes to Salt-Sensitive Hypertension in Rats

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Previous studies have shown that high salt induces artery stiffness by causing endothelial dysfunction via increased sodium influx. We used our unique split-open artery technique combined with protein biochemistry and *in vitro* measurement of vascular tone to test a hypothesis that bone morphogenetic protein 4 (BMP4) mediates high salt-induced loss of vascular relaxation by stimulating the epithelial sodium channel (ENaC) in endothelial cells. The data show that high salt intake increased BMP4 both in endothelial cells and in the serum and that exogenous BMP4 stimulated ENaC in endothelial cells. The data also show that the stimulation is mediated by p38 mitogen-activated protein kinases (p38 MAPK) and serum and glucocorticoid-regulated kinase 1 (Sgk1)/neural precursor cell expressed developmentally downregulated gene 4-2 (Nedd4-2) (Sgk1/Nedd4-2). Furthermore, BMP4 decreased mesenteric artery relaxation in a benzamil-sensitive manner. These results suggest that high salt intake stimulates endothelial cells to express and release BMP4 and that the released BMP4 reduces artery relaxation by stimulating ENaC in endothelial cells. Therefore, stimulation of ENaC in endothelial cells by BMP4 may serve as another pathway to participate in the complex mechanism of salt-sensitive (SS) hypertension.

1. Introduction

High dietary sodium chloride intake has been related to hypertension and its target organ damage [1]. It has long been known that a high-salt diet increases the stiffness of endothelial cells [2]. High salt-induced endothelial cell stiffness promotes increased arterial stiffness by elevating transforming growth factor (TGF)-β levels [3]. Recent clinical studies have also shown that high salt intake induces large artery stiffness in a salt-sensitive population [4]. Therefore, investigation of the molecular mechanism by which high salt causes endothelial cell dysfunction and artery stiffness has clinical significance for improving the management of salt-sensitive hypertension. Previous studies have shown that elevated sodium influx accounts for endothelial cell stiffness [5]. The pathways for sodium influx were unclear, until we recently recorded the single-channel activity of ENaC in endothelial cells [6]. However, the complex mechanism by which high dietary salt stimulates ENaC in endothelial cells remains to be further determined.

It has been well established that overproduction of TGF-β1 contributes to salt-sensitive hypertension [7]. Since TGF-β1 stimulates ENaC expression in cultured human cortical collecting duct principal cells [8], high salt may stimulate ENaC in endothelial cells by promoting TGF-β production. However, conflicting results also show that TGF-β1 inhibits renal and lung ENaC [9–11]. The conflicting results may be due to the different doses of TGF-β because TGF-β can

induce biphasic effects [12]. TGF-β consists of a complex superfamily that binds to its also complex receptors [13]. Among the various ligands of TGF-β receptors, the multifunctional growth factor BMP4, which belongs to the TGF-β superfamily [14], attracted our particular attention. We wanted to test the role of BMP4 in mediating high salt-induced ENaC activity in endothelial cells, because previous studies have shown that administration of BMP4 induces hypertension in mice [15]. Additionally, overexpression of BMP4 may mediate hypertension in patients with colorectal adenocarcinoma, because previous studies have shown that BMP4 is overexpressed in human colorectal adenocarcinoma cells [16, 17] and that hypertension often occurs in patients with adenocarcinoma [18, 19]. These studies suggest that BMP4 may act as a signaling molecule to mediate the development of hypertension.

In this study, we aimed to characterize the role and the potential mechanisms of BMP4-mediated ENaC channels in the regulation of blood pressure in salt-sensitive hypertension rats. We performed cell-attached patch-clamp, perforated whole-cell patch-clamp, isometric myography, and Western blotting experiments to address this question in salt-sensitive rats, using a preparation of the freshly isolated vascular endothelium. We hypothesized that excessive activation of ENaC by BMP4 is a central contributor to endothelial injury disease and salt-sensitive hypertension.

2. Materials and Methods

2.1. Animals. All animal care and experimental procedures were approved by the Harbin Medical University Animal Supervision Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [20, 21].

Male Sprague-Dawley (SD) rats and male Dahl salt-sensitive (SS) rats weighing 220-240 g (8 weeks) were used in the present study. The age of the animals in the various groups was matched. SD and SS rats were divided randomly into two groups: normal salt (NS) diet (0.3% NaCl, *w*/*w* for three weeks) and high-salt (HS) diet (5% NaCl, *w*/*w* for three weeks). Water and food were supplied *ad libitum*. The blood pressure of awake rats was measured weekly by tail-cuff plethysmography (CODA, 20310, Kent Scientific Corporation, USA). To minimize stress-induced fluctuations in blood pressure, the rats were trained to get used to daily blood pressure measurement for 3 days before the experiments began. Before detection of tail artery pulsations, the rats were placed in the holder for 15 min at 37°C. The higher and lower readings were discarded and the tail arterial blood pressure was obtained from the average of six measurements. Then, blood was collected from the main abdominal artery for serum BMP4 detection using an ELISA kit (LS-F23161, Lifespan Biosciences, USA).

2.2. Primary Culture of Rat Mesenteric Artery Endothelial Cells. Mesenteric artery endothelial cell (MAEC) primary culture was carried out using a previously described method [22, 23]. Briefly, SD and SS rats were euthanized by CO_2 suffocation. When the abdomen was opened, the whole mesenteric vascular bed was dissected out, and all the vein branches of the mesenteric bed were rapidly excised under a dissecting microscope. The remained arterial branched were digested with 0.2 mg/mL collagenase IA (C9891, Sigma-Aldrich, USA), for 50 min at 37°C with mild shaking. When the enzymes were digested, endothelial cells were collected by centrifugation at 1200 rpm for 5 min. The cells were resuspended in DMEM solution (100 U/mL penicillin, 100 μg/mL streptomycin, and 20% fetal bovine serum) and cultured in gelatin-coated Petri dishes. Nonadherent adherent cells were removed after 1 hour, and the adherent endothelial cells were cultured at 37°C with 5% CO_2 for 3-5 days. These cells were used for experiments or, respectively, treated with BMP4 (20 ng/mL, 314-BP-010, R&D, USA), noggin (100 ng/mL, 6057-NG-025, R&D, USA), SB202190 (10 μM, S7067, Sigma-Aldrich, USA), mannitol (80 mM), and additional NaCl (40 mM) in the DMEM culture medium (10-013-CVRC, Corning, USA) for 24 hours.

2.3. Electrophysiology Recording. As previously described, in situ cell-attached patch-clamp recordings of ENaC single-channel currents were performed using intact vascular endothelia [22–24], 3 weeks after feeding NS and HS diet. Mesenteric arteries and physiological saline solution (PSS) were added to a Petri dish for dissection. PSS contained (in mM): 137 NaCl, 5.4 KCl, 0.05 $CaCl_2$, 0.4 KH_2PO_4, 0.4 Na_2HPO_4, 4.4 $NaHCO_3$, and 10 HEPES (pH 7.4 with HCl). Second-order branches of mesenteric arteries were dissected and placed on a 5 × 5-mm cover glass coated with L-polylysine, or the primary culture of rat MAECs was transferred to a chamber mounted on an inverted Nikon microscope (Tokyo, Japan), allowing direct access to the endothelial cell layer. Single-channel ENaC currents were recorded in a cell-attached configuration with an Axon Multiclamp 200B amplifier (Axon Instruments; Foster City, CA, USA) at room temperature (22–24°C). Patch pipettes were pulled from borosilicate glass with a Sutter P-97 horizontal puller, and the pipette resistances ranged from 6 to 10 MΩ in the bath. The bath and the pipette solutions contained (in mM): 135 NaCl, 4.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, and 5 Na-HEPES (pH 7.4 with NaOH). After gigaseal formation, the single-channel currents were recorded immediately at least over 30 min. The data were acquired by application of 0 mV to the patch pipettes and then sampled at 5 kHz and low-pass filtered at 1 kHz with Clampex 10.2 software (Molecular Devices, Sunnyvale, CA, USA). Single-channel traces were further filtered at 30 Hz prior to analysis. The open probability (P_O) was calculated according to the following formula: $P_O = NP_O/N$; the apparent number of active channels in the patch was represented by N (N was estimated from the current amplitude histogram).

Perforated whole-cell recording was performed in MAECs, using pipettes of 1~2 MΩ resistance as previously described [6]. Bath saline was as the same described for single-channel recordings, but with the addition of 10 mM D-glucose. The pipette solution contained (in mM): 40 KCl, 100 K-gluconate, 1 $MgCl_2$, 1 $CaCl_2$, 0.1 EGTA, 4 Na_2ATP, 10 glucose, 10 HEPES, and 2 GTP (pH 7.2 with KOH). For perforated patch whole-cell recording, the pipette solution

was supplemented with 20 μg/mL amphotericin B (46006-100MG, Fluka, USA). Representative current sweeps evoked by a voltage ramp from 60 mV to -100 mV (holding potential at 40 mV) for a duration of 500 ms. The data were generated by an Axon Multiclamp 200B amplifier (Axon Instruments; Foster City, CA, USA) at room temperature (22–24°C) in MAECs isolated from SS rats. The cells were cultured with or without an additional 20 ng/mL BMP4 in the culture medium for 24 hours, in the presence or absence of 1 μM benzamil.

2.4. Wire Myograph Studies. Isolated mesenteric artery ring responsiveness was measured using an isometric myograph (Danish Myo Technology, Aarhus, Denmark), as previously described [22, 23, 25, 26]. Briefly, the isolated second-order mesenteric arteries were carefully cleaned of adherent adipose tissue and cut into 1.8-2 mm lengths. Before evaluating the relaxation effect of ACh and NTG, some rat mesenteric artery rings were incubated for 4 hours in DMEM solution (100 U/mL penicillin, 100 μg/mL streptomycin, and 20% fetal bovine serum) and were placed in a CO_2 incubator with 95% O_2 plus 5% CO_2 with BMP4 (50 ng/mL), noggin (a BMP4 antagonist, 200 ng/mL), or SB202190 (a p38 MAPK inhibitor, 20 μM). Then, changes in the isometric tone of mesenteric arteries were recorded in a wire myograph equilibrated in PSS at 37°C and bubbled with a mixture of 5% CO_2 in 95% O_2. The arterial rings were given a resting tension of 3 mN and then allowed to equilibrate for 60 min prior to experiment. The integrity of the functional endothelium was tested by obtaining a relaxation to ACh (1 μM) in rings precontracted with phenylephrine (10 μM). The endothelium was considered intact when such an ACh–induced relaxation was more than 85% of the precontraction value to phenylephrine. After a 60 min stabilization period, KPSS that contained (in mM) 82.4 NaCl, 60 KCl, 0.05 $CaCl_2$, 0.4 KH_2PO_4, 0.4 Na_2HPO_4, 4.4 $NaHCO_3$, and 10 HEPES (pH 7.4 with HCl) was added to the chambers and then washed out with PSS until a reproducible maximal contraction was achieved. Endothelium-dependent relaxation (EDR) and endothelium-independent relaxation were measured by testing concentration responses to the cumulative addition of acetylcholine (ACh, 0.1 nM to 100 μM) or nitroglycerin (NTG, 0.1 nM to 10 μM) to precontracted rings with phenylephrine (Phe, 10 μM). Some rings were incubated with benzamil (an ENaC blocker, 1 μM, B2417, Sigma-Aldrich, USA) for 1 hour before assessing their relaxation response to ACh and NTG.

2.5. Transfection of Plasmids. A lentivirus (LV) vector consisting of p38 MAPK short hairpin RNA (shRNA) and a control LV were constructed and packaged according to the manufacturer's protocols (GeneChem, Shanghai, China). When the cell confluence was approximately 20%-30%, the p38 MAPK shRNA LV (MOI = 10) or control LV (MOI = 10) was transfected in culture dishes with a HiTransG P infection enhancer (40 μL/mL). Sixteen hours after transfection, the complete medium was replaced, and the culture continued. After 48 hours, the transfection medium was removed, and the cells were incubated with or without BMP4 for 24 hours. Successful endothelial cell transfection was evaluated by quantitative real-time PCR (qRT-PCR) and Western blotting.

2.6. Quantitative Real-Time PCR. The p38 MAPK expression level was analyzed via qRT-PCR. Total RNA was extracted from endothelial cells that were sorted from rat mesenteric arteries using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RT system protocol for reverse transcription was performed in a 20 μL reaction mixture. Total RNA (1 μg) was used in the reaction, and a random primer was used to initiate cDNA synthesis. The reaction mixture was treated for 10 min at 25°C, 37°C for 120 min, and 85°C for 5 min. SYBR Green PCR core reagents (Applied Biosystems) and an ABI Prism 7500 sequence detection system were used for qRT-PCR. According to the manufacturer's recommendations, qRT-PCR was performed in a 20 μL reaction volume. GAPDH was used as an internal control for p38 MAPK. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative value compared to the control sample. MAECs were genotyped using the following primers: p38 MAPK-Rat-Forward (GTACCTGGTGACCCATCTCA), p38 MAPK-Rat-Reverse (TCCAATTCAGCATAATCTCG), GAPDH-Rat-Forward (TCAACGGCACAGTCAAGG), GAPDH-Rat-Reverse (ACTCCACGACATACTCAGC).

2.7. Western Blot. For Western blot analyses, cell extractions were cleared by 13300 × g for 15 min at 4°C. Protein concentration was determined by BCA Protein Assay Kit (APPLYGEN, Beijing, China). The protein samples were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane using a Trans-blot unit (Bio-Rad Laboratories) for 1.5 hour at 250 mA. The membranes were blocked with 5% (wt/vol) skim milk and 0.1% (vol/vol) Tween-20 in TBS (pH 7.4) for 1 hour at room temperature (25°C). Primary antibodies against BMP4 (1 : 500, ab39973, Abcam, UK), p38 MAPK (1 : 200, sc-7149, Santa Cruz Biotechnology, USA), phospho-p38 (p-p38) MAPK (1 : 1,000, 9216, Cell Signaling Technology, USA), Sgk1 (1 : 500, ab59337, Abcam, UK), p-Sgk1 (1 : 1,000, 44-1260G, ThermoFisher, USA), Nedd4-2 (1 : 500, 4013, Cell Signaling Technology, USA), p-Nedd4-2 (1 : 500, ab168349, Abcam, UK), and GAPDH (1 : 5,000, ab8245, Abcam, UK) were incubated with the membranes overnight at 4°C. After washing with TBS-T, the membranes were incubated for 1 hour at room temperature with the corresponding secondary antibodies (1 : 10,000). All membranes were washed with TBS-T, and the bands were quantified by using the Odyssey infrared imaging system (LI-COR) and Odyssey v3.0 software.

2.8. Chemical Reagents. Unless otherwise noted, all chemical reagents used in this study were purchased for Sigma-Aldrich.

2.9. Statistics and Data Analysis. All values were expressed as means ± SEM. All data were subjected to the Kolmogorov-Smirnov normality test, and then, parametric or nonparametric analyses were chosen accordingly. If the data were not normally distributed, a nonparametric test (Mann–Whitney test to compare two groups or Kruskal-Wallis test with Dunn's post hoc test to compare three groups) was

performed. If the data were found to follow a Gaussian distribution, parametric tests were done (Student's two-tailed t -test for comparisons between two groups or one-way ANOVA for multiple groups). Data subjected to ANOVA were followed by Bonferroni's post hoc tests only when the F value attained $P < 0.05$, and there was no significant inhomogeneity of variances. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. HS Diet Increases Blood Pressure, Impairs Vascular Relaxation, and Stimulates Endothelial ENaC in SS Rats but Not in SD Rats. Our previous studies have shown that the HS diet (8% NaCl) increases systolic blood pressure (SBP) in both SD and SS rats [22, 23]. Here, we found that SS rats fed HS diet (5% NaCl) resulted in a significant increase in SBP, diastolic blood pressure (DBP), and mean arterial pressure (MAP), in a time-dependent manner. However, there were no changes in either SD rats fed the HS diet or NS diet, or in SS rats fed with NS in a period of 3 weeks (Figures 1(a)–1(c)). To determine whether the HS diet altered vascular function in these rats, we used isometric myography to test vascular tone. The data showed that three weeks after feeding the HS diet to SS rats blunted endothelium-dependent artery relaxation (EDR) responses to ACh but had no effect on endothelium-independent artery relaxation responses to NTG. However, EDR was unchanged in HS diet-fed SD rats (Figures 1(d)–1(f)).

To test whether the HS diet enhanced ENaC activity in these rats, we performed cell-attached patch-clamp recordings of ENaC signal-channel currents from intact endothelial cells attached to isolated mesenteric arteries. ENaC activity was significantly higher in HS diet-fed SS rats than in NS diet-fed SS rats; HS diet did not affect ENaC activity in SD rats (Figures 1(g) and 1(h)). These results indicate that the HS diet increases both SBP and DBP, impairs endothelium-dependent vascular relaxation, and stimulates ENaC in SS rats but not in SD rats.

3.2. HS Diet Increases BMP4 Expression Both in the Serum and in Endothelial Cells from SS Rats but Not in those from SD Rats. We explored whether BMP4 content in HS diet-fed SD and SS rats was increased. The data showed that serum BMP4 levels were increased in HS diet-fed SS rats but not in HS diet-fed SD rats (Figure 2(a)). Moreover, the HS diet significantly enhanced BMP4 expression in endothelial cells from SS rats. In contrast, BMP4 expression in HS diet-fed SD rats showed no significant change (Figure 2(b)). Our results suggest that BMP4 may contribute to high salt-induced hypertension in SS rats.

3.3. BMP4 Mediates HS Diet-Induced Impairment of EDR and ENaC Activity in SS Rats. Other studies have indicated that BMP4 destroys EDR [15, 27] and that ENaC blockade ameliorates EDR [22]. Therefore, we reasoned that BMP4 may blunt EDR by stimulating ENaC in HS diet-fed SS rats. Incubation of isolated mesenteric artery rings with either a BMP4 antagonist, noggin (200 ng/mL for 4 hours) or with an ENaC blocker, benzamil (1 μM for 1 hour) significantly prevented HS-induced or exogenous BMP4-induced (50 ng/mL treatment for 4 hours) impairment of EDR. In contrast, endothelium-independent relaxation responses to NTG were unaffected (Figures 3(a)–3(c)). These data suggest that BMP4 may lead to endothelial dysfunction by stimulating ENaC in HS diet-fed SS rats.

We then next determined whether HS diet increases ENaC activity through BMP4. We detected a benzamil sensitive ENaC single-channel current in the endothelial cells of split-open SS rat arteries, which the biophysical features including the channel conductance were similar to what we have previously reported [22, 23]. Furthermore, the endothelial ENaC activity was significantly upregulated by HS diet feeding or BMP4 incubation. In contrast, noggin or benzamil treatment abolished the ENaC activity induced by HS diet feeding or BMP4 treatment (Figures 3(d) and 3(e)). Moreover, the data generated from perforated whole-cell patch-clamp recordings showed pretreatment of the primary cultured endothelial cells with BMP4 for 24 hours significantly increased the ENaC currents compared to those of control cells, and the BMP4-induced increase in whole-cell ENaC currents was inhibited by benzamil (1 μM) (Figures 3(f) and 3(g)). These data strongly suggest that the HS diet induces endothelial dysfunction by stimulating endothelial ENaC via BMP4.

3.4. HS Diet Results in Endothelial Dysfunction and Increases ENaC Activity through p38 MAPK in SS Rats. According to previous studies, p38 MAPK is involved in the pathogenesis of hypertension [28]. To determine whether p38 MAPK mediates HS diet-induced hypertension, endothelial cells were freshly isolated from the mesenteric arteries of HS or NS diet-fed SS rats and subjected to Western blotting. Our data showed that the expression levels of p-p38 MAPK but not those of p38 MAPK were significantly elevated in endothelial cells from HS diet-fed SS rats, but not in those from NS diet-fed SS rats (Figures 4(a) and 4(b)). Next, we determined whether p38 MAPK is involved in HS-induced impairment of EDR. The results showed that HS diet-induced impairment of EDR was reversed by treating the vascular rings with a p38 MAPK inhibitor, SB202190 (20 μM) for 4 hours in SS rats (Figures 4(c)–4(e)). Furthermore, HS-induced increase in endothelial ENaC activity was abrogated by treating the arteries with 20 μM SB202190 for 4 hours prior to performing cell-attached recordings (Figures 4(f) and 4(g)). These results suggest that the HS diet stimulates ENaC and causes endothelial dysfunction in SS rats via p38 MAPK.

3.5. HS-Induced Increase in BMP4 Stimulates Sgk1/Nedd4-2 via p38 MAPK. Since p-p38 MAPK in hypertensive rats is enhanced by BMP4 [28], we then examined whether BMP4 may stimulate the levels of active (phosphorylated) Sgk1/Nedd4-2 via p38 MAPK to elevate ENaC activity by reducing ENaC ubiquitination. To reduce the number of animals used, endothelial cells were freshly isolated from the mesenteric arteries of SS rats fed NS diet, amplified in culture, and treated with NaCl (additional 40 mM in culture media), NaCl (additional 40 mM in culture media) + noggin (100 ng/mL), or NaCl (additional 40 mM in

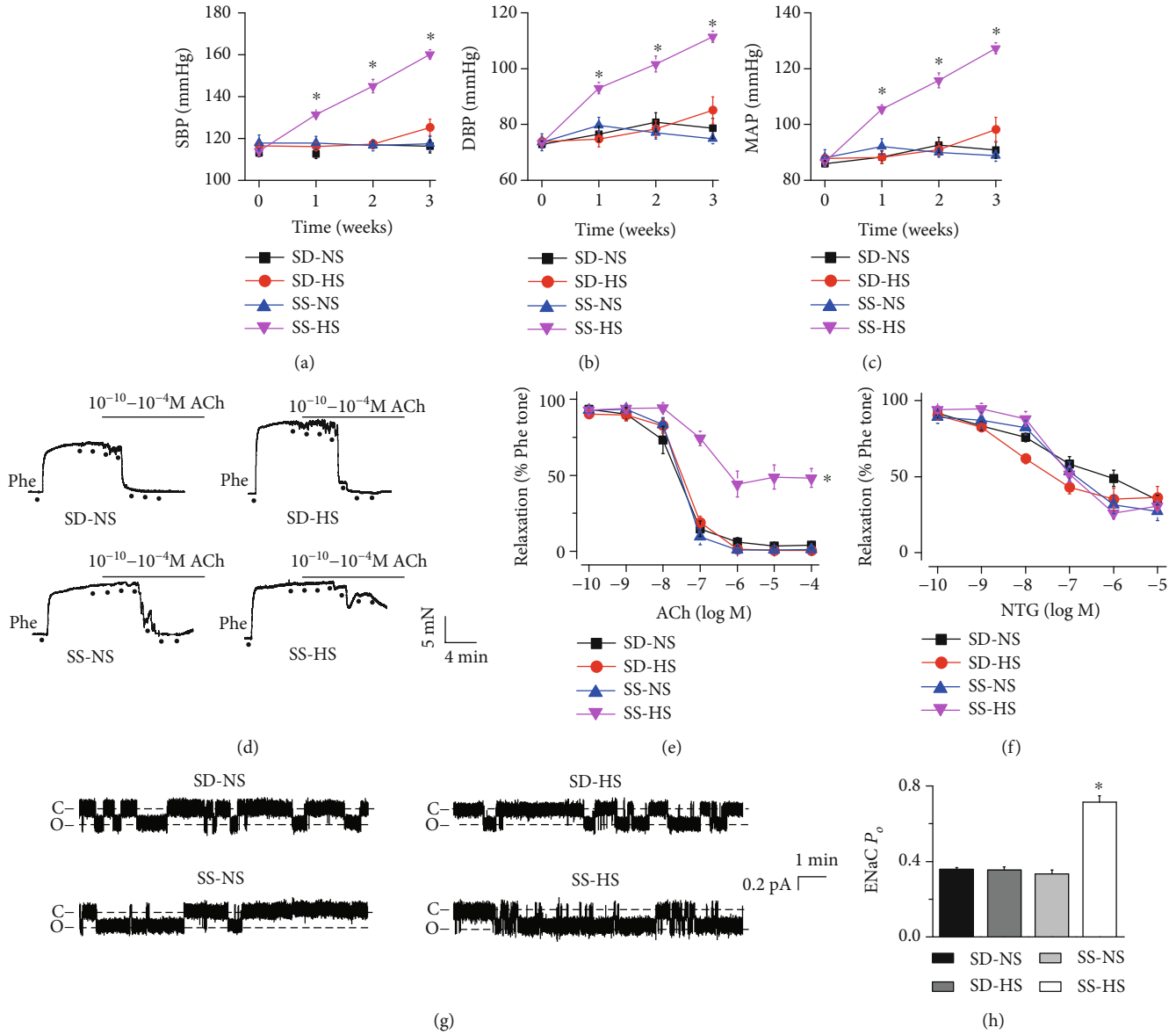

Figure 1: HS diet elevates blood pressure in a time-dependent manner, reduces endothelium-dependent artery relaxation, and stimulates endothelial ENaC in SS rats. SBP (a), DBP (b), and MAP (c) were measured in SD and SS rats fed NS (0.3%) or HS (5%) diet for 0, 7, 14, and 21 days. *represents $P < 0.05$ *vs.* SS-NS, SS rats fed NS ($n = 7$ rats for each group). (d, e) Representative traces of ACh-induced artery relaxation in NS or HS diet-fed SD and SS rats and summary of relaxation responses of mesenteric arteries to different doses of ACh under the conditions shown in (d). The first dot in each plot represents the time when Phe (10 μM) was added. The following dots indicate ACh concentrations gradually increased to 0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM. *indicates $P < 0.05$ *vs.* SS-NS ($n = 6$ for each group). (f) Summary of relaxation responses of mesenteric arteries from these rats to different doses of NTG ($n = 6$ for each group). (g) Representative ENaC single-channel currents generated by split-open artery technique in intact endothelial cells from NS or HS diet-fed SD and SS rats, under the indicated conditions. (h) Summary of calculated ENaC P_O obtained from single-channel recordings from the different experimental groups as shown in (g). *represents $P < 0.05$ *vs.* SS-NS ($n = 6$ rats for each group).

culture media) + SB202190 (10 μM) for 24 hours. Our data showed that BMP4 and p-p38 MAPK were significantly elevated by additional NaCl in the culture medium and that the increases in p-p38 MAPK were reversed by noggin (Figures 5(a)–5(b)). It has been revealed that enhanced phosphorylation levels of Sgk1/Nedd4-2 prevents Nedd4-2 mediated ENaC degradation and increases ENaC activity [29–31]. Therefore, we next explored whether p38 MAPK and its upstream signal BMP4 could increase the levels of phosphorylated Sgk1/Nedd4-2 in these endothelial cells. The data showed that total Sgk1, p-Sgk1, and p-Nedd4-2 were significantly increased in HS-treated primary cultured endothelial cells and that the increases were abolished by noggin or SB202190 (Figures 5(c)–5(d)). We then examined whether the effect of high salt on the expression of these proteins in endothelial cells was due to the change in osmolarity. As the osmolarity of 80 mM of mannitol equals the osmolarity of 40 mM of NaCl [32], 80 mM mannitol used to examine

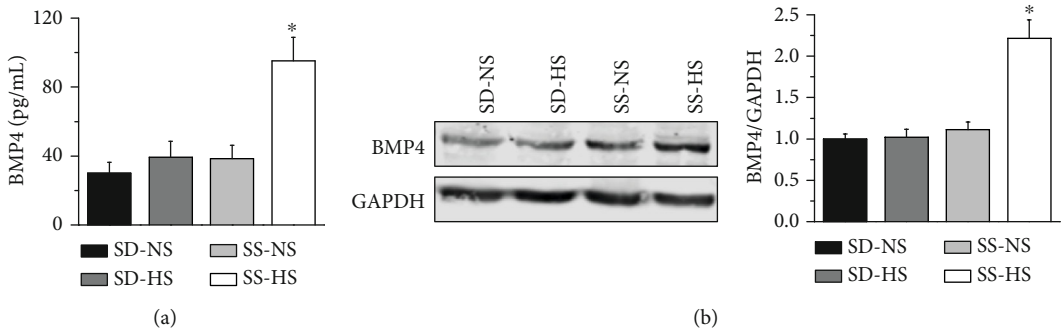

Figure 2: HS diet increases BMP4 levels both in the serum and in the endothelial cells of SS rats. (a) Serum BMP4 levels were measured at day 21 in NS or HS diet-fed SD and SS rats. *represents $P < 0.05$ *vs.* SS-NS ($n = 13$ for each group). (b) Representative Western blot and summarized data showing BMP4 protein levels under each condition in endothelial cells isolated from SD and SS rats fed NS or HS diets. *represents $P < 0.05$ *vs.* SS-NS ($n = 10$ for each group).

whether osmolarity can alter the expression levels of these proteins. The data showed that mannitol at a concentration of 80 mM did not affect the expression levels of BMP4, p-p38 MAPK, p38 MAPK, p-Sgk1, Sgk1, p-Nedd4-2, and Nedd4-2 (Figures 5(e)–5(h)), suggesting the effect of high salt on the expression levels of these proteins were not due to changes in osmolarity. These data suggest that HS stimulates Sgk1/Nedd4-2 signaling through BMP4 and p38 MAPK.

3.6. p38 MAPK Mediates BMP4-Induced ENaC Activity by Stimulating Sgk1/Nedd4-2. To confirm the role of p38 MAPK in mediating BMP4-induced ENaC activity, we knocked down p38 MAPK gene expression using a shRNA LV. We found that protein expression levels of p38 MAPK and p-p38 MAPK, p38 MAPK mRNA levels were reduced, whereas the negative control (NC) LV did not affect p38 MAPK or p-p38 MAPK (Figures 6(a)–6(c)). Moreover, Western blot data revealed that the expression levels of p-p38 MAPK were significantly increased by BMP4, and the effects of BMP4 on p-p38 MAPK were abolished by p38 MAPK shRNA transfection (Figures 6(d) and 6(e)). Furthermore, BMP4-induced in an increase in p-Sgk1, Sgk1, and p-Nedd4-2 levels were reversed p38 MAPK shRNA infection, whereas BMP4 did not affect Nedd4-2 expression (Figures 6(f)–6(i)). These results suggest that BMP4 stimulates Sgk1/Nedd4-2 through p38 MAPK. Furthermore, the data showed that knocking down p38 MAPK attenuated BMP4-induced increase in ENaC activity in the primary cultured endothelial cells isolated from SS rats (Figures 6(j) and 6(k)). These data together indicate that BMP4 increases ENaC activity by stimulating Sgk1/Nedd4-2 via p38 MAPK.

4. Discussion

In the present study, for the first time, we show that high salt intake induces overexpression of BMP4 in the serum and endothelial cells of salt-sensitive rats, that BMP4 stimulates ENaC in endothelial cells via a pathway associated with p38 MAPK and Sgk1/Nedd4-2, and that BMP4 decreases EDR by stimulating ENaC. These results may have clinical significance for managing hypertension in salt-sensitive populations and for improving the treatment of colorectal adenocarcinoma patients with hypertension.

We have previously shown that the HS diet (8% NaCl) increases SBP in both SD and SS rats [22, 23]. Since a diet containing approximately 0.3% NaCl is considered an NS diet [33], the HS diet we previously used 8% NaCl contains almost 30 times more salt than the NS diet. This substantial difference could be the reason that the HS diet increases BP in both SD and SS rats. Therefore, we used a reduced HS diet (5% NaCl) for the present study. According to a simple practice guide for dose conversion between rat and human [34], when rats feeding a HS diet with 5% NaCl content, it is equivalent to a person weighing 60 kg eating about 46.2 g of NaCl per day. The HS diet used in SS hypertension is much higher than the salt in human diet. However, such high concentrations have been extensively used to produce a SS hypertension model in SS rats [35–37]. Indeed, the data showed that 5% NaCl increases BP and BMP4 only in SS rats but not in SD rats.

We demonstrate that in SS rats BMP4 plays a crucial role in HS-induced ENaC activity and endothelial dysfunction via p38 MAPK-dependent activation of Sgk1/Nedd4-2. In contrast to TGF-β which causes endothelial dysfunction in SD rats in response to a relatively high salt (8%) diet [38], our data suggested that BMP4 may be a signaling molecule that mediates HS-induced hypertension specifically in SS populations. Indeed, BMP4 infusion by osmotic pumps increased systolic blood pressure in a time- and dose-dependent manner in both C57BL/6 mice and apolipoprotein E-null mice [15]. We favour the notion that increased BMP4 is a reason for rather than a result of hypertension, because the administration of BMP4 causes hypertension [15].

In the present study, we did not show the mechanism by which HS diet increases serum BMP4. Since our data showed that the HS diet also increases BMP4 in endothelial cells, we argue that increased serum BMP4 in response to HS challenge may come from endothelial cells as a paracrine signaling molecule. This argument is also supported by a previous study showing that BMP4 is expressed in endothelial cells and can induce endothelial dysfunction [39]. However, increased serum BMP4 may also come from other tissues, because HS intake stimulates the expression of BMP4 in renal cortex tissue [40]. It remains unclear how HS promotes

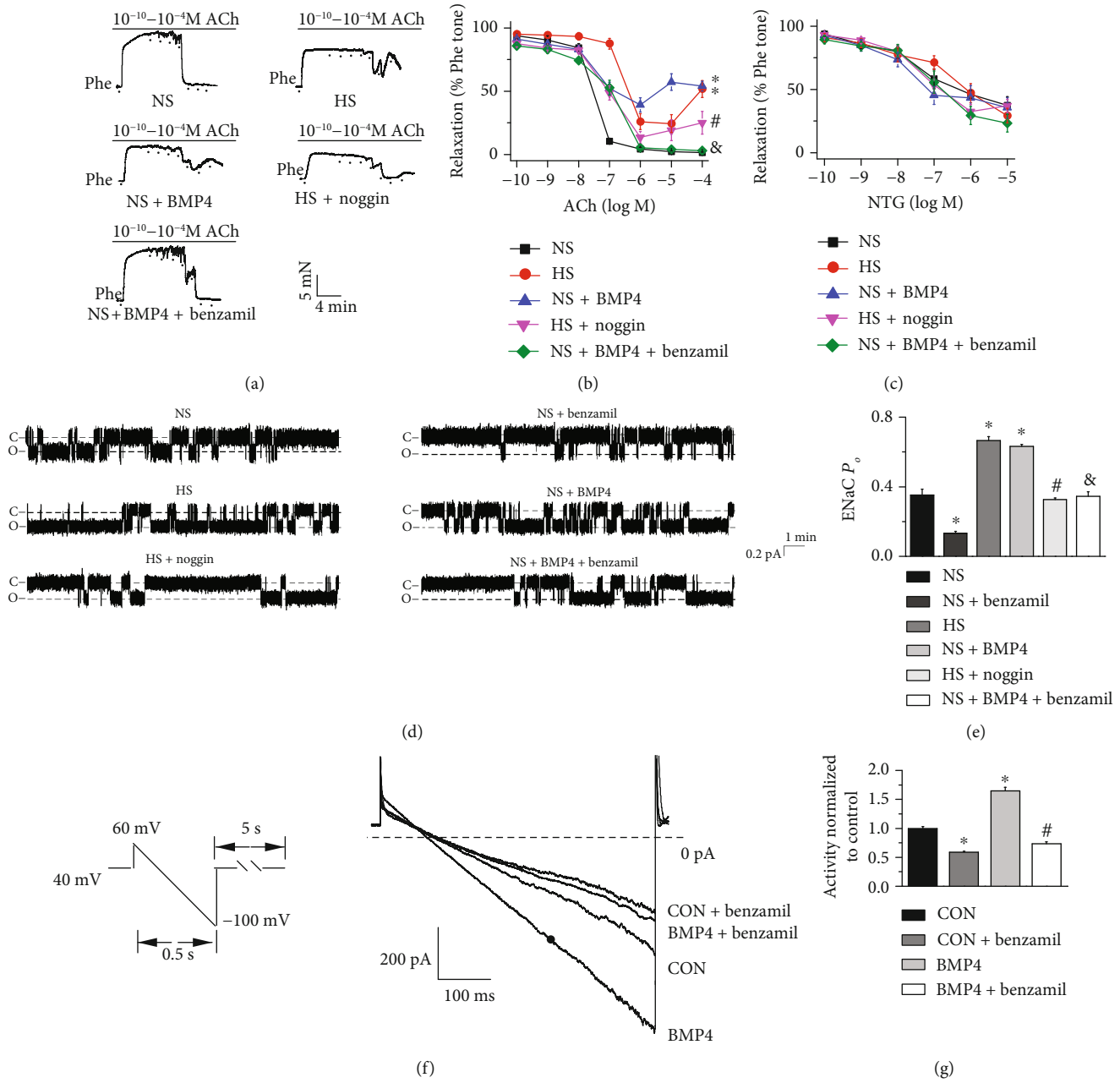

FIGURE 3: HS diet impairs EDR and stimulates ENaC via a pathway associated with BMP4 in SS rats. (a) Representative traces of ACh-induced artery relaxation in NS or HS diet-fed SS rats; the vascular rings were, respectively, treated with BMP4 (50 ng/mL) and noggin (200 ng/mL) for 4 hours and benzamil (1 μM) for 1 hour before experiments. The first dot in each plot represents the time when Phe (10 μM) was added. The following dots indicate ACh concentrations gradually increased to 0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM. (b) Summary of relaxation responses of mesenteric arteries to different doses of ACh under the conditions shown in (a). *indicates $P < 0.05$ *vs.* NS; #indicates $P < 0.05$ *vs.* HS; &represents $P < 0.05$ *vs.* NS + BMP4 ($n = 6$ for each group). (c) Summary of relaxation responses of mesenteric arteries from these rats to different doses of NTG ($n = 6$ for each group). (d) Representative ENaC single-channel currents in intact endothelial cells attached to a mesenteric artery isolated from NS or HS diet-fed SS rats; mesenteric arteries, respectively, treated with BMP4 (50 ng/mL) and noggin (200 ng/mL) for 4 hours and application of 1 μM benzamil prior to recording. (e) Summary of ENaC P_O obtained from different experimental groups as shown in (d). *$P < 0.05$ *vs.* NS, #$P < 0.05$ *vs.* HS, &$P < 0.05$ *vs.* NS + BMP4 ($n = 6$ rats for each group). (f–g) Representative perforated whole-cell recording setup and summarized data showing the ENaC currents in primary cultured endothelial cells isolated from SS rats. The cells were cultured with or without an additional 20 ng/mL BMP4 in the culture medium for 24 hours, in the presence or absence of 1 μM benzamil. *indicates $P < 0.05$ *vs.* control group; #represents $P < 0.05$ *vs.* BMP4 treated group ($n = 6$ individual cells for each group).

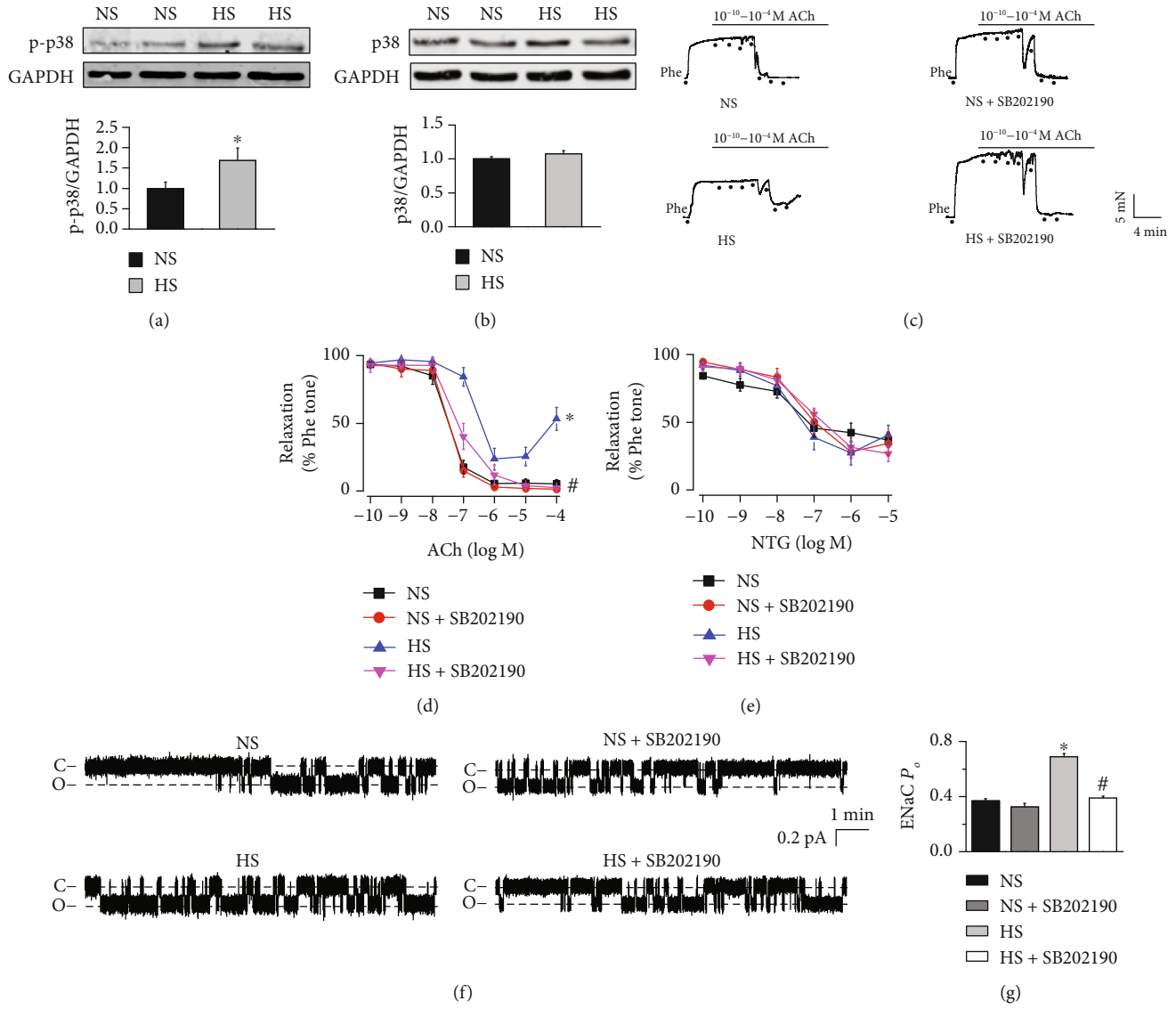

FIGURE 4: HS diet impairs endothelium-dependent relaxation and stimulates ENaC via p38 MAPK in SS rats. (a, b) Upper panels are representative Western blots and summarized data showing p38 MAPK and p-p38 MAPK protein levels under each condition in endothelial cells isolated from SS rats fed NS or HS diet. $^{*}P < 0.05$ *vs.* NS ($n = 6$ for each group). (c) Representative traces of ACh-induced artery relaxation in NS or HS diet-fed SS rats, in absence of or in the presence of 20 μM SB202190 for 4 hours. The first dot in each plot represents the time when Phe (10 μM) was added. The following dots indicate ACh concentrations gradually increased to 0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM. (d, e) Summaries of relaxation responses of mesenteric arteries to different doses of ACh (d) and to different doses of NTG (e). $^{*}P < 0.05$ *vs.* NS; $^{\#}P < 0.05$ *vs.* HS ($n = 6$ for each group). (f) Representative ENaC single-channel currents generated from the intact endothelial cells of mesenteric artery isolated from NS or HS diet-fed SS rats, in the absence of or in the presence of 20 μM SB202190 for 4 hours. (g) Summary of ENaC P_O obtained from single-channel recordings from the SS rats, under indicated conditions, as shown in (f). *indicates $P < 0.05$ *vs.* NS; $^{\#}$represents $P < 0.05$ *vs.* HS ($n = 6$ rats for each group).

BMP4 expression. Previous studies have shown that aldosterone promotes BMP4 expression in the kidney [40]. Interestingly, we showed that the HS diet elevates plasma aldosterone in SS rats [22]. These studies together suggest that HS may elevate serum BMP4 by elevating plasma aldosterone. However, inconsistent results also exist, showing that aldosterone decreases BMP4 levels in mouse mesangial cells [41]. Nevertheless, the present study suggests that BMP4 mediates HS-induced endothelial dysfunction and hypertension. Since previous studies have shown that BMP4 infusion induces hypertension in mice in a vascular NADPH oxidase-dependent manner and the subsequent endothelial dysfunction [15], BMP4 may act as a paracrine signaling molecule to modulate endothelium-dependent vascular relaxation in salt-sensitive hypertension.

The present study is the first evidence showing that BMP4 stimulates ENaC. We also showed that the downstream signaling transduction is associated with phosphorylation of p38 MAPK and then Sgk1/Nedd4-2. This pathway is not surprising, because it is known that p-p38 MAPK is elevated in hypertensive rats fed HS/high-fat diet [42], and phosphorylation of Nedd4-2 by Sgk1 increases ENaC

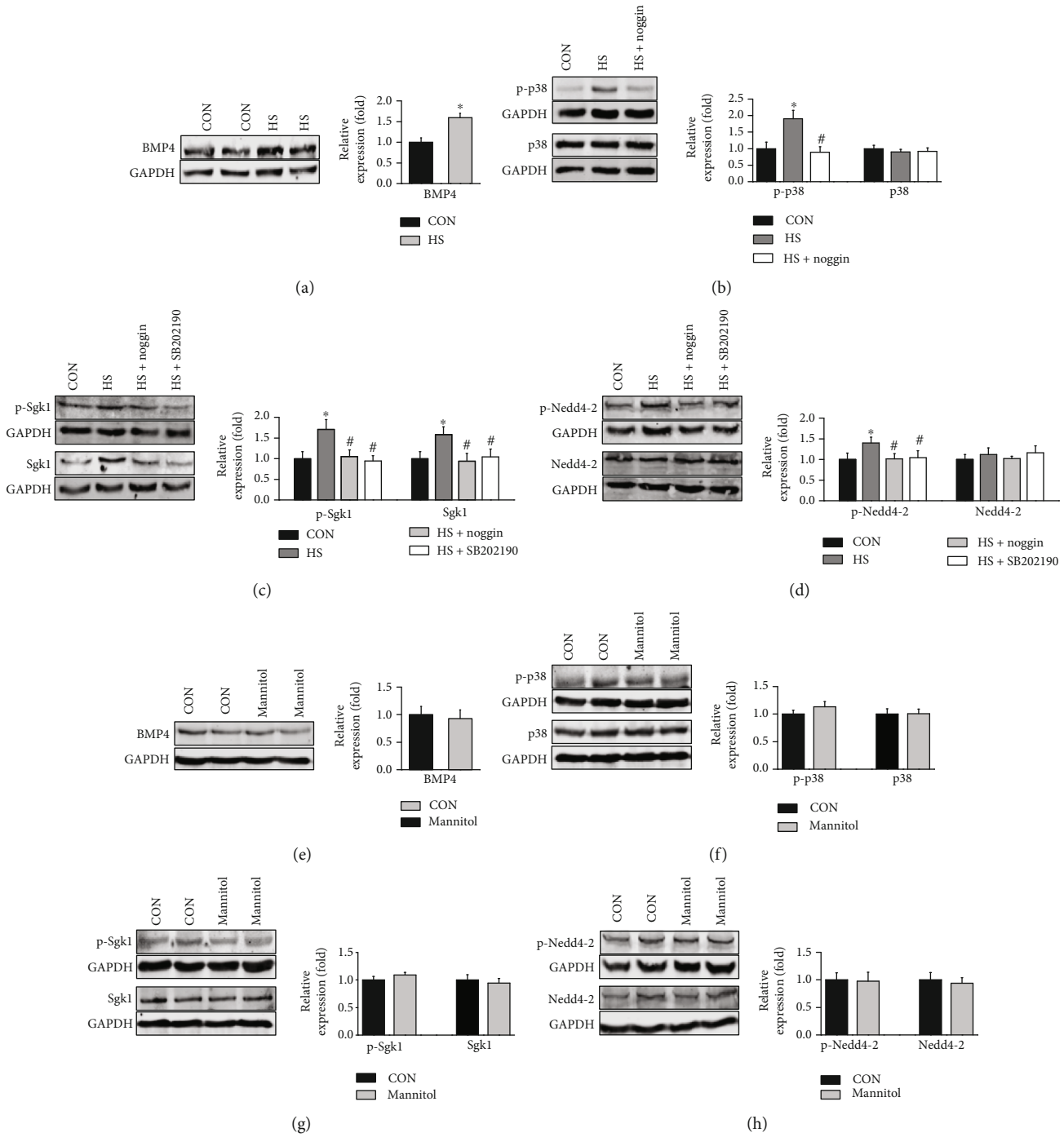

Figure 5: HS activates Sgk1/Nedd4-2 via BMP4 and p38 MAPK signaling. (a) Representative Western blots and summarized data showing BMP4 protein levels in endothelial cells isolated from SS rats either under control conditions or treated with an additional 40 mM NaCl in the culture medium for 24 hours. The data are the means ± SEMs of 6 experiments in each group. *indicates $P < 0.05$ *vs.* control. (b) Representative Western blots and summarized data showing p38 MAPK and p-p38 MAPK protein levels under control conditions or treated with HS additional 40 mM NaCl in the culture medium for 24 hours, in the absence of and in the presence of 100 ng/mL noggin. *indicates $P < 0.05$ *vs.* control; #represents $P < 0.05$ *vs.* HS ($n = 6$ for each group). (c–d) Representative Western blots and summarized data showing Sgk1, p-Sgk1, Nedd4-2, and p-Nedd4-2 protein levels under control conditions or treated with additional 40 mM NaCl in the culture medium for 24 hours, in the absence of and in the presence of either 100 ng/mL noggin or 10 μM SB202190 for 24 hours. *indicates $P < 0.05$ *vs.* control; #represents $P < 0.05$ *vs.* HS ($n = 6$ for each group). (e–h) Representative Western blots and summarized data showing BMP4, p-p38 MAPK, p38 MAPK, p-Sgk1, Sgk1, p-Nedd4-2, and Nedd4-2 protein levels in primary cultured endothelial cells isolated from SS rats either under control conditions or treated with an additional 80 mM mannitol in the culture medium for 24 hours. The data are the means ± SEMs of 6 experiments in each group.

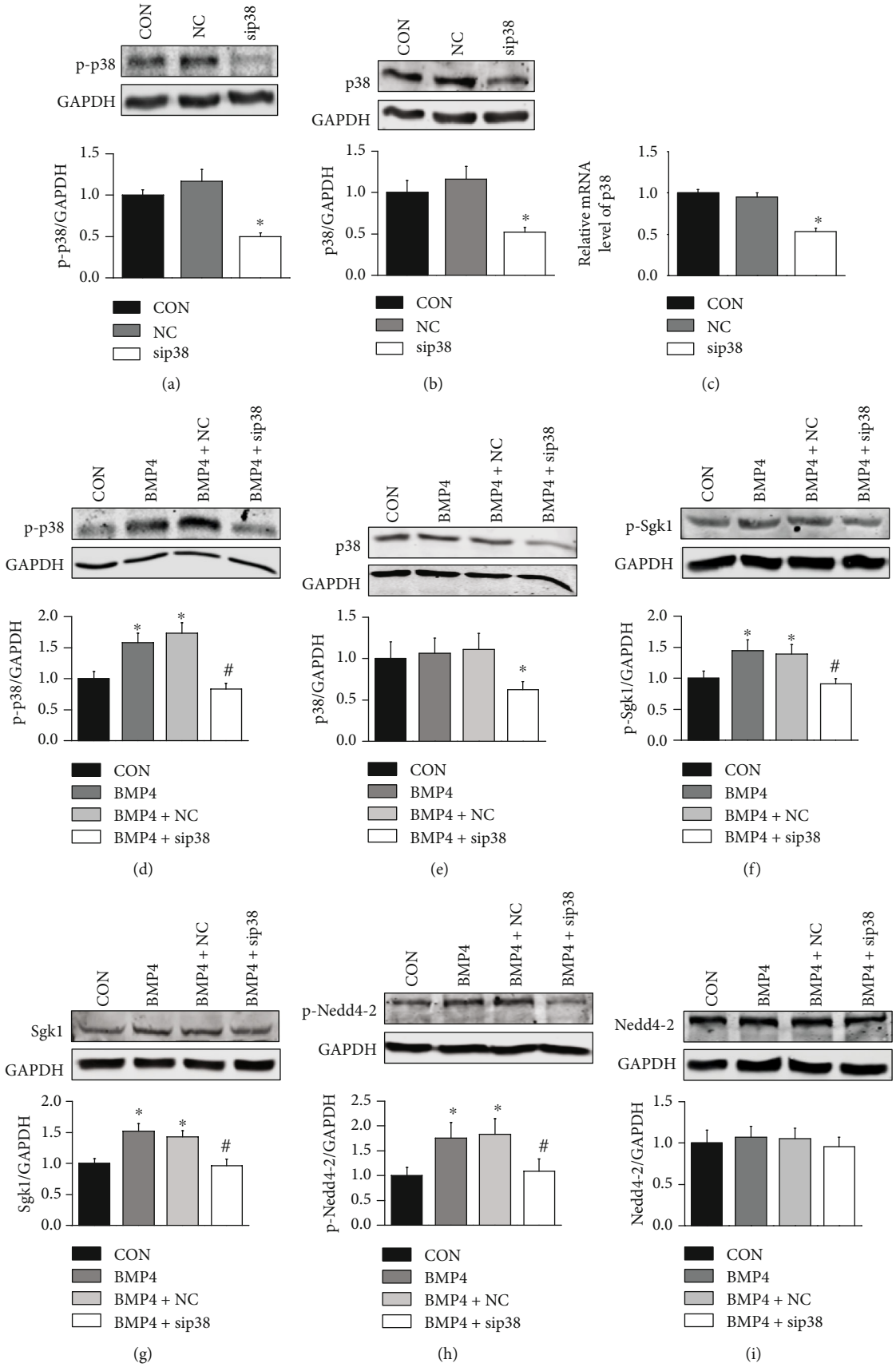

Figure 6: Continued.

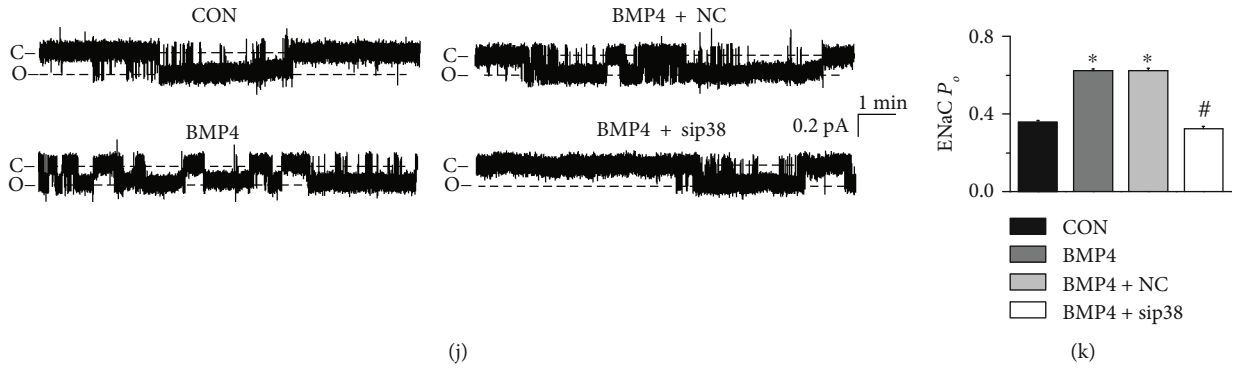

Figure 6: BMP4 stimulates ENaC by activating p38 MAPK and Sgk1/Nedd4-2. The experiments were conducted in the primary cultured endothelial cells (ECs) isolated from SS rats and the ECs were, respectively, transfected by p38 MAPK control LV (MOI = 10) and p38 MAPK shRNA LV (MOI = 10) to knockdown p38 MAPK. (a, b) Representative Western blots and summarized data showing p38 MAPK and p-p38 MAPK protein levels in primary cultured endothelial cells isolated from SS rats either under control conditions or treated with p38 MAPK control LV or p38 MAPK shRNA LV. $^*P < 0.05$ *vs.* control ($n = 6$ for each group). (c) Summarized data showing p38 MAPK mRNA levels in primary cultured endothelial cells isolated from SS rats either under control conditions or treated with p38 MAPK control LV or p38 MAPK shRNA LV. $^*P < 0.05$ *vs.* control ($n = 6$ for each group). (d–i) Representative Western blots and summarized data showing p38 MAPK, p-p38 MAPK, Sgk1, p-Sgk1, Nedd4-2, and p-Nedd4-2 protein levels in ECs or in p38 MAPK gene manipulated ECs under control conditions or treated with 20 ng/mL BMP4 for 24 hours. *indicates $P < 0.05$ *vs.* control; $^\#$represents $P < 0.05$ *vs.* BMP4 ($n = 6$ for each group). (j) Representative ENaC single-channel currents recorded from ECs or in p38 MAPK gene manipulated ECs, in the absence of or in the presence of 20 ng/mL BMP4 (24 hours). (k) Summary of ENaC P_O obtained from the experiments shown in (j). *indicates $P < 0.05$ *vs.* control; $^\#$represents $P < 0.05$ *vs.* BMP4 ($n = 6$ for each individual cells).

activity by reducing its degradation [29–31]. It is also known that BMP4 activates p38 MAPK in mouse endothelial cells via a ROS-dependent mechanism and that BMP4 causes endothelial dysfunction that can be reversed by a p38 MAPK inhibitor [25]. Furthermore, a p38 MAPK inhibitor can prevent HS-induced Sgk1 expression in inner medullary collecting duct cells [43]. However, inconsistent results also exist, showing that p38 MAPK reduces ENaC activity in mice [44]. We first showed that ENaC can be stimulated by the Sgk1/Nedd4-2 in endothelial cells. Since a p38 MAPK shRNA lentivirus could prevent BMP4-induced Sgk1, p-Sgk1, and p-Nedd4-2 activation, p38 MAPK should be the immediate signaling molecule after BMP4 but before Sgk1 and Nedd4-2 in the pathway. We argue that increased ENaC activity by BMP4 in response to HS diet may account for reduced vascular relaxation, because the data show that the relaxation can be abolished by benzamil, an ENaC blocker. This argument is also supported by our previous studies showing that increased ENaC activity impairs vascular relaxation in nitric oxide (NO)-dependent manner [22]. We have also shown that blockade of ENaC prevents ox-LDL-induced vascular dysfunction by increasing NO [24]. It has been shown in Liddle mice that activated ENaC is responsible for reduced NO production and contributed to vascular endothelial cell stiffness [45]. How ENaC in endothelial cells works with ENaC in the distal nephron to mediate the pathogenesis of SS hypertension must still be determined.

5. Conclusion

HS intake elevates BMP4 in endothelial cells and serum of SS rats. The elevated BMP4 stimulates ENaC in endothelial cells via a pathway associated with p38 MAPK and Sgk1/Nedd4-2. BMP4 induced by HS decreases endothelium-dependent artery relaxation by stimulating ENaC. These results may have clinical significance for the management of hypertension in SS populations.

Abbreviations

BMP4: Bone morphogenetic protein 4
ENaC: Epithelial sodium channel
SD: Sprague-Dawley
SS: Salt-sensitive
NS: Normal salt
HS: High salt
EDR: Endothelium-dependent relaxation
p38 MAPK: p38 mitogen-activated protein kinase
p-p38: Phospho-p38
Sgk1: Serum/glucocorticoid regulated kinase 1
Nedd4-2: Neural precursor cell-expressed developmentally downregulated protein 4-2
MAEC: Mesenteric artery endothelial cell
ECs: Endothelial cells
TGF-β: Transforming growth factor-β
PSS: Physiological saline solution
ACh: Acetylcholine
Phe: Phenylephrine
NTG: Nitroglycerin
LV: Lentivirus
shRNA: Short hairpin RNA
qRT-PCR: Quantitative real-time PCR
SBP: Systolic blood pressure
DBP: Diastolic blood pressure
MAP: Mean arterial pressure
NC: Negative control
NO: Nitric oxide
P_O: Open probability.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

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Research Article

Microarray Analysis of Small Extracellular Vesicle-Derived miRNAs Involved in Oxidative Stress of RPE Cells

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The aim of this study was to investigate the miRNA profiles of nanosized small extracellular vesicles (sEVs) from human retinal pigment epithelial (RPE) cells under oxidative damage. ARPE-19 cells were cultured with ox-LDL (100 mg/L) or serum-free medium for 48 hours, sEVs were then extracted, and miRNA sequencing was conducted to identify the differentially expressed genes (DEGs) between the 2 groups. RNA sequence results were validated using quantitative real-time PCR. The Gene Ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes pathway, and ingenuity pathway analyses (IPA) were performed for the DEGs. Results revealed that oxidative stress inhibited RPE cell viability and promoted sEV secretion. A total of 877 DEGs from sEVs were identified, of which 272 were downregulated and 605 were upregulated. In total, 66 enriched GO terms showed that the 3 most significant enrichment terms were cellular processes (biological processes), cell (cellular component), and catalytic activity (molecular function). IPA were used to explore DEGs associated with oxidation damage and further construct a miRNA-target regulatory network. This study identified several DEGs from oxidation-stimulated RPE cells, which may act as potential RNA targets for prognosis and diagnosis of RPE degeneration.

1. Introduction

Oxidative damage is one of the major contributors to retinal degenerative diseases such as age-related macular degeneration (AMD) [1]. AMD is a multifactorial disease in which oxidative stress serves as a key component. The retinal pigment epithelium (RPE) is a highly specialized, polarized epithelium, which is in intimate contact with the outer segments of the photoreceptor and Bruch's membrane [2]. PRE cells are particularly metabolically active, highly oxygenated, and vulnerable to oxidative stress under exposure to photosensitizers such as lipofuscin [3]. Oxidative stress induces cell apoptosis through reactive oxygen species, thereby leading to RPE dysfunction [4].

Exosome refers to one form of extracellular nanometer-sized vesicle, which mediates multiple extra- and intercellular activities, including cell-cell communication, immune modulation, extracellular matrix turnover, stem cell division/differentiation, neovascularization, and cellular waste removal [5]. RPE cells secrete extracellular vesicles (EVs) in response to oxidative stress, resulting in angiogenesis in endothelial cells [6]. Exosomal biological markers CD63 and LAMP2 have been found in the drusen of AMD patients and stressed RPE cells, which suggests that the drusen is initiated by intracellular proteins from RPE cells and becomes extracellular via the exosome [7]. Small extracellular vesicles (sEVs) contain multiple functional molecules such as mRNA, microRNA (miRNA), and proteases. miRNAs are small noncoding RNA molecules, which inhibit several targeting mRNA expressions. Genetic mutations of miRNAs induce pathophysiological and immunological dysfunction in RPE cells. A related study suggested exosomal miRNA variations as predictive biomarkers in AMD disease [8]. Here, we aimed to screen for differentially expressed miRNA profiles in sEVs derived from oxidative stress-stimulated RPE cells and identify potential functional miRNAs, which may be associated with RPE oxidation.

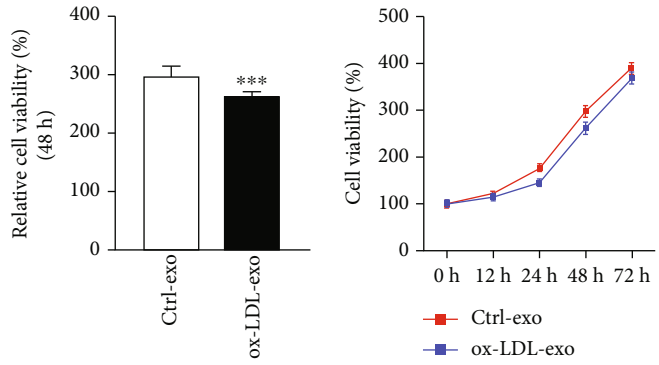


Figure 1: ox-LDL reduced RPE cell viability. ARPE-19 cells were treated with control (serum-free medium) or ox-LDL (100 mg/L) for 48 hours. Cell viability was tested by CCK8 assay. Data are expressed as mean ± SD ($n = 3$). Experiments were repeated 3 times. $^{***}p < 0.001$ vs. the control group.

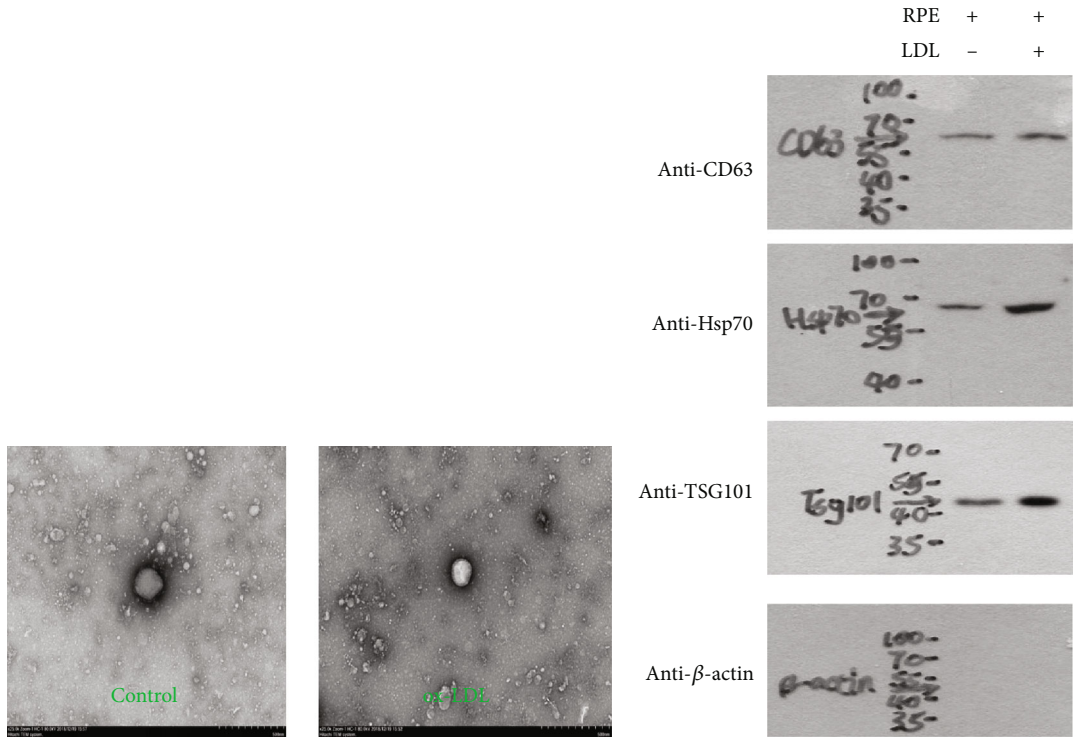


Figure 2: Transmission electron microscopy images of exosome in the ox-LDL and control groups (a) and western blot results of exosomal marker proteins (b).

2. Materials and Methods

2.1. Cell Culture and Oxidative Stress Induction. The human RPE cell line (ARPE-19) is transformed and maintained at 1×10^6 cells/mL culture in DMEM/F12 medium (Gibco Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; HyClone, Shanghai, China), penicillin/-streptomycin (1 : 100, Sigma, USA), 4 mM glutamine, and 0.19% HEPES (Sigma), in a humidified incubator at 37°C and 5% CO_2. Cells were seeded and grown to 70-80% confluence before being placed in a serum-free medium (SFM) for 24 hours, then randomized into SFM or human oxidized low-density lipoprotein (ox-LDL, 100 mg/L, AppliChem, Darmstadt, Germany) groups for 48 hours.

2.2. CCK8 Assay for RPE Cell Viability. ARPE-19 cells were seeded at a density of 1×10^4 cells/100 μL/well in 96-well plates. After the treatment mentioned above, 10 μL of XTT (BBI Life Sciences, China) solution was added into each well for 1 hour at 37°C. Cell viability was determined by measuring absorbance at 450 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

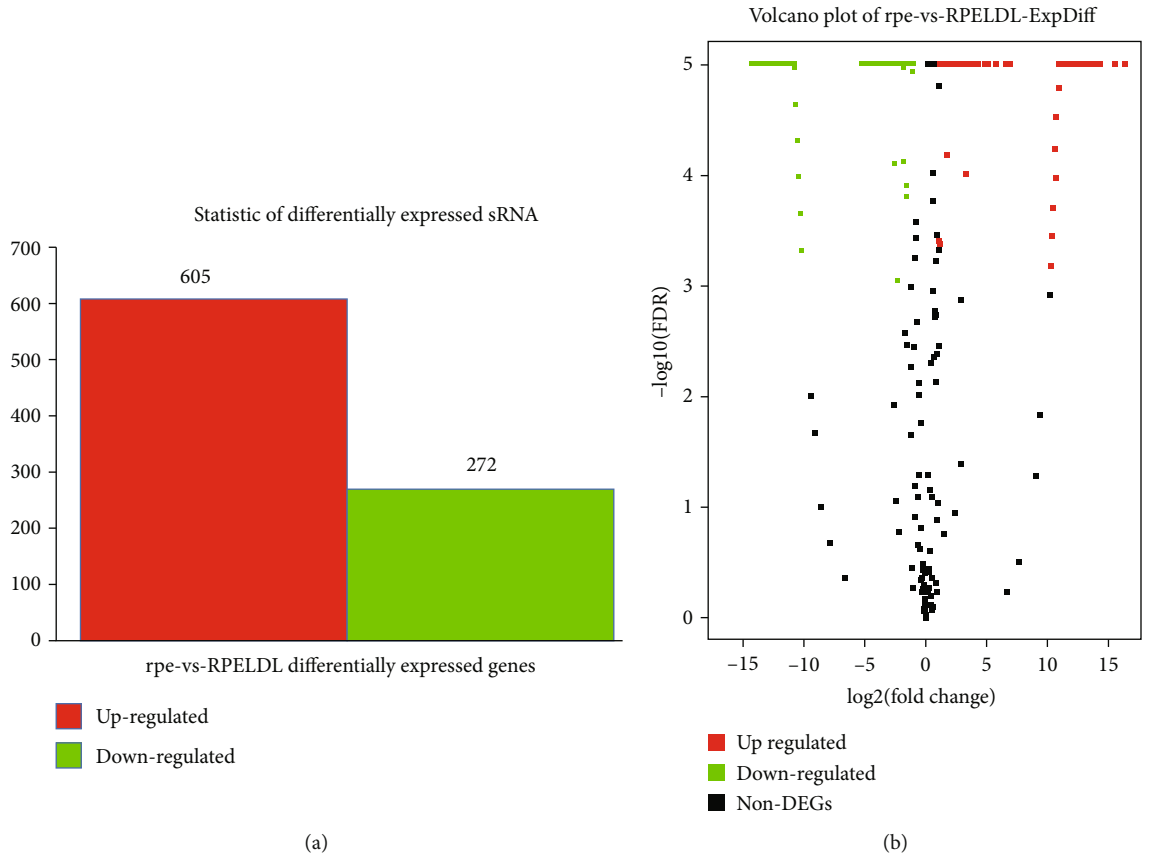


Figure 3: General RNA-Seq analysis of sEV-derived miRNAs and statistical analysis of differentially expressed genes between the ox-LDL and control groups. (a) Histogram of DEGs between 2 groups. (b) Volcano plot of DEGs. p value < 0.05 was considered significant.

Table 1: List of the top 10 DEGs between the ox-LDL and control groups.

Gene ID	log2Ratio (RPELDL/rpe)	p value	FDR	Description	Primer sequence ($5' \rightarrow 3'$)
hsa-miR-3184-3p	16.27193714	<0.001	<0.001	Up	TCCTCTTCTCCCTCCTCCCA
hsa-let-7e-5p	15.46467337	<0.001	<0.001	Up	AGCTGGTGTTGTGAATCAGG
hsa-miR-208a-5p	13.98992663	<0.001	<0.001	Up	CGCATCCCCTAGGGCATTGG
hsa-miR-138-5p	13.57222651	<0.001	<0.001	Up	TAGTGCAATATTGCTTATAG
hsa-miR-1228-3p	13.13089227	<0.001	<0.001	Up	AAAGTCTCGCTCTCTGCCCC
hsa-miR-423-5p	-14.52833201	<0.001	<0.001	Down	GGAGCGAGATCCCTCCAAAAT
hsa-miR-1910-5p	-13.02410078	<0.001	<0.001	Down	GAGCTTTTGGCCCGGGTTAT
hsa-miR-197-3p	-11.56985561	<0.001	<0.001	Down	GGCTGTTGTCATACTTCTCATGG
hsa-miR-877-3p	-10.27612441	<0.001	<0.001	Down	TCACAGTGGCTAAGTTCTGC
hsa-miR-324-5p	-9.409390936	<0.001	<0.001	Down	TGAGGGGCAGAGAGCGAG

FDR: false discovery rate.

2.3. Exosome Isolation and Transmission Electron Microscopy Imaging. Exosomes were isolated from ARPE-19 cells using multistep differential centrifugation [9]. ARPE-19 cells were centrifuged at 300 × g for 10 minutes at 4°C. Subsequently, the supernatant was subjected to the following centrifugation steps: 2000 × g for 10 minutes, 10,000 × g for 30 minutes, and 100,000 × g for 70 minutes. The resulting sEVs were finally resuspended in PBS and centrifuged at 100,000 × g for 70 minutes again. The morphology of sEVs was visualized using the Hitachi transmission electron microscope operated at 80 kV (Hitachi, Japan).

2.4. Western Blot Analysis. As described previously [10], after incubating for 5 min at 90°C with loading buffer (Life Technologies, Australia), 10 μg of exosomes in each group was electrophoresed on NuPAGE Novex 4–12% Bis-Tris Gels (Life Technologies, USA). Gels were transferred onto PVDF membranes using the Trans-Blot Turbo system. Membranes

were blocked in 2% BSA solution for 3 hours and then probed overnight with primary exosomal marker protein antibodies: anti-Hsp70 (ab134045, Abcam, Cambridge, UK), anti-CD63 (ab181606, Abcam), and anti-TSG101 (ab125011, Abcam) at 4°C, followed by incubation with a secondary antibody for 3 hours. The ChemiDoc XRS gel documentation system (Bio-Rad Laboratories, USA) was used to quantify the immune-reactive proteins, and β-actin was used as a loading control for each lane. Each indicated band was quantified and normalized to β-actin through ImageJ software.

2.5. miRNA Extraction and miRNA Sequencing. As reported previously [11], RNA extraction was performed using the Total Exosome RNA and Protein Isolation Kit (catalog # 4478545; Invitrogen, USA) according to the provided instructions. 200 ng-1 μg RNA in final volume of 30 μL solution was collected for each sample. Total RNA quantity and quality (260/280 absorbance ratio) were assessed using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer to test concentration and inorganic ions or polycarbonate contamination. miRNA sequence was isolated by BGI Company (China) based on previous instructions [12]. cDNA libraries were constructed using the Ion Total RNA-Seqv2 kit (Life Technologies, USA) ($n = 3$ for each group) and purified using AMPure beads (Beckman Coulter). Emulsion PCR and enrichment of cDNA-conjugated particles were performed with an Ion OneTouch 200 Template Kit v2 DL (Life Technologies). The final cDNA samples were sequenced single end on the HiSeq 2000 System with a 50 bp read length.

2.6. Bioinformatics Analysis of the Data. Raw data was filtered to eliminate low-quality reads, primers, adaptors, and other contaminants. Following this, we summarized the length distribution and common and specific sequences between samples. After filtering, the remaining tags were called clean tags and stored in FASTQ format. Bowtie2 was used to map clean reads to the reference genome and other sRNA databases. To identify differentially expressed genes (DEGs), differentially expressed miRNAs (DEMIs) were screened out using the limma package through the thresholds of fold change > 2 or <0.5 and adjusted p value of <0.05 [13].

To perform Gene Ontology (GO) enrichment analysis, we mapped all genes to GO terms in the database, which calculated the gene numbers for every term. The hypergeometric test was then used to find significantly enriched GO terms in the input gene list.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was used to perform pathway enrichment analysis. This analysis identified significantly enriched metabolic or signal transduction pathways from target genes of DEGs when compared with the whole genome background. The p value was corrected using the Bonferroni method; a corrected p value < 0.05 was considered significant.

In this study, experimentally verified miRNA-mRNA regulatory pairs were obtained using TargetScan and miRanda and a miRNA-target regulatory network was constructed by comparing the DEGs with obtained miRNA-gene regulatory pairs.

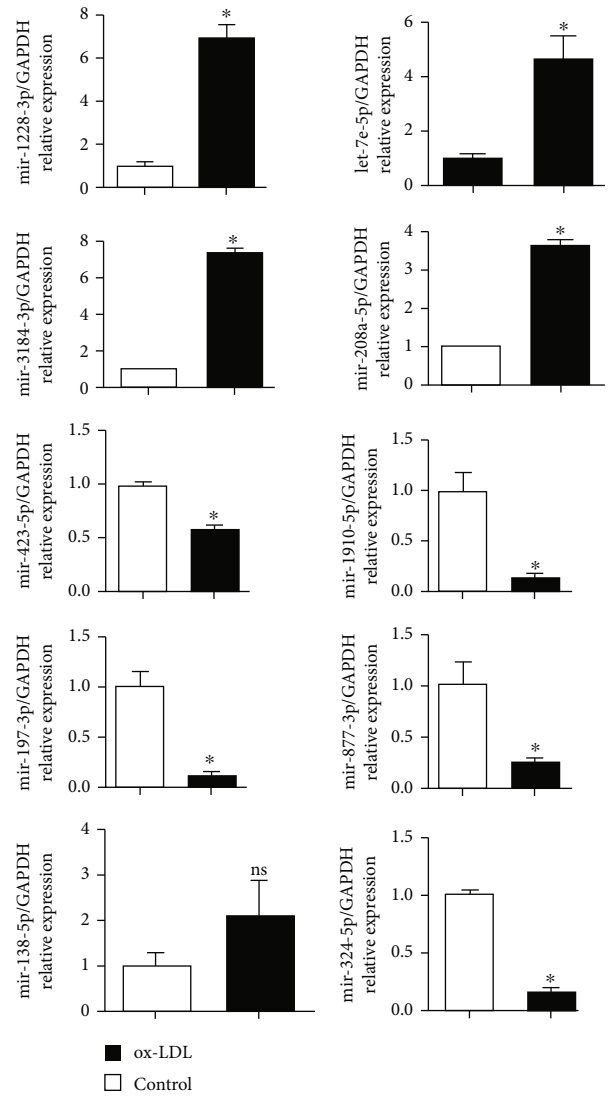


Figure 4: Validation of the top 10 selected DEGs screened from miRNA sequence by qRT-PCR tests. ARPE-19 cells were treated as before, and miRNAs were extracted from sEVs. Data was expressed as mean ± SD ($n = 6$). Experiments were repeated three times. $^*p < 0.05$ vs. the control group. ns: no significance.

2.7. Validation of miRNA Expression Using Quantitative Real-Time PCR (RT-PCR). In order to validate initial miRNA sequence results, the 10 most significant up- or downregulated miRNAs were selected for further RT-PCR tests as reported previously [14]. Total RNA was isolated using TRIzol reagent and the quality and quantity of RNA was measured using a NanoDrop 2000 spectrophotometer. Each reverse transcription reaction mixture contained 10 mL of SYBR Green Master Mix, 0.5 mL of miR-RT primers F (10 mM), 0.5 mL of miR-RT primers R (10 mM), and RNase-free H_2O. The RT-PCR reactions for the selected 10 miRNAs were performed using the ViiA 7 Real-Time PCR System (ABI, USA) under the following conditions: 95°C for 1 min, followed by 40 PCR cycles (95°C for 10 s and then 60°C for 20 s). miRNA expression was normalized to the endogenous reference gene *GAPDH*. Each sample was

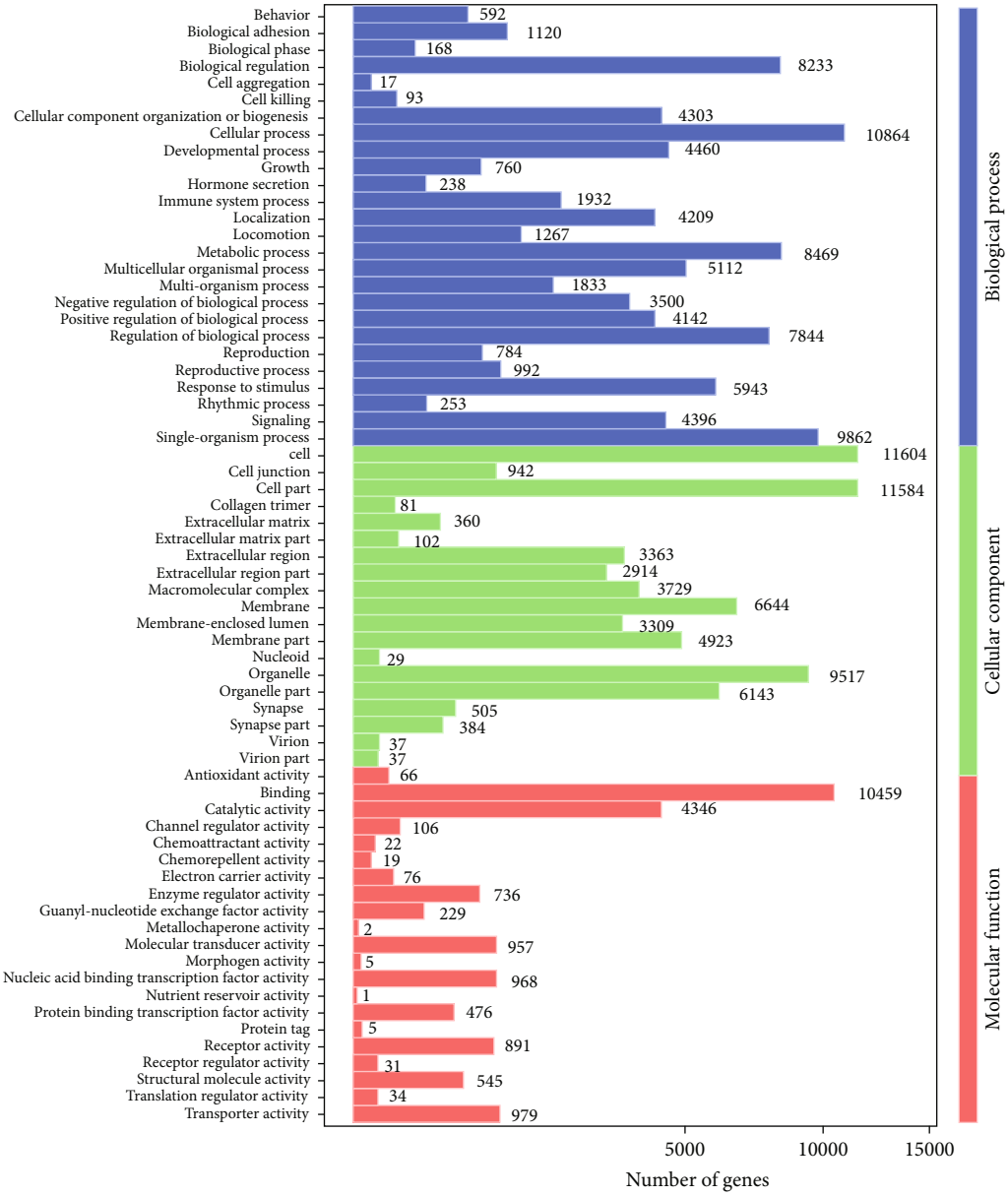


FIGURE 5: Go enrichment analysis of DEGs between 2 groups. Blue, green, and red bars represent the enrichment and numbers of DEGs in the biological process, cellular component, and molecular function, respectively.

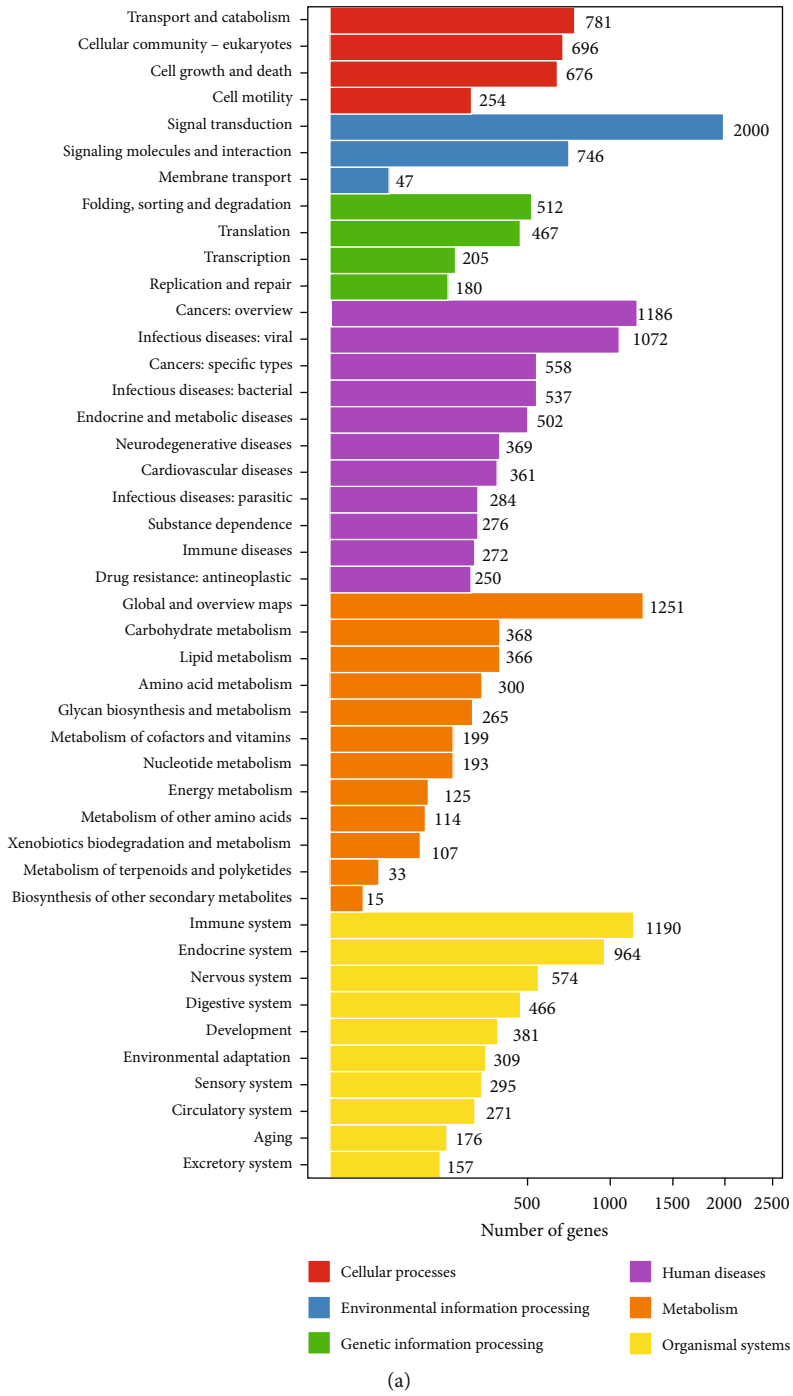
analyzed in triplicate. Specific primers were produced by BIOTNT Company (Shanghai, China). Relative quantification was achieved by the comparative $2^{-\Delta\Delta ct}$ method.

2.8. Statistics. The data were analyzed with a one-way analysis of variance (ANOVA) using the statistical program SPSS 17.0. All data were presented as mean ± SD. p value < 0.05 was considered statistically significant.

3. Results

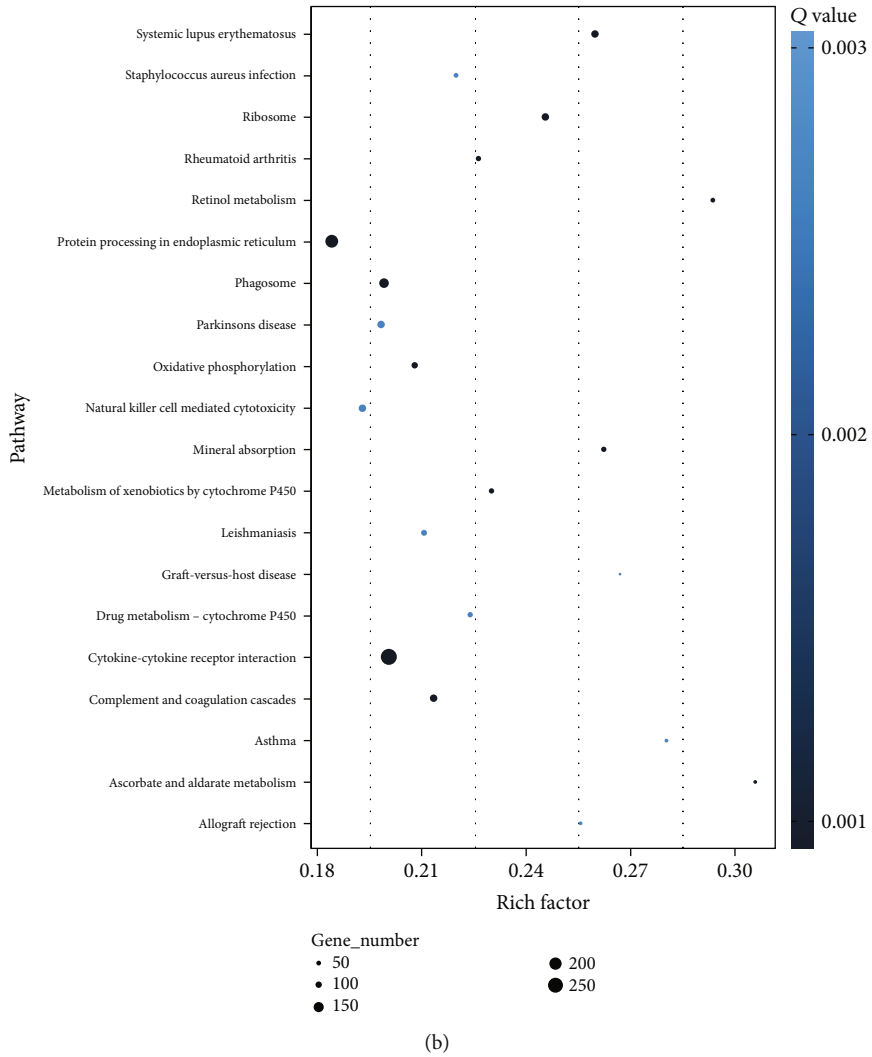
3.1. ox-LDL Decreases ARPE-19 Cell Viability. We first measured the cytotoxicity of ox-LDL to ARPE-19 cells after 48 hours. Figure 1 shows that cell viability in the ox-LDL group was significantly lower than that in the control group ($p < 0.05$), which indicated its cytotoxicity and aligned with previous conclusions [15, 16].

3.2. Characterization of sEVs and Biological Marker Protein Detection. Transmission electron microscopy images of sEVs derived from both groups revealed the presence of distinct vesicles with an average diameter of 106 ± 7.62 nm (Figure 2(a)). The vesicles were also positive for exosomal markers. CD63 is the general tetraspanin protein used as the exosomal "star marker" [17]. TSG101 and Hsp70 are also commonly used for exosome detection [18, 19]. As shown in Figure 2(b), we found that the expression levels of TSG101 and Hsp70 were statistically higher in the ox-LDL group than the control, but it did not reach a significant difference ($p > 0.05$).



(a)

Figure 6: Continued.



(b)

Figure 6: KEGG pathways analysis of DEGs between 2 groups. (a) 6 categories of biological functions and numbers of genes in different pathways. (b) Rich factors of the 20 most enriched pathways. The sizes of circles correspond to gene numbers. The colors correspond to the Q value.

3.3. Differential Expression of miRNA Profiles in sEVs Isolated from the Oxidative and Control RPE Cells. In order to identify the influence of oxidation on miRNA profiles from sEVs of RPE cells, miRNA sequence was sequenced after treatment for 48 hours. Figure 3 shows that 877 significantly differentially expressed miRNAs had been screened between the ox-LDL and control groups, among which 272 were downregulated and 605 were upregulated. The top 10 differentially expressed genes are listed in Table 1.

3.4. DEG Validation Using RT-PCR. The top 10 selected DEGs were further validated using RT-PCR (Figure 4). PCR tests revealed similar results of RNA sequence, except that miR-138-5p showed insignificant differences between the two groups ($p > 0.05$).

3.5. Gene Ontology Enrichment Analysis. The GO analysis contains three ontologies: biological processes, molecular function, and cellular components. We identified 66 enriched GO terms, among which 26 belong to biological processes, 21 belong to molecular function, and 19 belong to cellular components. The three most enriched biological process terms were cellular processes, single-organism processes, and metabolic processes. Meanwhile, cell, cell part, organelle, catalytic activity, transporter activity, and transporter activity were the most enriched GO terms of cellular components and molecular function, respectively (Figure 5).

3.6. KEGG Pathway Enrichment Analysis. KEGG analysis classified DEGs into 6 categories according to their biological functions: cellular processes (4 pathways), environmental information processing (3 pathways), genetic information processing (4 pathways), human diseases (11 pathways), metabolism (12 pathways), and organismal systems (10 pathways). The 20 most enriched pathways are presented in Figure 6.

3.7. Functional Exploration with Ingenuity Pathway Analysis. Based on the KEGG and Gene Ontology results, we further

Table 2: List of DEGs in IPA.

Function	Pathway or GOID	Name (Homo sapiens (human))	Count	Gene ID
AMD	hsa02010	ABC transporters	8	miR-345-5p, miR-210-5p, miR-34a-5p, miR-1908-5p, miR-485-5p, miR-1343-3p, miR-423-5p, miR-4488
	hsa03420	Nucleotide excision repair	5	miR-138-5p, miR-345-5p, miR-1908-5p, miR-1343-3p, miR-485-5p
	hsa04060	Cytokine-cytokine receptor interaction	12	miR-138-5p, miR-345-5p, miR-210-5p, miR-378a-5p, miR-34a-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p, miR-4488, miR-210-5p, miR-423-5p
	hsa04062	Chemokine signaling pathway	9	miR-138-5p, miR-345-5p, miR-210-5p, miR-378a-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p, miR-4488
	hsa04145	Phagosome	7	miR-138-5p, miR-210-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p, miR-4488
	hsa04620	Toll-like receptor signaling pathway	6	miR-345-5p, miR-210-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-4488
Lipid metabolism	hsa00561	Glycerolipid metabolism	10	miR-138-5p, miR-345-5p, miR-378a-5p, miR-34a-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p, miR-4488, miR-210-5p
	hsa00564	Glycerophospholipid metabolism	10	miR-138-5p, miR-345-5p, miR-210-5p, miR-378a-5p, miR-34a-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p, miR-4488
	hsa00565	Ether lipid metabolism	3	miR-34a-5p, miR-423-5p, miR-4488
	hsa00600	Sphingolipid metabolism	6	miR-34a-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p, miR-4488
Oxidative damage	GO:1902175	Regulation of oxidative stress-induced intrinsic apoptotic signaling pathway	0	
	GO:1900407	Regulation of cellular response to oxidative stress	9	miR-138-5p, miR-345-5p, miR-210-5p, miR-34a-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p, miR-4488
	GO:0001306	Age-dependent response to oxidative stress	9	miR-138-5p, miR-345-5p, miR-210-5p, miR-34a-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p, miR-4488
	GO:0036473	Cell death in response to oxidative stress	4	miR-138-5p, miR-210-5p, miR-1343-3p, miR-4488
	GO:1902882	Regulation of response to oxidative stress	9	miR-138-5p, miR-345-5p, miR-210-5p, miR-34a-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p, miR-4488
Cellular inflammation	GO:0002532	Production of molecular mediator involved in inflammatory response	8	miR-4488, miR-345-5p, miR-378a-5p, miR-34a-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p
	GO:0002534	Cytokine production involved in inflammatory response	8	miR-4488, miR-345-5p, miR-378a-5p, miR-34a-5p, miR-1908-5p, miR-1343-3p, miR-485-5p, miR-423-5p
	GO:0002537	Nitric oxide production involved in inflammatory response	0	
	GO:0002540	Leukotriene production involved in inflammatory response	0	
	GO:0002541	Activation of plasma proteins involved in acute inflammatory response	0	
Choroid angiogenesis	GO:0045765	Regulation of angiogenesis	7	miR-4488, miR-138-5p, miR-345-5p, miR-210-5p, miR-1908-5p, miR-1343-3p, miR-423-5p

searched for related functional genes and associated pathways by ingenuity pathway analysis (IPA) from DEGs. We identified several pathways and genes which related to AMD (6 pathways), lipid metabolism (4 pathways), oxidative damage (5 GO terms), cellular inflammation (5 GO terms), and choroidal neovascularization (GO:0045765) (Table 2).

3.8. miRNA-Target Regulatory Network Analysis. We used TargetScan and miRanda software to predict possible targeted mRNAs for DEGs and associated their intersections with the IPA results; a total of 10 miRNAs and 43 targeted mRNAs formed a miRNA-target regulatory network (Figure 7).

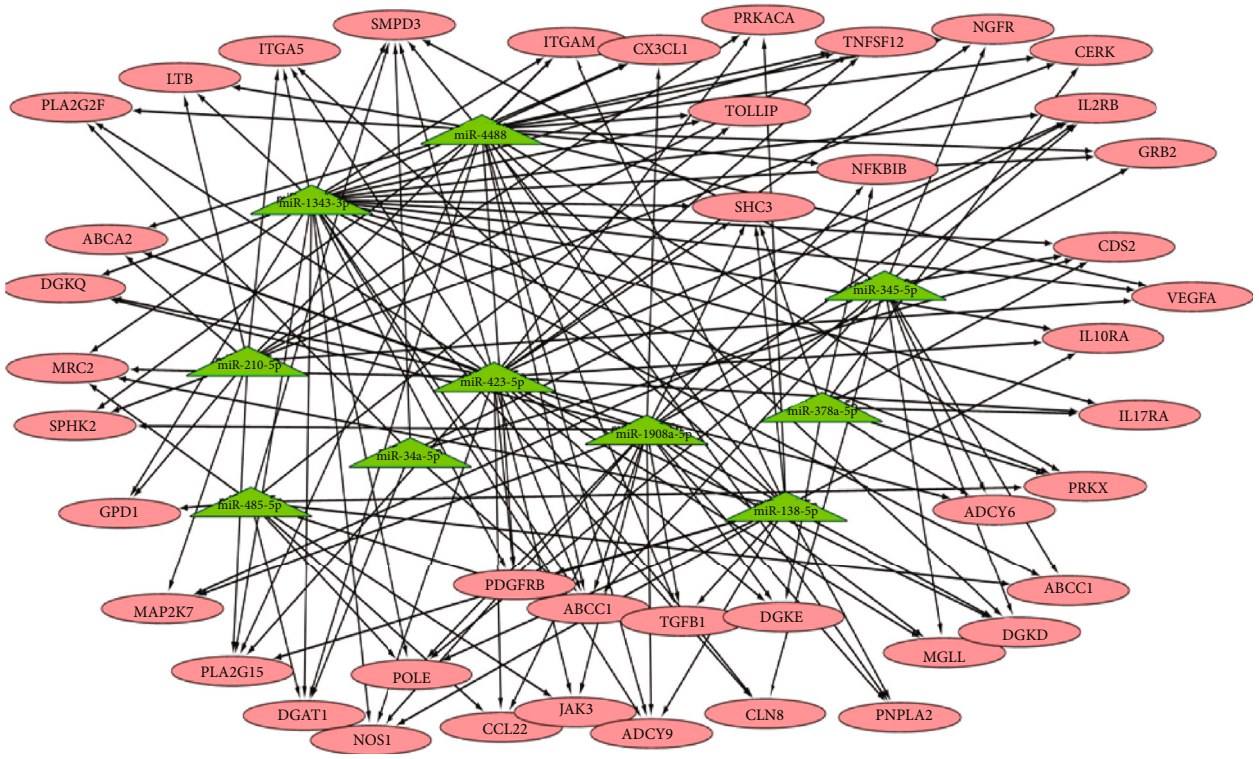


Figure 7: microRNA-target regulatory networks of differentially expressed genes (DEGs). Green triangles represent miRNAs; red circles represent targeting genes.

Table 3: List of DEGs related to AMD in previous studies.

Gene ID	log2Ratio (RPELDL/rpe)	*p* value	FDR	Description
hsa-*miR-192* [12]	-10.98	<0.001	<0.001	Down
hsa-*let-7c* [25]	5.60	<0.001	<0.001	Up
hsa-*miR-183* [24]	12.16	<0.001	<0.001	Up
hsa-*miR-27a* [25, 26]	12.10	<0.001	<0.001	Up
hsa-*miR-27b* [22]	4.99	<0.001	<0.001	Up
hsa-*miR-361-5p* [12]	-11.94	<0.001	<0.001	Down
hsa-*miR-335* [12, 22]	-12.06	<0.001	<0.001	Down
hsa-*miR-30c* [25]	10.51	<0.001	<0.001	Up

4. Discussion

Oxidative stress has been recognized as a major influence in AMD pathophysiology, and RPE appears to be the main site of damage [2]. Oxidative damage of the RPE layer originates from the digestion of photoreceptor outer segments and other reactive oxygen species. RPE damage occurs in multiple locations within the central part of the eye and finally forms a region of atrophy by the bystander effect, which is mediated via EVs [20]. In this research, ox-LDL decreased ARPE-19 cell viability and promoted sEV secretion. RNA sequences and RT-PCR tests confirmed a downregulation of *miR-1910-5p* in sEVs of the ox-LDL group, which is contrary to a similar study that found that H_2O_2 increases *miR-1910-5p* concentrations in ARPE-19 cells [21]. *miR-324-5p* was reported to be expressed in plasma of wet AMD patients, and we found a decreased expression in the ox-LDL group [22]. Desjarlais et al. [23] demonstrated an upregulation of *let-7g-5p* (>570%) in oxygen-induced retinopathy models during the neovascularization phase, which is consistent with our result. Other authors reported significant changes of *miR-192*, *let-7c*, *miR-183*, *miR-27a*, *miR-27b*, *miR-361-5p*, *miR-335*, and *miR-30c* in experimental AMD models, which were also observed in our study (see Table 3) [24–26]. KEGG analysis suggested cytokine-cytokine receptor interactions and phagosome and protein processing in the endoplasmic reticulum to be the most significant enrichment items. In agreement with other reports, cancer-related pathways are also involved in DEGs of sEVs in our study [15]. Further research is needed to explore the specific roles of these pathways.

IPA screened out 6 key pathways related to AMD and 4 GO terms related to oxidative damage. *miR-138-5p*, *miR-*

345-5p, *miR-210-5p*, *miR-34a-5p*, *miR-1908-5p*, *miR-1343-3p*, *miR-485-5p*, *miR-423-5p*, and *miR-4488* are associated with oxidative stress and AMD.

The miRNA-target regulatory network consists of several miRNAs and predicted targeting mRNAs. *miR-1343* was proved to be activated in response to stress in epithelial cells and targets both TGF-β receptors, which in turn contribute to the progression of angiogenesis in wet AMD [27, 28]. *miR-4488* was demonstrated to be involved in sphingolipid signaling and to modulate endoplasmic reticulum stress marker PERK in ARPE-19 cells [29]. *miR-345-5p* was found to be downregulated in ARPE-19 cells undergoing oxidative stress, which is also consistent with our findings [21]. A *miR-210-5p* variant was demonstrated to affect *CFB* expression in RPE cells and modulate the *CFB* level in AMD patients [30]. *miR-423-5p* is significantly increased in the proliferative diabetic retinopathy eyes and believed to modulate angiogenic signals [31]. In this study, it was downregulated Iafter ox-LDL treatment (FC = −14.52, $p < 0.05$). *miR-1908-5p* plays an important role in regulating lipid metabolism in blood, and *miR-378a-5p*/*138-5p*/*34a-5p* are important miRNAs mediating lipid metabolism, tumor angiogenesis, and oxidative stress [32–36]. According to the IPA results, miRNA-target mRNA network, and previous references, *miR-138-5p*, *miR-345-5p*, *miR-210-5p*, *miR-34a-5p*, *miR-1908-5p*, *miR-1343-3p*, *miR-485-5p*, *miR-423-5p*, and *miR-4488* may serve as potential RNA targets for prognosis and diagnosis of RPE degeneration.

Compared with previous attempts at this type of analysis, a lower number of identified DEGs coincided with this study, which is probably due to the use of different oxidative injury models in RPE cells. Our research investigated acute responses of RPE cells to oxidative stress, which could not represent pathogenesis of AMD since it is a long-term effect.

5. Conclusion

n conclusion, exploring oxidative stress-induced miRNA profiles has led us to potential prospects in evaluating RNA variation in sEVs, which may be useful as prognostic and diagnostic tools in the future.

Abbreviations

sEVs: Small extracellular vesicles
RPE: Retinal pigment epithelium
ox-LDL: Oxidized low-density lipoprotein
SFM: Serum-free medium
DEGs: Differentially expressed genes
GO: Gene Ontology
KEGG: Kyoto Encyclopedia of Genes and Genomes
IPA: Ingenuity pathway analyses
AMD: Age-related macular degeneration.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors report no conflicts of interest.

Authors' Contributions

Professor Xingwei Wu conceived the study, participated in its design and coordination, and helped draft the manuscript. Dr. Ke Mao participated in the design and operation of this experiment, performed the statistical analysis, and drafted the manuscript. All authors have read and approved the final manuscript.

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Research Article

Interplay between Nox2 Activity and Platelet Activation in Patients with Sepsis and Septic Shock: A Prospective Study

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Background. Although preclinical studies highlighted the potential role of NADPH oxidase (NOX) in sepsis, only few studies evaluated the oxidative stress in patients with sepsis and septic shock. The objective of the study is to appraise the oxidative stress status and platelet function in patients with sepsis and septic shock compared to healthy controls. *Methods and Results*. Patients with sepsis or septic shock admitted to the hospital Policlinico Umberto I (Sapienza University, Rome) underwent a blood sample collection within 1 hour from admission. Platelet aggregation, serum thromboxane B2 (TxB2), soluble NOX2-derived peptides (sNox2-dp), and hydrogen peroxide breakdown activity (HBA) were measured and compared to those of healthy volunteers. Overall, 33 patients were enrolled; of these, 20 (60.6%) had sepsis and 13 (39.4%) septic shock. Compared to healthy controls ($n = 10$, age 67.8 ± 3.2, male 50%), patients with sepsis and septic shock had higher platelet aggregation (49% (IQR 45-55), 60% (55.75-67.25), and 73% (IQR 69-80), respectively, $p < 0.001$), higher serum TxB2 (77.5 (56.5-86.25), 122.5 (114-131.5), and 210 (195-230) pmol/L, respectively, $p < 0.001$), higher sNox2-dp (10 (7.75-12), 19.5 (17.25-21), and 33 (29.5-39) pg/mL, respectively, $p < 0.001$), and lower HBA (75% (67.25-81.5), 50% (45-54.75), and 27% (21.5-32.5), respectively, $p < 0.001$). Although not statistically significant, a trend in higher levels of serum TxB2 and sNox2-dp in patients who died was observed. *Conclusions*. Patients with septic shock exhibit higher Nox2 activity and platelet activation than patients with sepsis. These insights joined to better knowledge of these mechanisms could guide the identification of future prognostic biomarkers and new therapeutic strategies in the scenario of septic shock.

1. Introduction

Sepsis is an increasing cause of admission to the Emergency Department (ED) and is associated with mortality rates potentially exceeding 50% [1–4]. Pathophysiological derangements occurring during sepsis, such as endothelial dysfunction, increased nitric oxide and arachidonic acid derivative synthesis (such as thromboxane and isoprostanes), and activation of inflammatory patterns, are responsible for the dysregulated host response and development of organ damage [5]. Platelets play a strategic role in the elicitation of the immune response to sepsis, being equipped with specific receptors able to respond to pathogens and enzyme systems capable of producing reactive oxygen species (ROS),

such as NADPH oxidase (NOX) [6]. The interplay between the oxidative stress and platelet activation [7–9] may contribute to the alterations occurring during sepsis and septic shock, and its appraisal may be useful for both identification of prognostic markers and future exploration of new therapeutic approaches. Although some preclinical evidences highlighted the potential role of the NOX signaling pathway in animal models of sepsis [10, 11], few studies evaluated the oxidative stress levels and platelet function in patients with sepsis and septic shock.

The aim of our study is to evaluate the oxidative stress status in patients with sepsis and in those with septic shock compared to healthy controls.

2. Methods

2.1. Study Design. This is a prospective study including nonconsecutive patients with sepsis and septic shock admitted to the ED of the tertiary care hospital Policlinico Umberto I, Sapienza University (Rome, Italy), from April 2017 to April 2018. The study has been performed in accordance with the ethical principles of the Declaration of Helsinki. The institutional review board at the Sapienza University of Rome approved the study protocol (Prot no. 560/17, ref 4550).

The primary goal was to evaluate the oxidative stress status and platelet function in patients with sepsis and in those with septic shock compared to healthy controls.

The secondary objective of the study was

(i) to compare the oxidative stress status and platelet function between septic patients who died and who survived within 30 days from the sepsis onset

In addition, we tried to identify differences in echocardiographic patterns in patients with sepsis and in those with septic shock.

Patients aged >18 years admitted to ED with sepsis or septic shock during the study period were eligible for inclusion in the study. Exclusion criteria were as follows: previous documented myocardial infarction (within 30 days), history of documented heart failure (according to ESC guidelines) [12], other causes of shock (hypovolemic, obstructive, and cardiogenic), severe heart valve disease, preexisting severe renal failure, pregnancy, active major bleeding, or Hb < 7 g/L or platelets $< 20 \times 10^3/\text{mm}^3$. Patients were finally included in the study in the study if the collection of a blood sample was possible within 1 hour from ED admission and before fluid replacement therapy and inotrope administration. When possible, patients underwent transthoracic echocardiography (TTE) during the first 12 h following the diagnosis of sepsis, as previously described [13]. All included patients underwent medical history record, physical examination, and blood sample collection within 1 hour from the diagnosis of sepsis/septic shock. Sepsis and septic shock have been defined according to the current definitions [14]. Demographic data, underlying diseases, and severity of illness of patients were reported on a standardized report form. Clinical data were assessed and recorded at the onset of signs of sepsis or septic shock. Data about the use of inotropes, the source of infection, and the administered antimicrobial therapy has been also recorded [15]. Study investigators prospectively followed all included patients with bedside visits every day. All patients were followed up for 30 days from the episode of sepsis or until death.

2.2. Laboratory Methods. Blood samples were obtained from all patients (within 1 hour from the diagnosis of sepsis/septic shock) and from 10 healthy volunteers and stored at -80°C until use with the antioxidant butylated hydroxytoluene (BHT) at a final concentration of 20 mmol/L. To obtain platelet-rich plasma (PRP), citrated blood samples (9 parts of blood and 1 part of 3.8% Na citrate) were centrifuged for 15 minutes at 180 g. To avoid leukocyte contamination, only the top 75% of the PRP was collected. In each PRP sample, platelet count was adjusted to 2×10^8 platelets/mL. Platelet aggregation was evaluated on a Bio/Data 8-channel platelet aggregometer (PAP-8E, Bio/Data) using siliconized glass cuvettes under continuous stirring at 1200 rpm. Serum thromboxane B2 (TxB2) was measured by ELISA and expressed as picomoles per liter (pmol/L). Intra-assay and interassay coefficients of variation were 4.0% and 3.6%, respectively. Serum Nox2 activity (sNox2-dp) was measured by ELISA as previously described [16]. Finally, serum H_2O_2 breakdown activity (HBA) was measured with an HBA assay kit (Aurogene, code HPSA-50) as previously reported [17]. The % of HBA was calculated according to the following formula: $\%\text{of HBA} = [(\text{Ac} - \text{As})/\text{Ac}] \times 100$, where Ac is the absorbance of H_2O_2 1.4 mg/mL and As is the absorbance in the presence of the serum sample.

2.3. Statistical Analysis. According to the aim of the study, a comparison between parameters of oxidative stress and platelet function among patients with sepsis, patients with septic shock, and healthy controls was performed. Moreover, we performed a comparison of the same variables between patients who survived and those who died after the episode of sepsis and septic shock.

Continuous variables were compared by Student's t-test if normally distributed and the Mann–Whitney U test if nonnormally distributed. Categorical variables were evaluated using χ^2 or the two-tailed Fisher's exact test. Analysis of variance (ANOVA) was used to analyze the differences among groups. Multivariate analysis was performed to identify factors independently associated with 30-day mortality. Variables significant in the univariate analysis and those deemed to have clinical significance were included in the multivariate model. Platelet aggregation, serum TxB2, sNox2-dp, and serum HBA were also included to test their potential association with death. A stepwise multiple logistic regression model was finally used to identify predictors of mortality. Odds ratio (OR) and 95% confidence intervals (CI) were calculated.

Statistical significance was established at ≤ 0.05. All reported p values are 2-tailed. The results obtained were analyzed using commercially available statistical software packages (SPSS, version 20.0; SPSS, Inc., Chicago, IL, and R, version 3.0.2; R Development Core Team, Vienna, Austria).

Table 1: Clinical characteristics of all patients and comparison between patients with sepsis and those with septic shock.

Variables	Healthy controls ($N = 10$)	Overall ($N = 33$)	Patients with sepsis ($N = 20$)	Patients with septic shock ($N = 13$)	p
Demographics					
Male	5 (50%)	16 (48.5%)	9 (45%)	7 (53.8%)	0.619
Age, median (IQR)	68.5 (62-73)	75 (54-85)	74 (54-82.5)	79 (52.5-86.5)	0.888
Previous hospitalization, last 3 months	—	14 (42.4%)	7 (35%)	7 (53.8%)	0.284
Previous antibiotic therapy, last 30 days	—	7 (21.1%)	6 (30%)	1 (7.7%)	0.126
Comorbidities					
Cardiovascular disease	—	19 (57.6%)	11 (55%)	8 (61.5%)	0.710
Chronic heart failure	—	9 (27.3%)	4 (20%)	5 (38.5%)	0.245
Diabetes	—	7 (21.1%)	5 (25%)	2 (15.4%)	0.509
Chronic kidney disease	—	15 (45.5%)	7 (30%)	8 (61.5%)	0.073
Hepatic disease	—	4 (12.1%)	1 (5%)	3 (23.1%)	0.120
Neurologic disease	—	8 (24.2%)	5 (25%)	3 (23.1%)	0.9
COPD	—	9 (27.3%)	6 (20%)	3 (23.1%)	0.833
Solid cancer	—	7 (21.1%)	5 (25%)	2 (15.4%)	0.509
Splenectomy	—	1 (3%)	0	1 (7.7%)	0.208
Aspirin use	—	10 (30.3%)	7 (35%)	3 (23.1%)	0.466
Charlson Comorbidity Index	—		4.5 (1-7)	8 (2-9)	0.298
Source of infection					
Respiratory tract	—	9 (27.3%)	6 (30%)	3 (23.1%)	0.663
Genitourinary tract	—	8 (24.2%)	4 (20%)	4 (30.8%)	0.481
Abdomen	—	8 (24.2%)	4 (20%)	4 (30.8%)	0.481
Skin and soft tissue	—	1 (3%)	1 (5%)	0	0.413
Meningitis	—	1 (3%)	1 (5%)	0	0.413
Bone	—	1 (3%)	1 (5%)	0	0.413
Intravascular device	—	1 (3%)	0	1 (7.7%)	0.208
Others*	—	4 (12.1%)	3 (15%)	1 (7.7%)	0.530
PICC	—	3 (9.1%)	2 (10%)	1 (7.7%)	0.822
CVC	—	2 (6.1%)	1 (5%)	1 (7.7%)	0.751
NIV	—	4 (12.1%)	2 (10%)	2 (15.4%)	0.643
Severity scores of sepsis					
SAPS-II	—	34 (23.5-42)	32 (17.5-38)	37 (31-51)	0.024
qSOFA	—	2 (1-2)	1 (0-2)	2 (2-2.5)	0.002
SOFA score	—	6 (4-10)	4.5 (2-6)	10 (7.5-12)	<0.001
Use of inotropes	—	9 (27.3%)	—	9 (69.2%)	<0.001
Mortality	—	9 (27.3%)	3 (15%)	6 (46.2%)	0.050

COPD = chronic obstructive pulmonary disease; CVC = central venous catheter; IQR: interquartile range; NIV = noninvasive ventilation; PICC = peripheral inserted central catheter. *Others include 1 malaria (sepsis), 1 mononucleosis (sepsis), and 2 bacteremia with an unknown source of infection (1 sepsis and 1 septic shock).

3. Results

A total of 33 patients were included in the study. Thirteen (39.4%) patients had septic shock. Overall, 9 (27.3%) patients had pneumonia, 8 (24.3%) a genitourinary tract infection, 8 (24.3%) an intra-abdominal infection, 1 (3%) a skin and soft tissue infection, 1 (3%) osteomyelitis, 1 (3%) an intravascular device-related infection, and 1 (3%) meningitis. Two (6.1%) patients had a bloodstream infection from an unknown source of infection. In 23 cases, the etiological pathogen was detected. Supplementary Table 1 summarizes the sources of infection with relative etiological diagnosis.

No differences in age, comorbidities, and source of infection have been found between the two groups (Table 1). A trend to higher mortality rates was observed in patients with septic shock (46.2%) compared to those with sepsis (15%, $p = 0.05$).

Platelet aggregation was higher in patients with septic shock (73%) compared to those with sepsis (60%) and healthy controls (49%, $p < 0.001$). A similar trend has been

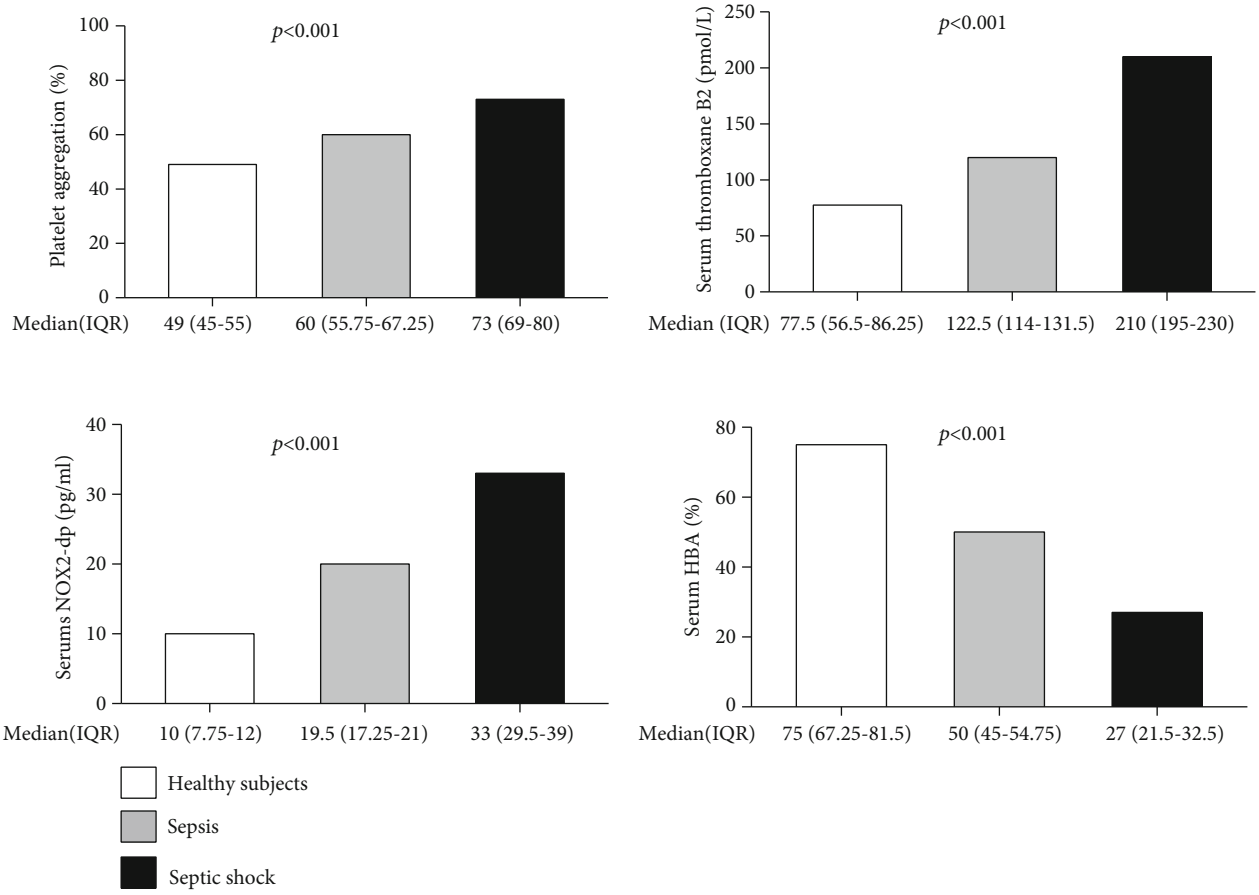

Figure 1: Comparison of platelet aggregation percentage, serum thromboxane B2, Nox2 activity, and serum HBA among healthy controls, patients with sepsis, and those with septic shock. Legend: $^{*}p < 0.001$ among the 3 groups (one-way ANOVA for k samples). HBA = hydrogen peroxide breakdown activity; IQR = interquartile ranges; TBx2 = thromboxane B2.

observed for TxB2 (septic shock 210 pmol/L, sepsis 122.5 pmol/L, and healthy controls 77.5 pmol/L, $p < 0.001$) and serum Nox2-dp (septic shock 33 pg/mL, sepsis 19.5 pg/mL, and healthy controls 10 pg/mL, $p < 0.001$). Conversely, antioxidant status, evaluated by hydrogen peroxide breakdown activity (HBA), was 75% in healthy controls and lower in patients with sepsis (50%) and in those with septic shock (27%, $p < 0.001$) (Figure 1). Although not statistically significant, platelet aggregation, serum TxB2, and sNox2-dp tend to be higher in patients who died, while serum HBA levels were higher in survivors (Table 2). Multivariate analysis identified the SOFA score on admission (OR 1.34, 95% CI 1.03-1.88, $p = 0.03$) and Charlson Comorbidity Index (OR 1.48, 95% CI 1.05-2.06, $p = 0.02$) as independent predictors of 30-day mortality.

TTE was performed in 27 patients (17 with sepsis and 10 with septic shock). No significant differences were found in echocardiographic parameters, except for the inferior vena cava (IVC) collapsibility index, which was higher in patients with sepsis compared to those with septic shock ($p = 0.035$) (Supplementary Table 2). Similarly, no significant differences were found among patients who survived and did not probably due to a small sample effect (Supplementary Table 3). The only significantly different parameters were the right ventricular diameter (33 mm (30.8, 35.0) vs. 38 mm (36.5, 41.0), $p = 0.033$) and E/E' ratio (5.8 (5.6, 6.8) vs. 7.8 (7.2, 13.2), $p = 0.037$) being both higher in patients who died.

4. Discussion

Patients with septic shock exhibit higher Nox2 activity and impaired systemic scavenging capacity of H_2O_2 when compared to patients with sepsis and to healthy controls. Moreover, they have higher serum TxB2, a reliable marker of platelet aggregation.

The increasing upregulation of these parameters from septic patients to those with septic shock suggests that oxidative stress may be implicated in the cellular damage observed during sepsis. The Nox family can be responsible for inflammation, injury, and possibly tissue repair occurring in septic shock [18]. In an LPS-induced septic model, Nox4-knockdown mice had decreased production of inflammatory mediators compared to mice overexpressing Nox4, data supporting the crucial role of Nox4 as a key component of the inflammatory response to sepsis [11]. In patients with pneumonia, Nox2 activation is implicated in oxidative stress and subsequent myocardial damage [19].

Table 2: Comparison of parameters of the redox status and platelet function among healthy controls, septic patients who survived, and those who died during 30 days after the septic episode.

Variables	Septic patients who survived ($N = 24$)	Septic patients who died ($N = 9$)	p
Platelet aggregation (%)	66.5 (58.5-71.5)	73 (60-80)	0.166
Serum thromboxane B2 (pmol/L)	129.5 (120-193.75)	190 (132.5-220)	0.094
Serum sNox2-dp (pg/mL)	20.5 (18-28)	29 (19-32.5)	0.309
Serum H_2O_2 scavenger (HBA) (%)	45 (29.25-53.5)	35 (24.5-52.5)	0.619

Nox: NADPH oxidase; HBA: hydrogen peroxide breakdown activity; sNox2-p: soluble NOX2-derived peptides.

The upregulation of Nox2 and the related oxidative stress in patients with sepsis and septic shock admitted to ED can have different clinical implications. First, the levels of sNox2-dp and serum TxB2 and the percentage of HBA may be considered suitable markers of the disease. A trend in higher serum sNox2-dp and serum thromboxane B2 between septic patients who survived and those who died has been observed. It did not achieve the statistical significance, probably due to the low sample size. Their prognostic role should be confirmed in further larger studies. These parameters are low-cost and easy to be implemented in the emergency room and may provide both diagnostic and prognostic information in septic patients. Finally, the identification of specific pathways may guide future targeted and individualized therapy in patients with sepsis and high redox state activation.

No significant differences in echocardiographic parameters between patients with sepsis and with septic shock were found, except for IVC collapsibility, maybe due to a small sample size effect, or for the differences in hemodynamic optimization including volume administration in patients with septic shock.

Even if echocardiography has been proposed as the fifth pillar in bedside physical examination, its value in terms of mortality reduction in sepsis and septic shock has not been definitely proven [20].

Nevertheless, bedside echocardiography is extremely useful in septic patients to further assess the hemodynamic state and fluid responsiveness, as different forms of cardiac dysfunction may be present: LV systolic and/or diastolic dysfunction, right ventricular dysfunction, and hyperdynamic LV function with or without LVOT outflow obstruction [21].

This study has some limitations. First, the sample size is low, but it is the largest study evaluating the redox status and platelet function in patients with sepsis/septic shock. Moreover, some drugs such as aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) may affect platelet activation; however, no differences in previous aspirin, NSAID, and statin use have been detected between patients with sepsis and those with septic shock. We cannot exclude the idea that other factors may have impacted on the oxidative stress status of some included patients; however, data about all concomitant medications have been collected and did not differ between the groups of patients. Finally, not all patients with sepsis or septic shock consecutively admitted to ED of our hospital were included in the study; the collection of a blood sample for the detection of oxidative stress parameters was not possible before fluid replacement therapy and inotrope administration for all patients with sepsis/septic shock admitted to the ED of our hospital.

However, our study has also some strengths. All patients underwent blood sample collection for the dosages of parameters of the redox status and platelet aggregation before volume expansion and inotrope administration to avoid potential interferences with the results. Moreover, they were prospectively observed until 30 days from the ED admission. Finally, we were able to compare the redox status and platelet aggregation of septic patients to healthy volunteers, highlighting the differences in these parameters in the healthy status and in the presence of sepsis.

In conclusion, the oxidative stress status and platelet function in patients with sepsis and septic shock might be easy-to-use disease markers in the ED and may have a prognostic value, allowing the early identification of patients at high risk at 30-day mortality. Regulating Nox-mediated signaling and platelet function may effectively modulate inflammatory responses: further studies are needed to investigate the potential role of Nox signaling as a therapeutic target.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflict of interest.

Authors' Contributions

Giusy Tiseo and Elena Cavarretta contributed equally to this work.

Supplementary Materials

Supplementary Table S1: source of infection and etiology of the 33 episodes of sepsis. Supplementary Table S2: echocardiographic parameters in patients with sepsis and with septic shock. Supplementary Table S3: echocardiographic parameters in septic patients who survived and those who died. *(Supplementary Materials)*

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Research Article

Increased Intraplatelet ADMA Level May Promote Platelet Activation in Diabetes Mellitus

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Background. Antiplatelet therapy has become a standard therapeutic approach in the secondary prevention of cardiovascular system disorders of thrombotic origin. Patients with concomitant diabetes mellitus (DM) obtain fewer benefits from this treatment. Hence, the pathophysiology of altered platelet function in response to glucose metabolism impairment should be of particular interest. *Objectives*. The aim of our study was to verify if the platelet expression of the asymmetric dimethylarginine (ADMA) in diabetic patients differs in comparison to the nondiabetic ones. The correlation of platelet-ADMA with platelet activation and aggregation as well as with other risk factors was also investigated. *Material and Methods*. A total of 61 subjects were enrolled in this study, including thirty-one type 2 diabetic subjects without diabetes-related organ damage. Physical examination was followed by blood collection with an assessment of platelet aggregation, traditional biochemical cardiovascular risk factors, and evaluation of nitric oxide bioavailability parameters in plasma and thrombocytes. Subsequently, the assessment of endothelial function using Peripheral Arterial Tonometry and Laser Doppler Flowmetry (LDF) was performed. *Results*. In the DM group, elevated concentration of intraplatelet ADMA and higher ADMA/SDMA ratio compared to the control group was observed. It was accompanied by higher ADP-mediated platelet aggregation and lower microvascular response to a local thermal stimulus measured by LDF in the diabetes group. *Conclusions*. Type 2 diabetes is related to higher intraplatelet concentration of asymmetric dimethylarginine (ADMA), which may result in impaired platelet-derived nitric oxide synthesis and subsequent increased platelet activity, as assessed by the ADP-induced aggregation. Laser Doppler Flowmetry, compared to EndoPAT 2000, appears to be a more sensitive indicator of the impaired microvasculature vasodilation in diabetics without the presence of clinically significant target organ damage.

1. Introduction

Antiplatelet therapy, after several decades following its implementation to clinical practice, has become a standard therapeutic approach in preventing cardiovascular events of thrombotic origin 1. Based on numerous clinical studies, it has been clearly demonstrated that it markedly lowers the morbidity and mortality in the high-risk population 2. Nevertheless, patients with concomitant diabetes mellitus, being at least at high cardiovascular risk, obtain fewer benefits from this treatment, when compared to the nondiabetics. This partially explains the higher incidence of cardiovascular events in this subpopulation 3. Among numerous factors (Figure 1), increased platelet activity, platelet aggregation, and altered platelet-endothelial cell interaction may play a key role in developing vascular diabetic complications 4, 5. Hence, the pathophysiology of altered platelet function in response to the presence of glucose metabolism abnormalities

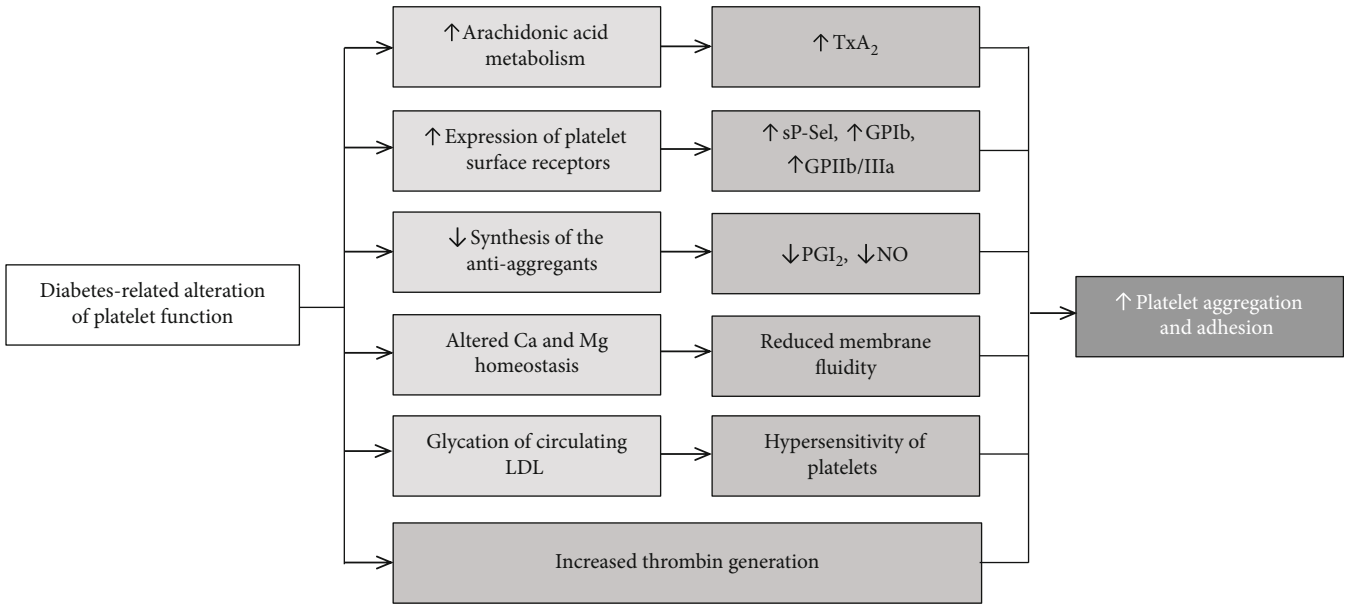

Figure 1: Summary of diabetes-related alterations of platelet function. TXA2: thromboxane A2; sP-Sel: P-selectin soluble form; GPIb: platelet glycoprotein Ib; GPIIb/IIIa: platelet glycoprotein IIb/IIIa; PGI2: prostacyclin; NO: nitric oxide; LDL: low density lipoprotein. Modification based on [12,13]

should be of particular interest in developing novel therapeutic approaches minimizing the occurrence of acute cardiovascular events in the diabetic population.

Nitric oxide (NO) and the regulation of its synthesis play a crucial role in the pathogenesis of cardiovascular disorders. Over the last three decades, numerous studies described the exact role of NO and its metabolic pathway components in developing endothelial dysfunction and its contribution to further cardiovascular events of ischemic origin6. A competitive nitric oxide synthase inhibitor, asymmetric dimethylarginine (ADMA), appears to be a key element regulating the NO bioavailability, and its elevated plasma level was shown in numerous studies to be an early predictor for increased cardiovascular risk and future cardiovascular events 7–9. ADMA was demonstrated to be present not only in plasma but also in the intracellular compartment, including endothelial cells and human platelets 10.

Platelet-derived nitric oxide (PDNO) reduces the platelet degranulation, decreases thrombocyte and leukocyte adhesion to endothelium, and finally limits the aggregation process 11. Differences in regulation and alterations in its metabolism may result in increased platelet aggregation and contribute to their resistance to the antiplatelet treatment, observed among the populations at high risk for cardiovascular events 12, 13. As the role of PDNO appears to be partially different from the endothelial-derived NO (primarily associated with vasodilation), it might be considered as the autonomous system involved in the maintenance of intravascular homeostasis 11. The determination of the intraplatelet parameters of the NO metabolic pathway and their relation to the same parameters measured in plasma may contribute to a better understanding of pathophysiological processes associated with increased cardiovascular risk in diabetes.

The population of patients with diabetes mellitus is continuously growing. Recently, the heterogeneity of this group and the need for an individual approach have been increasingly emphasized. It is expected that in almost 30% of cases a 5-year CVD risk in diabetic patients is similar to the general population 14. Therefore, a more precise estimation of the long-term risk for cardiovascular events is required. In this purpose, the methods for the assessment of endothelial function, which are proven to have an additional predictive value comparing to traditional risk factors15, such as EndoPAT 2000 and Laser Doppler Flowmetry, are used. Although both methods examine the endothelial function by evaluating changes in peripheral microcirculation, recent reports indicate their dependence on different mechanisms regulating its vasodilatory function 16.

Hence, the aim of our study was to verify if the platelet expression of the competitive nitric oxide synthase inhibitor, ADMA in diabetic patients, differs in comparison to the nondiabetic ones. The relations between intraplatelet ADMA levels and platelet activation properties were verified. The possible cross-talk between platelet ADMA and the occurrence of other cardiovascular risk factors as well as the presence of endothelial vasodilatory dysfunction were other goals investigated in this study.

2. Material and Methods

2.1. Bioethics Approval. The study protocol and all experiments were investigated and approved by the local Bioethics Committee. All of the volunteers agreed to participate in the study by signing a written informed consent, which was also verified by the Bioethical Committee. The project procedures are consistent with the principles of the Declaration of

Helsinki (Seventh Revision, 64th World Medical Association meeting, Fortaleza, 2013).

2.2. Subject Recruitment and Examination. 87 subjects were considered to participate in this study. After careful analysis of the inclusion and exclusion criteria followed, a total of 61 subjects were enrolled in this study, including thirty-one type 2 diabetic subjects aged 35-70, in the phase of oral treatment and without significant diabetes-related target organ damage. The control group constituted 30 healthy volunteers demographically matched to the study group in whom the glucose metabolism alterations, including diabetes, impaired fasting glycemia, impaired glucose tolerance, and insulin resistance, were excluded. All of the study participants underwent a standard detailed physical examination. Afterwards, 44 ml of peripheral venous blood was collected atraumatically by the Sarstedt S-Monovette® (Sarstedt Ag & Co., Nümbrecht, Germany) aspiration and vacuum kit for subsequent biochemical analyses. In both groups, these procedures were followed by biometric measurements and assessment of endothelial vasodilatory function using the Laser-Doppler Flowmetry (PeriFlux 5000, Perimed AB, Järfälla, Sweden) and Peripheral Artery Tonometry (EndoPAT 2000, Itamar Medical, Caesarea, Israel). From collected blood, biochemical characterization of both groups, including the determination of basic cardiovascular risk factors, aggregometric tests, and evaluation of selected parameters involved in the nitric oxide biotransformation pathway, was performed. The study protocol is summarized in Figure 2.

2.3. Platelet Preparation for LC-MS Analysis. The collected whole blood was supplemented with prostacyclin (PGI_2) at the final concentration of 0.06 μg/ml and centrifuged for 20 minutes at 230 × g at 21°C to obtain platelet-rich plasma. Subsequently, PRP was supplemented with PGI_2 (final concentration 0.3 μg/ml) and centrifuged for 10 minutes at 1000 × g at 21°C. The plasma was discarded, and the platelet pellet was carefully washed three times with 1 ml of Tyrode HEPES buffer pH 7.4. Rinsed platelets were suspended in 4 ml of Tyrode HEPES buffer pH 7.4 supplemented with $CaCl_2$ (final concentration 1 mM). The resulted suspension was immediately analyzed for platelet count and contamination with WBC and RBC (Sysmex device, Sysmex, Norderstedt, Germany). The pure PLT suspension was adjusted with Tyrode HEPES buffer pH 7.4 containing $CaCl_2$ to a final concentration of 2.5×10^8 ml. Samples containing platelets in amounts of 5.0×10^8 cells were preserved for LC-MS analysis. The samples were obtained by centrifugation of the suspension of known concentration for 5 min, 10000 × g at 4°C, and stored at −80°C until further analyses.

2.4. Evaluation of Intraplatelet Parameters of the Nitric Oxide Bioavailability. Into platelet pellet, 10 μl of internal standard solution and 700 μl of cold extraction solution consisting of methanol and water (7 : 3) were added. Samples were then vortexed (5 min, 2000 rpm, 4°C), frozen (-20°C, 15 min), and centrifuged (9 min, 15000 rcf, 4°C). Subsequently, supernatants were transferred to the new polypropylene microtubes and dried at 55°C using SpeedVac evaporator (Thermo Savant). Dried samples were then derivatized as bellow.

LC-MS/MS analysis was performed using the Acquity UPLC system (Waters, Milford, MA, USA) equipped with a cooled autosampler. The sample temperature in the autosampler was set to 6°C, and the injection volume was 4 μl. The Waters BEH Shield C18 column (1.7 μm, 2.1 × 10 mm) was thermostatted in a column oven at 60°C. The flow rate was 0.350 ml/min with a total run time of 8 min. Eluents: A: water with 0.1% formic acid (FA), B: methanol with 0.1% FA. The following gradient was used: 0.0 min–3% B, 2.5 min–14% B, 4.6 min–60% B, 4.8 min–90% B, 6.1 min–3% B. MS analysis was performed using SYNAPT G2 Si mass spectrometer (Waters, Milford, MA, USA) equipped with electrospray ionization source (ESI) in positive ionization mode. The spray voltage, source temperature, and the desolvation temperature were set at 0.5 kV, 140°C, and 450°C, respectively. Data acquisition was performed using MassLynx software (Waters) for the following ions (m/z): 150.0919, 156.1295, 237.1239, 243.1339, 263.1090, 267.1382, 279.1457, 286.1897, 307.1770, and 314.2209 for DMA, D6-DMA, ornithine, D6-ornithine, citrulline, D4-citrulline, arginine, D7-arginine, ADMA, SDMA, and D7-ADMA, respectively. Standard calibration curves were prepared using the following concentration ranges: 0.3 to 15 μM for ornithine, 0.5 to 25 μM for arginine, 0.005 to 0.25 μM for ADMA and SDMA, 0.1 to 5 μM for citrulline, and 0.014 to 0.7 μM for DMA.

2.5. Evaluation of Parameters of the Nitric Oxide Bioavailability in Plasma. The plasmatic level of metabolites involved in NO synthesis was measured according to the method described by Fleszar et al. 17. Briefly, 100 μL of plasma, 50 μl of borate buffer, and 10 μl of internal standard solution (100 μM D7-arginine, 20 μM D7-ADMA, 25 μM D6-DMA, 100 μM D6-ornithine, and 50 μM D4-citrulline) were transferred into 2 ml polypropylene tubes and mixed (1 min, 1200 RPM, 25°C). Then, 400 μl of acetonitrile and 10 μl of 10% BCl in acetonitrile were added and mixed (10 min, 1200 RPM, 25°C). Subsequently, samples were centrifuged (7 min, 4°C, 22,000 RCF) and 100 μl of clear supernatant was diluted four times with water, transferred to chromatographic glass vials, and analyzed. LC-MS/MS analysis was performed using the equipment and settings described above with 10-fold higher concentrations ranges of standard calibration curves.

2.6. Aggregometry. Platelet function was assessed using the impedance aggregation method in whole blood using the four-channel optical aggregometer (Chrono-log 700, Chrono-Log, Pennsylvania, USA). This method is based on multiple platelet aggregation on the electrodes and changing of the electrical resistance between their two wires. Whole blood was collected to the polypropylene tubes for 10% sodium citrate using the Sarstedt S-Monovette® (Sarstedt Ag & Co., Nümbrecht, Germany) aspiration and vacuum kit. After collection, the tubes were kept at room temperature for a maximum of 90 minutes before engaging the test. Two different aggregation activators were used: adenosine

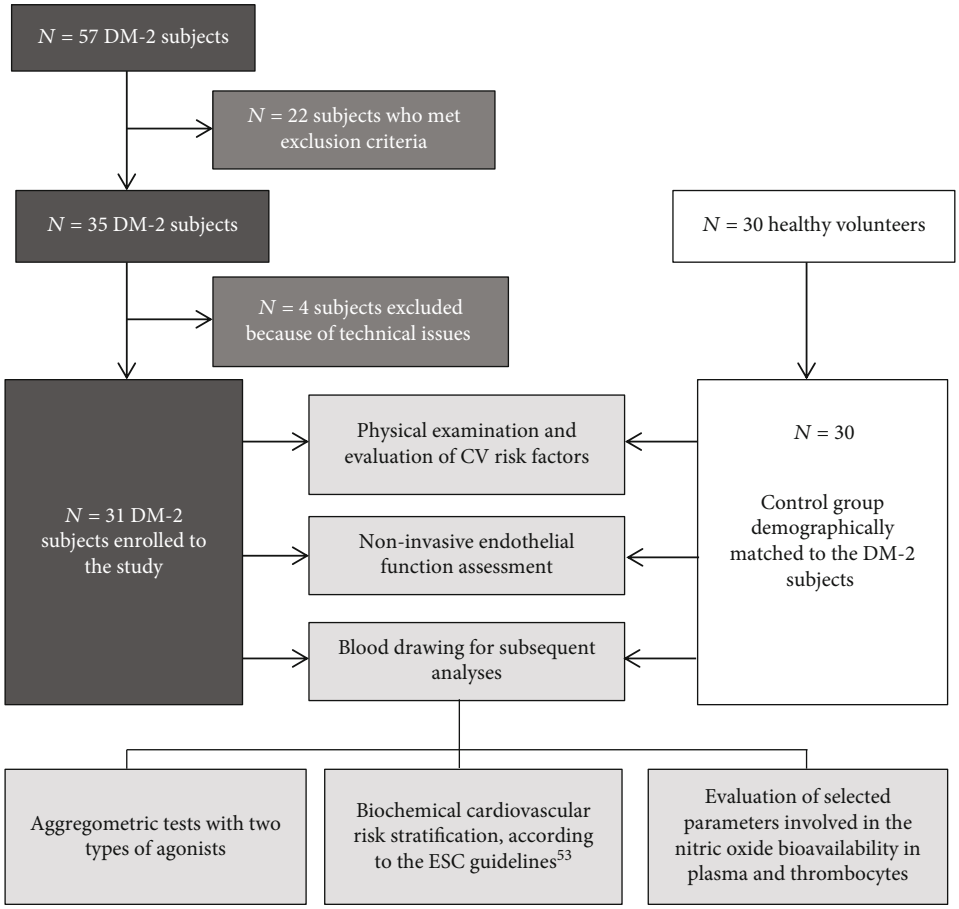

Figure 2: Study protocol. DM-2: type 2 diabetes mellitus; CVD: cardiovascular diseases; NOS: nitric oxide synthase.

diphosphate (ADP) and arachidonic acid (AA). The 1 : 1 solution of whole blood in room temperature with 0,9% natrium chloride was placed in the test chambers. Then, a certain number of agonists, necessary to obtain appropriate concentrations (0,5 mg/ml for AA, 20 μmol for ADP), was added to the prepared solution. After 6 minutes, aggregation curves were recorded, measured, and analyzed by dedicated software (Aggrolink®, Chrono-Log, Pennsylvania, USA). The increase in electrical impedance was given in aggregation units.

2.7. Endothelial Function Assessment. The ability of the endothelium to dilate blood vessels through the synthesis of nitric oxide was measured by two devices following two different protocols. Laser Doppler Flowmetry allows for dynamic monitoring of changes in the microcirculation of superficial skin layers under the effect of stimuli: chemical (like acetylcholine or pilocarpine) or thermal—through a heated thermostatic probe. The wavelength of the laser beam changes when it hits in the moving blood cells and the changes in its frequency and distribution are associated with the number and speed of blood morphotic elements flowing through the vessels. In this study, the local thermal stimulus was used. Standard recording during local heating consists of two main phases: peak phase (up to several minutes)—dependent on stimulation of local sensory nerves followed by substance P excretion, and plateau phase (after 20 minutes)—conditioned mostly by nitric oxide and partially by norepinephrine and neuropeptide Y [18]. The probe of the LDF device (Periflux 5000, Perimed, Järfälla, Sweden) was placed on the forearm skin with no visible superficial vessels. The studied limb was immobilized with a vacuum pillow provided by the device manufacturer. After 10 minutes of baseline record with a temperature of 33°C, heating was set to 44°C for the next 30 minutes. To prevent the impact of baseline flow variability, the results were shown as total hyperemia index (THI) and maximum hyperemia index (MHI) (Figure 3).

Simultaneously, the endothelial response to transient hypoxemia was measured by Peripheral Arterial Tonometry (EndoPAT 2000, Itamar Medical, Caesarea, Israel). Microvascular blood flow in both hands' middle fingers was measured by dedicated pressure probes. These sensors are equipped with a latex cuff which, when inflated, through the pressure change generated by the blood flow, generates a signal that is converted by dedicated computer software into a pulse waveform. This signal was recorded for 6 minutes before and after five minutes of ischemia induced by inflation of a sphygmomanometer cuff minimum 50 mmHg over the systolic blood pressure value as described in the protocol developed by the device manufacturer. At least 15 min prior and during the examination patient remained in the isolated quiet room, with constant air

$$\text{THI} = \frac{\text{mean flow after heating}}{\text{mean flow before heating}}$$

$$\text{MHI} = \frac{\text{mean flow after 20 minutes of heating}}{\text{mean flow before of heating}}$$

Figure 3: Calculation of microvascular flow parameters changes measured by Laser Doppler Flowmetry. THI: total hyperemia index; MHI: maximum hyperemia index.

temperature, and was instructed to move as little as possible to prevent signal disturbances. As a result, the software created by Itamar calculates the reactive hyperemia index (RHI) and augmentation index (AI).

2.8. Statistical Analysis. The data is presented as the mean ± SD. The differences between two continuous parameters were assessed using a Mann-Whitney *U*-test or a Student's *t*-test, following the Shapiro-Wilk test and Levene's test as appropriate. For comparison of more than two groups, an ANOVA followed by Tukey's test or a Friedman test (for nonparametric statistics) was performed as appropriate. All calculations were made with Statsoft® Statistica 13.3 software.

3. Results

3.1. Baseline Characteristics. The characteristics of the diabetic and control groups are presented in Table 1. The groups were matched in both age and sex. Regarding demographical measurements, higher BMI and WHR ratios were observed in the DM group. In morphological parameters, only WBC was significantly higher in the DM group, but its value did not exceed the normal range. The difference in HBA_{1C}, fasting glucose, and insulin level with higher rates in DM subjects was also observed. According to biochemical characteristics, differences involved only eGFR, hsCRP, serum sodium, and magnesium, but the mean values did not extend the reference limits. Higher HOMA-IR and lower QUICKI indexes were noted is the DM group.

3.2. Assessment of Endothelial Function. There were no significant differences between groups in the Reactive Hyperaemia Index (RHI) and Augmentation Index (AI), as measured by the EndoPAT® 2000. However, the change in superficial blood flow following the local thermal stimulus revealed significantly lower values of both calculated hyperemia indexes in diabetic, as examined by Laser Doppler Flowmetry. A summary of the endothelial function assessment is shown in Table 2.

Abbreviations: RHI: reactive hyperemia index (EndoPAT 2000); AI: augmentation index (EndoPAT 2000); THI: total hyperemia index (Laser Doppler Flowmetry); MHI: maximum hyperemia index (Laser Doppler Flowmetry).

3.3. Parameters of the Platelet and Plasma NO Bioavailability. In the DM group, elevated concentrations of intraplatelet ADMA (0,091 ± 0,038 μM versus 0,068 ± 0,037 μM, p = 0,003), DMA (0, 58 ± 0, 26 versus 0, 44 ± 0, 33 μM, p = 0,049), and higher ADMA/SDMA ratio (0, 80 ± 0, 26 versus 0, 72

Table 1: Demographic and biochemical characteristics between studied groups including cardiovascular risk stratification parameters. Results are presented as mean ± SD.

Parameter	Diabetes group $N = 31$ (mean ± SD)	Control group $N = 30$ (mean ± SD)	p value
Age (y)	58, 48 ± 8, 17	54, 67 ± 6, 08	NS
Women (%)	10 (32%)	13 (43%)	NS
BMI (kg/m^2)	30, 18 ± 3, 94	26, 02 ± 3, 87	<0,05
WHR	0, 98 ± 0, 08	0, 92 ± 0, 12	<0,05
WBC (k/μl)	7, 18 ± 2, 13	5, 89 ± 1, 57	<0,05
RBC (mln/μl)	4, 72 ± 0, 48	4, 88 ± 0, 50	NS
Haemoglobin (g/dl)	14, 35 ± 1, 25	14, 68 ± 1, 26	NS
Haematocrit (%)	42, 15 ± 3, 62	43, 70 ± 3, 75	NS
MCV (fL)	89, 4 ± 4, 64	89, 69 ± 3, 94	NS
MCH (pg)	30, 47 ± 2, 00	30, 16 ± 1, 32	NS
MCHC (g/dL)	34, 07 ± 1, 08	33, 63 ± 0, 98	NS
Platelets (k/μl)	250,81 ± 57, 36	244 ± 40, 59	NS
PDW (fL)	13, 07 ± 2, 63	12, 51 ± 1, 85	NS
Glucose (mg/dl)	115,73 ± 40, 91	93, 82 ± 8, 79	<0,05
$Hb1_{AC}$ (%)	6, 13 ± 0, 60	5, 56 ± 0, 30	<0,05
Insulin (uU/mL)	8, 79 ± 4, 93	6, 71 ± 3, 60	<0,05
Total cholesterol (mg/dl)	194,06 ± 52, 17	220,76 ± 49, 55	NS
LDL (mg/dl)	114,29 ± 44, 03	136,31 ± 39, 25	NS
HDL (mg/dl)	50, 13 ± 12, 05	57, 24 ± 14, 50	NS
Triglycerides (mg/dl)	164,10 ± 104,45	135,93 ± 68, 79	NS
hsCRP (mg/l)	2, 46 ± 2, 53	1, 05 ± 0, 82	<0,05
Creatinine (mg/dl)	0, 91 ± 0, 15	0, 96 ± 0, 16	NS
eGFR (ml/min/1,73m^2)	84, 57 ± 14, 52	77, 86 ± 10, 24	<0,05
Urea (mg/dl)	33, 97 ± 6, 83	34, 20 ± 8, 74	NS
Uric acid (mg/dl)	6, 05 ± 1, 50	5, 54 ± 1, 44	NS
TSH (μIU/mL)	1, 79 ± 0, 92	1, 60 ± 0, 56	NS
Troponin I (pg/ml)	3, 08 ± 2, 06	2, 32 ± 0, 98	NS
BNP (pg/ml)	28, 98 ± 36, 89	21, 36 ± 15, 36	NS
Sodium (mmol/l)	139,33 ± 1, 82	140,55 ± 2, 36	<0,05
Potassium (mmol/l)	4, 33 ± 0, 28	4, 29 ± 0, 31	NS
Magnesium (mg/dl)	1, 94 ± 0, 23	2, 14 ± 0, 16	<0,05
Calcium (mmol/l)	9, 72 ± 0, 38	9, 63 ± 0, 32	NS
HOMA IR	2, 49 ± 1, 31	1, 68 ± 0, 89	<0,05
HOMA-β (%)	89, 57 ± 126,66	91, 34 ± 51, 32	NS
QUICKI	0, 60 ± 0, 09	0,668 ± 0, 10	<0,05

Abbreviations: NS: result statistically nonsignificant; BMI: body mass index; WHR: waist-hip ratio; WBC: white blood cells; RBC: red blood cells; MCV: mean (red blood) cell volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; PDW: platelet distribution width; eGFR: estimated glomerular filtration rate; HDL: high-density lipoprotein; LDL: low-density lipoprotein; hsCRP: high-sensitivity C-reactive protein; TSH: thyroid-stimulating hormone; BNP: brain natriuretic peptide.

Table 2: Assessment of endothelial function by EndoPAT 2000 and Laser Doppler Flowmetry in studied groups.

Parameter	Diabetes group	Control group	p value
RHI	2, 35 ± 0, 87	2, 23 ± 0, 55	NS
AI (%)	29, 48 ± 19, 84	21, 18 ± 17, 18	NS
THI	7, 77 ± 4, 34	11, 21 ± 6, 87	<0,05
MHI	8, 98 ± 4, 88	14, 63 ± 8, 56	<0,05

± 0, 19, $p = 0{,}046$) compared to the control group were shown (Figure 4.). There were no differences in other studied intraplatelet parameters of the nitric oxide bioavailability (L-Arg, SDMA, and L-Arg/ADMA) (Figure 4) and in all investigated plasma parameters (Figure 5.).

3.4. Aggregometry. Significantly higher aggregation in response to ADP was observed in diabetic subjects when compared to the controls (83, 58 ± 23, 68 versus 68, 43 ± 18 , 97 U, $p = 0{,}017$) (Figure 6.). There was no difference in aggregation stimulated by arachidonic acid between studied groups.

In the diabetic group, a positive correlation between platelet ADMA and serum calcium ($r = 0, 41$), potassium ($r = 0, 44$), as well as platelet SDMA concentration ($r = 0, 67$) and age ($r = 0, 41$) were noted. What is more, a moderate negative correlation with serum magnesium ($r = –0, 55$) was demonstrated. The positive correlation was present between ADP stimulated aggregation with serum calcium level ($r = 0, 41$) and platelet SDMA concentration ($r = 0, 43$). A negative correlation was shown between diabetes-related parameters (glycemia, HbA_{1C}) and platelet SDMA concentration. In the control group, platelet ADMA concentration significantly correlated with platelet SDMA ($r = 0, 88$), DMA ($r = 0, 69$), and plasma ADMA (0,55).

4. Discussion

This is the first study to assess the parameters of the L-arginine: nitric oxide metabolic pathway in diabetic human platelets in comparison to the non-diabetic control. The enrolled groups were homogenous in all, except for diabetes-related biochemical parameters (glucose and insulin concentration, HbA_{1C}). Discrepancies in other parameters (hsCRP, eGFR, and sodium) did not extend the normal values.

Numerous studies link elevated plasma ADMA concentration with both type 1 and 2 diabetes. As it is reported, a higher ADMA level in this group of patients is related to the development of retinopathy 19, progression of nephropathy 20, and may be a predictor of future cardiovascular events 21. However, our results show no significant difference in the plasma concentration of ADMA and other measured nitric oxide metabolic pathway components in diabetic subjects, compared to the control group. According to Xiong et al., plasma ADMA concentrations are not dependent on the duration of the disease but are related to the existence of macroangiopathy 22. Other reports underline the predictive value of increased plasmatic ADMA and hsCRP concentrations in cardiovascular events in diabetes, however, the values of these parameters were higher than in our study (>0,63 μmol/l for ADMA, >6,0 mg/l for hsCRP) 21. Thus, the lack of difference observed in our study can be described by the early stage of the disease and the absence of diabetes-related organ damage.

In contrary to plasmatic ADMA, we demonstrated that intraplatelet ADMA concentration was significantly higher in patients with type 2 diabetes. This result was accompanied by a significantly higher concentration of dimethylamine (DMA)—a product of ADMA degradation catalyzed by the dimethylaminohydrolase (DDAH). As ADMA is the most potent inhibitor of all isoforms of nitric oxide synthase 23, which lower intraplatelet activity was recognized in previous studies 24, 25, there is a high probability that this mechanism is responsible for platelet-derived NO deficiency in diabetic subjects. What is more, PDNO-derived activation of intraplatelet cyclic guanidine monophosphate (cGMP) can cause platelet degranulation and aggregation 26, instead of inhibiting thrombocytes' activation. This statement is supported by the increased aggregation observed in the c-GMP-dependent phosphokinase A- (PKA-) knockout mice 26. Inhibition of platelet degranulation occurs with higher intraplatelet concentrations of NO by nitrosylation of soluble N-ethylmaleimide-sensitive factor (NSF) which prevents from the attachment to its receptors (SNAREs) involved in the process of platelet granules exocytosis 27. Considering that, it could be another mechanism affecting platelet function dependent on intraplatelet NO deficiency. Despite the fact that higher concentrations of L-arginine enhance nitric oxide synthesis 28 by increasing the substrate availability and the L-arginine/ADMA ratio, our results demonstrated no significant difference in intraplatelet concentrations of these parameters between studied groups. It indicates that the substrate/inhibitor relationship is not the sole determinant of its bioavailability without considering the membrane migration and degradation processes. Given the complexity of the intraplatelet nitric oxide synthesis pathway, more detailed research including the determination of all components involved in intraplatelet L-arginine transport, NOS activity regulation, and NO/cGMP pathway is necessary.

Interestingly, we observed no correlation between intraplatelet and plasma ADMA concentrations. The growing data suggests that these two compartments are independent of each other 11. Hence, it appears that the intraplatelet ADMA accumulation, potentially decreasing platelet-derived nitric oxide production, occurs at the earlier stages of diabetes than in plasma. As intraplatelet ADMA concentration correlates with the concentration of serum magnesium and calcium, the transport alterations might be involved in this process, but these mechanisms have yet to be explored.

As results from our study, a significant difference in aggregation mediated by ADP between groups with higher values in the diabetic one was observed, which is in line with the work of other authors 29, 30. What is more, some studies indicate that almost two-thirds of diabetic subjects were resistant to antiplatelet therapy with ADP-antagonist—clopidogrel

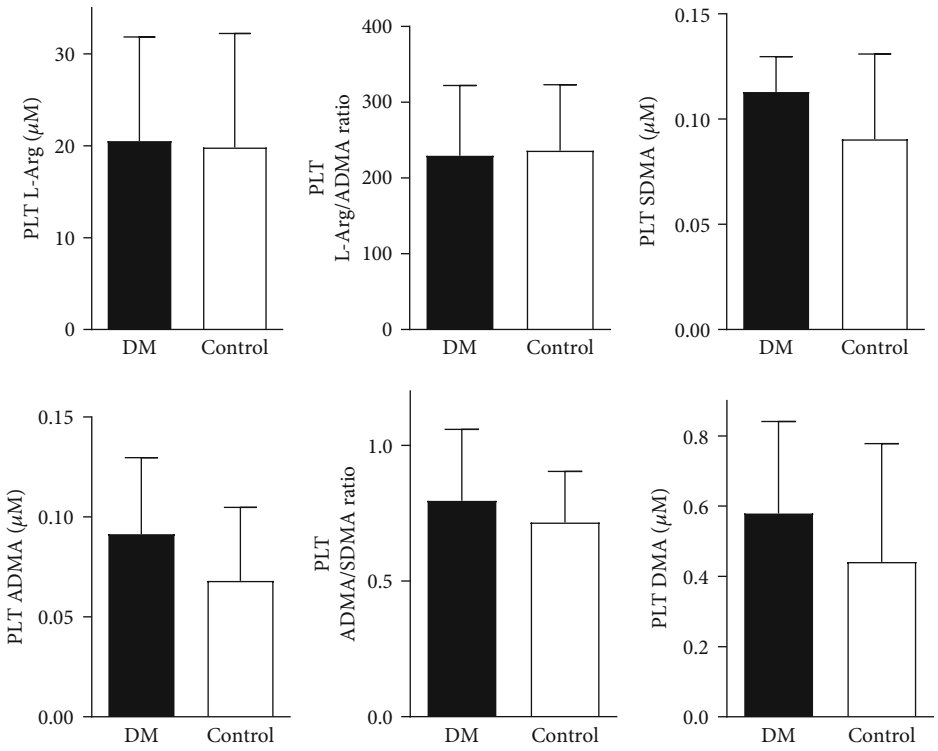

Figure 4: The comparison of intraplatelet parameters of nitric oxide bioavailability. $^{*}p < 0,05$ versus control group. Abbreviations: PLT L-Arg: intraplatelet L-arginine; PLT ADMA: intraplatelet asymmetric dimethylarginine; PLT SDMA: intraplatelet symmetric dimethylarginine; PLT DMA: intraplatelet dimethylamine; DM: diabetes mellitus group; Control: control group; PLT ADMA: intraplatelet asymmetric dimethylarginine.

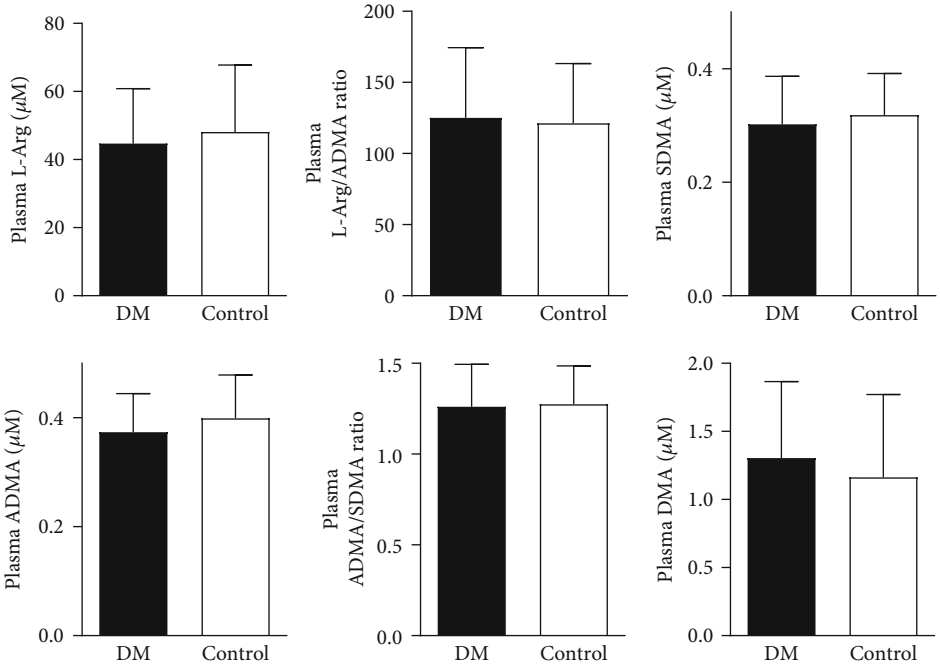

Figure 5: The comparison of plasmatic parameters of nitric oxide bioavailability. Abbreviations: Plasma L-Arg: plasmatic L-arginine; Plasma ADMA: plasmatic asymmetric dimethylarginine; Plasma SDMA: plasmatic symmetric dimethylarginine; Plasma DMA: plasmatic dimethylamine; DM: diabetes mellitus group; Control: control group;

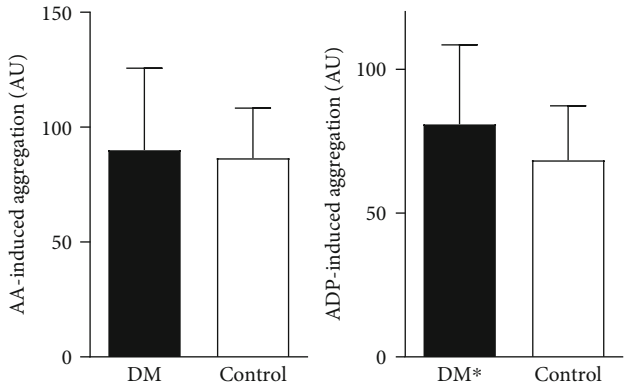

Figure 6: The comparison of platelet aggregation induced by AA and ADP. $^{*}p < 0,05$ versus control group. Abbreviations: AA: arachidonic acid; ADP: adenosine diphosphate; DM: diabetes mellitus group; Control: control group.

31, 32. The impairment of the PDNO production is postulated to be one of the main reasons for platelet hyperaggregability 11, thus elevated intraplatelet ADMA concentration, by competitive eNOS inhibition, may be considered as a primary cause of this condition. A potential cross-talk between these two processes may occur in the level of cGMP and cAMP interaction. Fleming et al. investigated the effect of insulin on activation of intraplatelet eNOS and the production of nitric oxide and demonstrated it to stimulate both cyclic GMP and AMP formation 33, which the action inhibits thrombocyte aggregation. Stimulating the $P2Y_{12}$ receptor with ADP, *via* the Gi protein action, lowers the concentration of intraplatelet cAMP leading to platelet aggregation 34. Therefore, lower PDNO concentration due to intraplatelet ADMA accumulation could be the reason for lower basal cAMP concentration, which results in increased aggregation with ADP whose activity is conducted by interaction with the same intracellular signal transmitter. On the contrary, despite the theoretical consistency, we found no significant correlation between intraplatelet ADMA concentration and aggregation induced by both ADP and arachidonic acid. However, a close relationship between the tested parameters cannot be excluded, possible involvement of other mechanisms of increased platelet aggregability in diabetes, for example, reduced platelet membrane lipid fluidity 35 or the action of advanced glycation end products (AGEs) 36, should be considered.

Noteworthy, we found a moderate negative correlation between serum magnesium and intraplatelet ADMA. The difference in serum magnesium concentration among studied groups may be the result of the increased Mg^{2+} transport from extracellular to the intracellular compartment in insulin-enhanced mechanism 37, 38. What is more, hypomagnesemia has been connected with impaired vasodilation and decrease in NO release 39, which would be consistent with the results of the current work, where lower serum magnesium levels are correlated with higher intraplatelet ADMA concentrations inhibiting the nitric oxide synthase. However, some authors showed that Mg^{2+}-induced vasodilation is independent of NO40, and others suggest that low magnesium prevents only from nitric oxide release, not production39, so its precise role in this pathway remains unclear.

In the current study, diabetic subjects were also characterized by elevated PLT ADMA/SDMA ratio in comparison with the control group. These results are accompanied by the difference between intraplatelet ADMA and SDMA in both studied groups. In both groups, these parameters significantly correlated with each other. In the control group, the correlation is very strong when in diabetic subjects, it decreases to moderate. What is more, the intraplatelet SDMA concentration negatively correlates with both glucose and HBA_{1C} level. It allows for careful speculation, that hyperglycemia, by inducing the protein glycation, may change the proteins' affinity to methyltransferases (PMRT-I, PMRT-II) and alter the process of their arginine residues methylation. The possible influence of platelet protein glycation on thrombocyte function has been repeatedly highlighted in scientific reports 25, 41, and its potential impact on NO production was also considered 42. Besides, glycation occurs between carbonyl groups and free amino groups present in polypeptide chain 43. Since arginine belongs to basic amino acids, its free amino group is a potential site of nonenzymatic glucose and another monosaccharide attachment. In the same location, the methyl group is attached by the action of PMRT 44. Therefore, the balance of the proteins' methylation may be disturbed to the side of asymmetrical methylation at the expense of symmetrical one, which results in the formation of more asymmetrical arginine derivatives after proteolysis causing the increase of ADMA/SDMA ratio and intraplatelet ADMA concentration, which inhibits NOS activity and thus reduces PDNO production. This theory is supported by the study of Lee et al. that arginine residue methylation is altered during the development of diabetes 45. Changes in arginine methylation are not only related to the formation of methylated arginine-derivates but also are known as an epigenetic regulator of gene transcription, cell signalling, protein stability, and translation 46, 47

In the current study, we assessed endothelial vasodilatory function using EndoPAT 2000 and Laser Doppler Flowmetry (LDF). According to Jakubowski et al., these methods evaluate independent mechanisms regulating the profile of endothelial vasodilatory function 16. We have shown reduced microvascular response to local peripheral thermal stimulus measured by LDF in diabetic subjects in comparison to the control group with no significant difference in the indexes provided by EndoPAT 2000. These results did not correlate with any of the demographical or biochemical risk factors what is in line with the findings of previously cited author. As vasodilation caused by the thermal stimulus applied locally is dependent not only on the activity of nitric oxide produced by eNOS isoform 48 but also on sensory nerve stimulation and release of sympathetic nervous system neurotransmitters 18. Both described mechanisms might be involved in impaired response to thermal stimulus in diabetic patients 49, 50. In diabetes, changes in microvascular flow measured by LDF, as shown in current and other studies 49, 51, were detected in earlier stages of the disease compared to EndoPAT 2000 52, which allows considering Laser Doppler Flowmetry as a more sensitive indicator of microvascular status. However, the exact role of the LDF as an independent predictor of cardiovascular events requires further investigation.

To conclude, type 2 diabetes is related to higher intraplatelet concentration of asymmetric dimethylarginine (ADMA), which may result in impaired platelet-derived nitric oxide synthesis and subsequent increased platelet activity, as assessed by the ADP-induced aggregation. In the early stage of well-controlled diabetes type 2, the accumulation of intraplatelet ADMA occurs prior to its increase in plasma. What is more, the Laser Doppler Flowmetry, but not Endo-PAT 2000, showed decreased microvascular skin flow after local heating in the diabetic group, hence LDF appears to be a more sensitive indicator of the impaired microvasculature vasodilation in diabetics without the presence of clinically significant target organ damage.

Data Availability

The original data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interests.

Acknowledgments

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Review Article

Impact of Lifestyles (Diet and Exercise) on Vascular Health: Oxidative Stress and Endothelial Function

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Healthy lifestyle and diet are associated with significant reduction in risk of obesity, type 2 diabetes, and cardiovascular diseases. Oxidative stress and the imbalance between prooxidants and antioxidants are linked to cardiovascular and metabolic diseases. Changes in antioxidant capacity of the body may lead to oxidative stress and vascular dysfunction. Diet is an important source of antioxidants, while exercise offers many health benefits as well. Recent findings have evidenced that diet and physical factors are correlated to oxidative stress. Diet and physical factors have debatable roles in modulating oxidative stress and effects on the endothelium. Since endothelium and oxidative stress play critical roles in cardiovascular and metabolic diseases, dietary and physical factors could have significant implications on prevention of the diseases. This review is aimed at summarizing the current knowledge on the impact of diet manipulation and physical factors on endothelium and oxidative stress, focusing on cardiovascular and metabolic diseases. We discuss the friend-and-foe role of dietary modification (including different diet styles, calorie restriction, and nutrient supplementation) on endothelium and oxidative stress, as well as the potential benefits and concerns of physical activity and exercise on endothelium and oxidative stress. A fine balance between oxidative stress and antioxidants is important for normal functions in the cells and interfering with this balance may lead to unfavorable effects. Further studies are needed to identify the best diet composition and exercise intensity.

1. Introduction

Obesity has become an epidemic and represents the major risk factor for several chronic diseases, including diabetes, cardiovascular diseases, and cancer [1]. Dietary modifications and physical exercise are popular among individuals who want to prevent overweight and keep fit. However, some recent studies have also suggested that the enthusiasm for the potential benefits of specific diets may exceed the current evidence supporting their implications [2, 3]. Therefore, it is very important to reappraise the risks and benefits of different diets to avoid unnecessary side effects.

The imbalance between prooxidants and antioxidants is linked to cardiovascular and metabolic diseases [4]. In normal conditions, homeostatic reactive oxygen species (ROS) act as secondary messengers in various intracellular signaling pathways in the cardiovascular system [5]. However, cellular oxidative stress is developed when the production of ROS and other oxidants exceeds the antioxidant defense [6]. Oxidative stress may lead to the subsequence oxidative modification or damage lipids, proteins, and DNA with deleterious consequences for metabolic and cardiovascular diseases [5]. Indeed, it has been shown that dietary and physical factors play an important role in modulation oxidative stress and endothelial function. Diet is a very important source of antioxidants, while exercising offers many health benefits, especially to cardiovascular system and muscle. Recent studies and media have suggested some specific diets to prevent overweight and improve cardiovascular health, including Mediterranean diet, ketogenic diet, and calorie restrictions [7–9]. However, different diets and physical factors have debatable roles in modulating oxidative stress and effects on the vascular system. The knowledge about the role of the behaviors and factors which are protective or harmful to the endothelium is still growing, and the newest information is recently summarized [10]. Since the endothelium and oxidative stress play critical roles in cardiovascular and metabolic diseases, appropriate choice of dietary and physical

factors could have significant implications in the prevention of cardiovascular and metabolic diseases.

In this review, we summarize current knowledge on the impact of diet modification (including different diet styles, calorie restriction, and nutrient supplementation) and physical factors on endothelium and oxidative stress. Besides, we further discuss the friend-and-foe roles of dietary on endothelium and oxidative stress, focusing on cardiovascular and metabolic diseases.

2. Endothelium

Endothelium is a single layer of flat, polygonal endothelial cells that rest on the inner walls of blood vessels. Endothelium plays an important role in modulating vascular function by sensing the shear or frictional force between blood flow and vascular endothelium. Upon stimuli, such as blood flow and receptor-mediated stimulants, endothelial cells release important vasoactive substances including both vasodilating [such as endothelium-derived hyperpolarizing factors (EDHFs), prostacyclin (PGI_2), and nitric oxide (NO)] and vasoconstricting factors [such as endothelin-1 (ET-1), thromboxane A2 (TXA2), and angiotensin II (Ang II)] to regulate vascular tone and architectures [11–13]. The activity of endothelial-derived NO or endothelium-derived relaxing factor (EDRF) plays an important role in the regulation of vascular function, blood pressure, and blood flow and has been widely used as a clinical marker of endothelial function [14, 15]. Mechanical forces elicited by the blood flow (shear stress) and pressure (cyclic strain) stimulate the gene expressions in endothelial cells and activate endothelial nitric oxide synthase (eNOS), which produces NO to regulate vascular function [16, 17]. In addition, it is known that laminar shear stress can also regulate antioxidant enzymes [18].

Vascular endothelium is the primary site of dysfunction in metabolic and cardiovascular diseases. Moreover, endothelial dysfunction is a hallmark of vascular aging [19]. Risk factors including hypertension, hypercholesterolemia, diabetes, and smoking are all associated with endothelial dysfunction [20]. Endothelial dysfunction is mainly characterized by the impairment in endothelium-dependent relaxation of blood vessels and the induction of a proinflammatory or prothrombotic state [16]. While NO inhibits platelet aggregation, smooth muscle cell proliferation, and the adhesion of monocytes to endothelial cells, depletion of NO leads to endothelial dysfunction and abnormal vascular remodeling [21]. Apart from pathological conditions, the anticontractile ability of endothelium is also significantly reduced during aging [19, 22], partly due to the decreased eNOS expression, NO bioavailability, or the soluble guanylyl cyclase (sGC) presence in the endothelium of aged arteries [23, 24]. Several pharmacological strategies including statins, angiotensin II receptor antagonists, and angiotensin-converting enzyme (ACE) inhibitors have been demonstrated to improve endothelial function in different studies [25]. Nonpharmacological interventions, such as physical activity and nutritional factors also play an important role in maintaining normal endothelial function [10].

3. Oxidative Stress in the Endothelium

Oxidative stress occurs when the cellular production of oxidant molecules, such as ROS, exceeds antioxidants' ability to defeat these insults. Generation of ROS is a normal physiological process in aerobic organisms. Vascular ROS are important intracellular signaling messengers that regulate vascular contractility, cell growth, and vascular remodeling [26]; however, oxidative stress can trigger the pathogenesis of related cardiovascular diseases [27]. Under normal conditions, deleterious ROS are mostly removed by cellular antioxidant systems (Figure 1). Excessive ROS are known to cause lipid peroxidation and oxidative modifications of proteins and nucleic acids that cause endothelial dysfunction [28].

Oxidative stress and the associated oxidative damage are mediators of vascular injury and inflammation in many cardiovascular diseases, especially when complicated with hypertension, hyperlipidemia, and diabetes [29, 30]. The major source of oxidative stress in the arterial wall is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) [31], which is implicated in the generation of ROS and the scavenging of NO [32]. In endothelium, increased generation of ROS reduces NO bioavailability, resulting in promotion of vasoconstriction and endothelial dysfunction. Other sources of ROS in the vascular wall include mitochondrial respiratory chain and other enzymatic reactions such as cyclooxygenase (COX), xanthine oxidase (XO), lipooxygenase (LOX), cytochrome P450, and dysfunctional eNOS [33–35]. On the other hand, vascular wall contains various enzymes that can reduce the ROS burden and act as antioxidant defense systems. These include superoxidase dismutase (SOD), catalase, glutathione peroxidase (GPx), heme oxygenase (HO), thioredoxin peroxidase (TPX), and paraoxonase (PON) [33, 36, 37]. Oxidative stress can lead to the oxidation of low-density lipoprotein (LDL), which inhibits the release of EDRF more than native or unoxidized LDL [14]. Moreover, oxidized LDL (ox-LDL) is cytotoxic to endothelial cells and chemotactic for monocytes, leading to accumulation of inflammatory cells and ROS in vasculature [38, 39]. Since vascular oxidative stress is the main pathophysiological mechanism leading to blunt NO bioavailability and endothelial dysfunction, attentions to potential treatment or prevention by dietary antioxidant substances have been drawn.

4. Diet Effect on Endothelial Function and Oxidative Stress

Healthy lifestyle and diet are associated with significant reduction in risk of obesity, type 2 diabetes, and cardiovascular diseases [40–42]. Due to the important role of ROS in CVD as mentioned above, there has been enormous interest in the application of naturally occurring antioxidants and diet interventions to ease or prevent metabolic and cardiovascular diseases.

It has been demonstrated that dietary factors may induce significant changes on vascular reactivity [15, 43–45]. Epidemiological evidence supports the concept that diets rich in fruits and vegetables promote health and prevent the development of cardiovascular diseases [46, 47]. A variety of nutrients

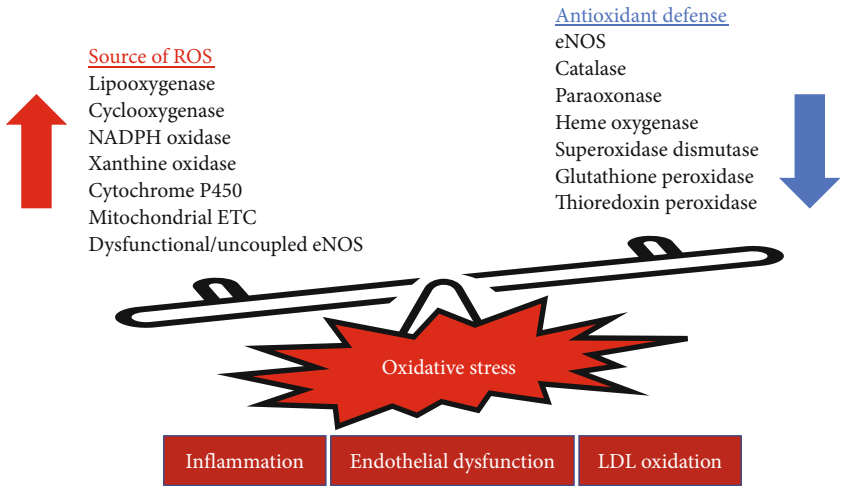

Figure 1: Oxidative stress occurs when the ROS production exceeds antioxidant defense. Generation of ROS is a normal physiological process. Under normal conditions, deleterious ROS are mostly removed by cellular antioxidant systems including functional endothelial nitric oxide synthase (eNOS), superoxidase dismutase, catalase, glutathione peroxidase, heme oxygenase, thioredoxin peroxidase, and paraoxonase. Sources of ROS including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cyclooxygenase, xanthine oxidase, lipooxygenase, cytochrome P450, and dysfunctional eNOS are augmented resulting in oxidative stress and related endothelial dysfunction.

have shown to improve endothelial function and prevent cardiovascular diseases. Recently, attention has been focused on dietary patterns in populations with lower prevalence of cardiovascular disease, for example, Mediterranean diet. A longitudinal investigation on human subjects suggests that a healthy diet containing more lean fish, raw vegetables, and fewer high-fat dairy products is associated with less endothelial dysfunction [48]. In addition, consumption of plant-derived foods which contain micronutrients, such as fiber, antioxidant, and phytochemicals, may inhibit intracellular inflammatory signaling pathways and reduce oxidative stress [49]. Here, we summarize the recent results from the animal studies and randomized trials on diet interventions and supplements that modulate vascular functions and discuss on both their beneficial and side effects.

4.1. High-Fat Diet. According to American Heart Association (AHA) guidelines, diet with more than 35% of total calories from fat is regarded as high-fat diet (HFD) [50]. HFD is a common experimental diet model used to induce obesity in animals. In general, there is no doubt that HFD is associated with an increased risk of cardiovascular diseases. Early researches, working on HFD and cardiovascular risk, focused on metabolic and lipid profile abnormalities [51], while more recent studies have indicated a potential and direct effect of endothelial dysfunction induced by dietary fat intake [52, 53]. HFD is considered as a risk factor for cardiovascular disease and causes endothelial dysfunction mostly due to its association with obesity and insulin resistance [54, 55]. Indeed, single high-fat meal can already impair endothelial function transiently, in terms of flow-dependent vasoactivity in normocholesterolemic volunteers [56]. Two consecutive fat-rich meals can impair endothelial function and elevate oxidative stress markers in healthy man [57]. Four-day HFD intake can induce endothelium-dependent vasodilator dysfunction associated with diminished NO bioavailability in healthy adults [53]. These suggest that HFD could have a direct negative effect on endothelium. Nevertheless, HFD can impair endothelial function in mice and reduce the local antioxidant defense in aorta [58]. HFD may induce endothelial dysfunction, at least partly, due to triacylglycerols that reduce NO bioavailability and increase oxidative stress [52, 57].

Indeed, not all HFDs have negative effects on endothelial function, but rather depend on different types of fat [59]. High intake of saturated fat increases the risk of cardiovascular diseases and decreases endothelial fibrinolytic capacity [60]. Diet high in saturated fat has been shown to induce cholesterol-independent endothelial dysfunction and increase markers of oxidative stress in rat [61]. The habitual consumption of diet high in saturated fat is strongly associated with impaired endothelial function (reactive hyperaemia index) in young overweight adults [62]. On the other hand, diets enriched in unsaturated fatty acids seem to show beneficial effects on endothelial function [63]. Obesity induced by high unsaturated-fat diet in rat has improved vascular reactivity to leptin and does not generate endothelial dysfunction, possibly due to the increase of vascular sensitivity to leptin and leptin-induced NO bioavailability [64]. In addition, high intake of trans fatty acids can adversely affect endothelial function and increase plasma inflammatory marker including C-reactive protein (CRP), interleukin-6 (IL-6), and soluble cell adhesion molecules (sICAM-1 and sVCAM-1) according to a cross-sectional study of 730 women [65]. High trans-fat diet has also been shown to reduce endothelial function (flow-mediated dilation) [66, 67] as well as endothelial cell activation [65]. Trans-fat may cause endothelial dysfunction, at least partially, by increasing NF-κB activation and impair insulin-mediated NO production in endothelial cells [68].

While HFD is relatively an experimental diet, cafeteria diet (CAF) containing a variety of highly palatable, high-salt, high-fat, and low-fiber energy dense foods, which is accessible in Western societies, is more accurately reflecting an obese diet [69]. CAF is a robust model of human metabolic syndrome with liver and adipose inflammation [70]. Indeed, both HFD and CAF can induce obesity, glucose intolerance, and insulin resistance to a comparable extent [71]. CAF can induce endothelial dysfunction in the absence of insulin resistance in rats [72], as well as vascular contractile dysfunction associated with increased oxidative stress and morphological remodeling in a hamster model [73]. In addition, CAF is more effective than HFD in causing PVAT-induced vascular dysfunction, associated with a significant reduction of vasodilatory response to acetylcholine in both mice [71] and rat [72]. Therefore, diet consisting of high fat, especially trans-fat and saturated fat can cause endothelial dysfunction and significantly increase the risk of cardiovascular diseases.

4.2. High-Sugar Diet. There is consensus that overconsumption of added sugar foods is positively associated with the risk for obesity and cardiovascular diseases [74, 75]. A prospective study has shown that people who got 17% to 21% of their calories from added sugar had a 38% higher risk of dying from cardiovascular disease compared with those who consumed 8% of their calories as added sugar [74]. Indeed, sugar added to foods and drinks supplies considerable extra calories without any benefits and may compromise the attainment of adequate dietary vitamin and mineral intake from the diet.

The new paradigm views overconsumption of sugar as an independent risk factor in cardiovascular diseases and other metabolic diseases [75]. High sugar intake may also cause endothelial dysfunction. Indeed, high glucose level promotes ROS formation, oxidative stress, and cellular death [76, 77]. Acute hyperglycemia caused by high glucose ingestion acutely deteriorates endothelial function in both human and animal studies, attributed by hyperglycemia-induced oxidative stress [78–81]. Exposure to high glucose increases eNOS activity and causing ROS formation due to eNOS uncoupling and aldose reductase activation in endothelial cells [82]. On the other hand, a recent double-blind randomized crossover trial has demonstrated that fructose load in healthy young subjects leads to unfavorable modifications of metabolic parameters, increased systolic blood pressure, and decreased endothelial NO production comparing to the same amount of glucose [83].

Another possible mechanism of how high-sugar diet induces endothelial dysfunction is attributed by advanced glycation end products (AGEs), a group of modified proteins and/or lipids that become glycated after exposure to sugars. AGEs can be ingested with high-temperature processed foods and also endogenously formed by nonenzymatic glycoxidation as a consequence of a high dietary sugar intake [84]. Chronic hyperglycemia promotes the formation of AGEs [85, 86], which have significant proinflammatory and prooxidant effects that contribute towards the pathology of diabetic and aging-related complications [87, 88]. In diabetic patients, high-AGE diet causes a significant increase in serum inflammatory markers [CRP and tumor necrosis factor α (TNF-α)] and endothelial dysfunction marker VCAM-1, whereas a low-AGE diet leads to a suppression of all these markers [89].

Another interesting mechanism by which high-sugar diet could promote oxidative stress is via protein phosphatase 2A (PP2A), a serine/threonine phosphatase that is responsible for the dephosphorylation of a wide range of substrates involved in cellular signaling, including p66Shc. High level of sugar has been shown to activate PP2A and NF-κB [90, 91]. P66shc is a longevity protein that regulates ROS and apoptosis, while dephosphorylated p66Shc may translocate into mitochondria and trigger oxidative stress [92]. Conversely, inhibition of high glucose-mediated PP2A expression prevents oxidative stress and increases NO production, thus reducing endothelial dysfunction [90].

4.3. Ketogenic Diet/Low-Carbohydrate, High-Fat Diet. The ketogenic diet is a low-carbohydrate, high-fat and adequate protein diet described a few decades ago for the management of children with epilepsy [93]. A classic ketogenic diet consists of a ratio of 4 : 1 fat to carbohydrate and protein [94]. In recent years, ketogenic diet or low-carbohydrate, high-fat diet (LCHFD) is suggested to be a successful weight-loss tool for obese subjects [95, 96] and popular among healthy people to maintain bodyweight. The rationale behind this diet is to induce ketosis, which the shift to fatty acids as the main respiratory substrate leads to increased production of ketone bodies (acetone, acetoacetate, and β-hydroxybutyrate) [97]. Currently, there are some evidence showing that ketogenic diet possesses anti-inflammatory effects and leads to short-term improvements in some cardiovascular risk factors and reduction in blood pressure [98–100]. However, there are still lacks of promising results to show that ketogenic diet or LCHFD is beneficial to vascular function.

In endothelial cells, ketone bodies significantly induce the expression of genes involved in the cellular antioxidant defense system (Nrf2 and HO-1) and reduce DNA damage against oxidative insult [101]. Ketogenic diet can also reduce glucose metabolism and improve heart function in a mice model with endothelial-specific Notch inhibition. [102]. However, the beneficial effect of ketogenic diet or LCHFD is still questionable, especially in human studies. Indeed, in ApoE knockout mice, LCHFD has no effect on oxidative stress markers, but accelerates atherosclerosis and reduces endothelial progenitor cells [103]. A study of Swedish women suggests that low-carbohydrate diets are associated with an increased risk of cardiovascular diseases [104]. A case-control study shows that ketogenic diet can promote arterial stiffening and endothelial damage in children and young adults with epilepsy [105]. In addition, LCHFD shows no improvement of endothelial function (flow-mediated dilation) in normal weight, young, healthy women [106], while LCHFD may lead to a reduction in flow-mediated dilation and predispose the endothelium to hyperglycemia-induced damage in healthy young adults [107]. Indeed, the detrimental effect of ketogenic diet may be attributed to the formation of advanced glycation end (AGE) products, which promote vascular damages [108]. Although the beneficial

effects of ketogenic diet or LCHFD on metabolic parameters are relatively promising, further researches are warranted to investigate the effect of ketogenic diet or LCHFD on cardiovascular health.

4.4. Mediterranean Diet. Mediterranean diet is characterized by high plant proteins, monounsaturated fat, and low animal products and saturated fat. The Mediterranean diet refers to an eating pattern of the olive growing areas surrounding the Mediterranean Sea. Numerous epidemiologic and intervention studies have shown that Mediterranean diet characterized by high consumption of vegetables, fish, olive oil, and moderate wine consumption is associated with a positive effect on endothelial function and a lower incidence of cardiovascular diseases [109–111]. Close adherence to a Mediterranean diet improves endothelial function (increased flow-mediated dilation) in obese subjects [109].

Indeed, Mediterranean diet is encompassed of nutrition and cultural behavior such as lifestyle and physical exercise [112]. The exact mechanism of which the Mediterranean diet has cardioprotective effects is thereby uncertain, but it is suggested that antioxidant and anti-inflammatory effect, improvement in endothelial function, and lipid profile are possible mechanisms [111]. Mediterranean diet has been shown to reduce blood pressure in hypertensive women and associate with increased plasma levels of NO and ET-1 and upregulated eNOS and ET-1 receptor in the endothelium [113]. Also, consumption of Mediterranean diet induces a reduction in endothelial damage and dysfunction, which is associated with an improvement in the regenerative capacity of the endothelium in healthy elderly subjects [114]. In randomized clinical trials, Mediterranean diet has been shown to significantly reduce circulating ox-LDL and inflammation markers in high cardiovascular risk subjects [115], as well as reduce oxidative stress and endothelial senescence in elderly subjects [116]. However, contradictory result has also been shown about the effect of the Mediterranean diet on endothelial function. In a clinical trial, treatment for 4 weeks with a Mediterranean-inspired diet fails to show beneficial effect in vascular function and reduce oxidative stress in healthy individuals with a low-risk profile for cardiovascular disease [117].

One special feature of Mediterranean diet is the consumption of olive oil, which is characterized by the high ratio monounsaturated to saturated fat. Consumption of olive oil-rich diet can decrease blood pressure and improve endothelial function in young women, which is associated with reduction in oxidative stress and inflammation mediators, such as ox-LDL and CRP levels [118]. However, our recent study suggests that olive oil, when compared to red fruit (*Pandanus conoideus Lam*) oil, has no significant effects on eNOS phosphorylation, NO production, and ROS levels in endothelial cells [119]. The favorable effect of Mediterranean diet on endothelial function might also be attributed to other components of this diet such as red wine and fish oil, as well as exercise which will be discussed below. Nevertheless, Mediterranean diet may represent a therapeutic strategy to reduce oxidative stress and inflammation and improve the associated metabolic and cardiovascular risk.

4.5. Calorie Restriction. Calorie restriction is the most potent and reproducible dietary interventions that shows beneficial effect in extending lifespan and attenuating aging-related chronic diseases including obesity and endothelial dysfunction [120].

In animal studies, caloric restriction is achieved by reducing calorie intake by 20-50% of *ad libitum* intake without altering the proportion of nutrients and inducing malnutrition [120]. Calorie restriction has been shown to improve life span, as well as physiological functions in most experimental models from yeast to nonhuman primates [121–123]. Short-term caloric restriction can prevent aging-induced endothelial dysfunction, at least partially by reversing altered iNOS/eNOS ratio, reducing oxidative stress, and increasing SOD enzyme activity in rat [124]. In both young and old mice, caloric restriction can reverse endothelial dysfunction by enhancing eNOS activity and NO production [125–127].

In human studies, lifelong caloric restriction has been shown to prolong lifespan, reduce atherosclerosis, and improve endothelial function [124, 128]. The most widely explored mechanisms of the beneficial effects of calorie restriction include improving cardiovascular risk-factor profile, reducing superoxide production and vascular oxidative stress, lowering circulating inflammatory cytokines, and upregulating sirtuin 1 (SIRT1) expression [129]. SIRT1 is a known longevity protein which can stimulate eNOS expression and activity [126, 130]. In response to caloric restriction, SIRT1 is activated and upregulates the activity of eNOS via deacetylating eNOS on lysine 496 and 506 residues [126, 131]. On the other hand, inhibition of SIRT1 prevents endothelium-dependent vasodilation and reduces NO bioavailability, suggesting that NO-mediating effect of caloric restriction is regulated via SIRT1 [132].

Although the translatability of caloric restriction to humans can be debatable, randomized trial has shown that a two-year 25% caloric restriction can attenuate biological aging and reduce the risk of cardiovascular diseases in nonobese young and middle-aged adults [133, 134]. Low-calorie diet can also improve endothelium-dependent vasodilation in obese patients with existing hypertension [135]. Long-term (>6.5 years) of caloric restriction has significantly reduced blood pressure comparing to that prior to the initiation [136]. Alternate day fasting with a low-fat diet for 12 weeks can effectively reduce weight and improve endothelial function (flow-mediated dilation) in both normal weight and overweight adults [137]. Collectively, these data suggest that short to long-term caloric restriction or reduced calorie intake may be able to prevent as well as reverse endothelial dysfunction and cardiovascular complications that caused by aging and obesity.

4.6. Diet Components and Supplements

4.6.1. Alcohol and Wine. A large number of clinical trials and epigenetics studies have strongly correlated the long-term consumption of polyphenol-rich diet with protection against chronic diseases such as cancers and cardiovascular diseases [138–140]. Red wine contains numerous amounts of polyphenols which possess antioxidant and anti-inflammatory

properties [141]. Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a plant polyphenol found mainly in grape fruits and red wine [142, 143]. Resveratrol is well-studied for its beneficial effect in cardiovascular protection by increasing NO production in the endothelium. Resveratrol can upregulate eNOS expression, stimulate eNOS activity, and prevent the uncoupling of eNOS [144]. In addition, resveratrol can improve endothelial function by activating SIRT1 [145]. Also, resveratrol has been shown to attenuate ox-LDL-induced cytotoxicity, apoptosis, ROS generation, and intracellular calcium accumulation in endothelial cells [39]. Therefore, red wine has been shown to improve endothelial function and reduce oxidative stress and the risk of cardiovascular events in both human and animal studies [146–148]. The beneficial effects of long-term consumption of moderate amounts of red wine can be attributed to increased HDL and reduction of ET-1 expression [149], as well as upregulation of sGC [150] and antioxidant enzymes (SOD and HO-1) [148]. Nevertheless, human clinical trials have shown that the beneficial effects of polyphenol are not always achievable within the context of moderate alcohol consumption as the bioavailability of the polyphenolic metabolites that reach the human body are always very low.

Indeed, recent studies suggest the potential influence of ethanol on endothelial functions [151, 152]. Both cross-sectional and randomized clinical trials have suggested that moderate alcohol consumption can improve endothelial function in healthy subjects [151, 153–155]. Chronic low ethanol consumption has also been shown to reduce systolic blood pressure and improve endothelial function in rat [156]. Moderate alcohol exposure (up to 1 drink or 12.5 g alcohol per day for women and 2 drinks or 25 g alcohol per day for men [157]) increases the activity of eNOS and NO production from the endothelium *in vitro* [158–160]. These studies suggest that ethanol may increase eNOS gene and protein expressions in endothelial cells. However, there are still controversies in the finding from the human studies. Flow-mediated dilation is impaired in either moderate or excessive alcohol consumption group according to a Japanese study [161]. Although the results from clinical studies of moderate/-habitual alcohol consumption remain controversial, one must bear in mind that excessive alcohol consumption is unquestionably harmful to human health at several levels.

4.6.2. Coffee. Coffee is one of the most popular pharmacologically active beverages, which is rich in plant phenolic compounds [162]. Various epidemiologic studies have already shown inverse associations of regular consumption of coffee with metabolic diseases including obesity and type 2 diabetes, as well as with cardiovascular diseases [163–166]. The beneficial effect of coffee in improving vascular function appears to be attributed by reducing the ROS production and enhancing NO bioavailability [167]. In a randomized, placebo-controlled, cross-over study, flow-mediated dilation response is significantly improved in the subjects taking caffeinated coffee compared to both groups taking decaffeinated coffee or water, suggesting that the consumption of caffeinated coffee can improve endothelial function [168]. Moreover, the acute administration of caffeine augments endothelium-dependent vasodilation in healthy young men associated with an increased NO production [169]. Indeed, coffee has been described as probably the most relevant source of dietary antioxidants [170]. A few studies have documented that a single serving of coffee may increase plasma antioxidant capacity by around 5% [170–172]. Caffeine may enhance endothelial cell migration and reendothelialization in part through an AMPK-dependent mechanism [173]. The most abundant polyphenol in coffee, chlorogenic acid (CGA), has been shown to increase the production of NO and reduce oxidative damages in isolated mice aortic ring [174]. However, the effects of coffee consumption on endothelial function are still controversial. Some studies have suggested an adverse effect of coffee consumption in endothelial function and blood pressure [172, 175]. These controversies may be attributed by the different additional supplements added to the coffee, including sugar and milk. Nevertheless, as coffee is a popular beverage worldwide, the effect of caffeine on endothelial function warrants detailed researches.

4.6.3. Gluten. Gluten consists of two classes of proteins, prolamin, and glutenin and can be found in several kinds of cereal. Gliadin, the wheat prolamin, is not fully digested by intestinal enzymes and is metabolized to biologically active peptides with cytotoxic activities, including increased intestinal permeability and modulation of the immune system [176]. Celiac disease is an autoimmune and systemic disease that develops in genetically susceptible individuals as a result of a permanent intolerance to gluten [177]. Celiac disease is also associated with endothelial dysfunction [178]. Gluten-free diet can reduce the risk for endothelial dysfunction [179] and reduce oxidative stress [180] in patients with celiac disease.

In individuals without celiac disease, the physiological effect of gluten intake is relatively unknown. Wheat alpha-amylase/trypsin inhibitors (ATIs) are activators of the innate immune system via activating toll-like receptor 4 (TLR4) in myeloid cells, triggering several autoimmune/inflammatory diseases. Gluten-containing cereals have been shown to contain the high concentration of ATIs that activate TLR4 [181]. Wheat ATIs are implicated in the pathogenesis of celiac disease as well as nonceliac wheat sensitivity [182]. Mice on a gluten-free diet show significantly attenuated clinical parameters of kidney dysfunction (proteinuria, haematuria, and hemoglobinuria) and serum inflammatory cytokines (IL-6 and TNF-α) compared to mice on a gluten-containing diet [183]. Increased gluten intake is associated with increased concentrations of plasma inflammatory marker, α_2-macroglobulin, in young adults without celiac disease [184]. Also, gluten diet can exacerbate vascular oxidative stress and inflammation and accelerate atherosclerosis in ApoE knockout mice [185]. Gluten feeding has been shown to elevate the rate of superoxide and nitrotyrosine production in the aortic root lesion [185]. These results suggest that the potential use of gluten-free diet is an alternative to ameliorate cardiovascular diseases independent of celiac disease.

4.6.4. Dark Chocolate. The main compound in chocolate is cocoa, which contains high content of polyphenols, while dark chocolate contains a greater amount of cocoa compared

with milk chocolate [186]. Chocolate and cocoa have been described as one of the most important sources of flavonoids in the human diet [187, 188]. Data currently available suggests that daily consumption of cocoa-rich chocolate may partially reduce the risk of cardiovascular disease [189].

Several mechanisms of how chocolates or cocoa flavonoids are protective against cardiovascular disease have been proposed including improving endothelial function, decreasing blood pressure, possessing antioxidant, antiplatelet, and anti-inflammatory effects, as well as a positive modulation of insulin resistance [187, 190–192].

Cocoa has been shown to reduce oxidative stress via lowering the activation of NADPH oxidase and stimulating NO-mediated vasorelaxation [193, 194]. In a cross-sectional study, administration of dark chocolate (>85% cocoa), but not milk chocolate (<35% cocoa), can significantly improve flow-mediated dilation and NO bioavailability in patients with nonalcoholic steatohepatitis [195]. In another study, dark chocolate, but not milk chocolate, inhibits platelet activity and oxidative stress in smokers [194]. And both studies suggest that dark chocolate reduces oxidative stress by downregulating NOX2.

Administration of a single dose of cocoa is dose dependently associated with significant increases in circulating flavanols and flow-mediated dilation in medicated type 2 diabetes patients [190]. In a randomized trial, the acute ingestion of either solid dark chocolate or liquid cocoa improves endothelial function and reduces blood pressure in overweight adults, while sugared cocoa may have attenuated these beneficial effects [196]. These studies suggest that the consumption of sugar-free dark chocolate would be the ideal choice to prevent endothelial dysfunction and cardiovascular diseases.

4.6.5. Omega 3 Polyunsaturated Fatty Acids. The correlation between high omega 3 polyunsaturated fatty acids (n-3 PUFAs) and low incidence of cardiovascular disease has been long recognized since an epidemiological study in the 1970s [197]. The broad range of beneficial effects of n-3 PUFAs is well known, including antiatherogenic, antithrombogenic, and blood pressure lowering [198]. Replacement of saturated fat with unsaturated fat has been suggested to improve endothelial function and show beneficial effect on endothelial repair and maintenance [199]. One of the mechanisms by which n-3 PUFAs influence endothelial function is mediated by their incorporation into biological membrane phospholipids and modulation of membrane composition and fluidity [200]. Caveolae-associated receptors mediate important pathways such as the NO-cGMP pathway, and the NADPH oxidase and TNF-α-NF-κB mediated COX-2 and PGE_2 activation pathway [201, 202]. n-3 PUFAs may exert their beneficial effects in the endothelium, by modulating the composition of membrane caveolae, similar as other lipids [203].

n-3 PUFAs including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to protect the cardiovascular system, in part, by enhancing eNOS activity and NO production [204, 205]. In various animal models, n-3 PUFAs have been shown to increase NO production by directly stimulating eNOS gene and protein expression, which improve vasodilation [206–208]. In addition to enhancing NO production, n-3 PUFAs also decrease oxidative stress. In endothelial cells, n-3 PUFAs can reduce the levels of inflammatory cytokines (IL-6 and TNF-α) and oxidative stress markers (ROS and malondialdehyde (MDA)) and increase the activity of SOD [209]. EPA and DHA have been shown to attenuate oxidative stress-induced DNA damage in endothelial cells through the upregulation of NRF2-mediated antioxidant response [210]. In epidemiology studies and clinical trials, n-3 PUFA supplementation prevents the development of atherosclerotic diseases [211, 212]. Moreover, n-3 PUFA supplementation has been shown to improve endothelial function in patients with primary antiphospholipid syndrome [213], type 2 diabetes [214], peripheral arterial disease [215], heart failure [216], and hypertriglyceridemia [217].

Recently, a ratio of EPA : DHA 6 : 1 has been recognized as a potent formulation to improve endothelial functions in different studies. Intake of omega-3 formulation EPA : DHA 6 : 1 can restore endothelium-dependent NO-mediated relaxations, most likely, by preventing the upregulation of the local angiotensin system, NADPH oxidase, and the subsequent formation of ROS in old rats [218]. Also, EPA : DHA 6 : 1 can prevent endothelial senescence in middle-aged or old rats by limiting both the shedding of endothelial microvesicles and their prosenescent, prothrombotic, and proinflammatory effects in endothelium [218]. However, a recent clinical study suggests that high-dose n-3 PUFA treatment in very high-risk patients with atherosclerotic cardiovascular disease and type 2 diabetes cannot improve the endothelial function indices [219]. Therefore, stronger evidence is needed before large-scale prescription of n-3 PUFAs in very high-risk patients. In overall, n-3 PUFAs have high potential to beneficially impact endothelial function and cardiovascular outcome.

4.6.6. Vitamins. Antioxidant vitamins (vitamins C, vitamin E, and β-carotene) appear to have beneficial effects on vascular endothelial function [220, 221]. β-carotene belongs to the family of carotenoids which are lipid-soluble plant pigments. β-carotene has been demonstrated to reduce inflammatory response and oxidative stress in TNF-α-treated endothelial cells *in vitro* [222], to prevent LDL oxidation [223], and to reduce the risk of atherosclerosis [224] and the incidence of cardiovascular diseases [223, 225, 226]. It has been shown that vitamin C (a water soluble antioxidant) and vitamin E (a lipid-soluble antioxidant) improve endothelial function by normalizing the expression of eNOS in hypercholesterolemic pigs [227]. Simultaneous administration of vitamins C and E can prevent the deleterious effects of postprandial hypertriglyceridemia on endothelial-dependent vasodilation [77].

In a randomized, placebo-controlled study, supplementation containing vitamin C (1000 mg/d) and vitamin E (400 IU/d) shows beneficial effects on glucose and lipid metabolism, blood pressure, and arterial elasticity in patients with cardiovascular risk factors [228]. Possible mechanisms for the beneficial effects of vitamins C and E on endothelial function could be attributed by eliminating superoxide and scavenging lipid hydroperoxyl radicals, and thereby maintaining NO bioavailability [220]. Long-term consumption of vitamin C in diabetic patients can improve certain

echocardiographic parameters and enhance vascular endothelial function (flow-mediated dilation) [229]. Chronic consumption of vitamin E partially prevents hyperglycemia-induced endothelial dysfunction in diabetic rat, while vitamin E deficiency enhances diabetic endothelial dysfunction dramatically *in vivo* [230].

Combined treatment with vitamins C and E has beneficial effects on endothelium-dependent vasodilation and arterial stiffness, which are associated with changes in plasma markers of oxidative stress in essential hypertensive patients [231]. However, one should be cautious about the long term or high-dose usage of vitamin. Vitamin C has been shown to increase the production of AGE products in diabetic patients [232]. Long-term treatment with 1,800 IU of vitamin E has no beneficial effects on endothelial or left ventricular function in diabetic patients, and some vitamin E-treated patients has a worsening in some vascular reactivity measurements when compared with control subjects [231].

5. Physical Exercise

Sedentary lifestyle and overconsumption of energy-rich food have been identified as a risk factor for the development of some cardiovascular complications [233, 234]. In comparison, regular physical activity (child and adolescents: 60 mins of moderate-to-vigorous physical activity daily; adults: 150 to 300 mins of moderate intensity per week [235]) has long been considered necessary for the achievement and maintenance of optimal health. Prospective studies provide direct evidence that a physically active lifestyle delays all-cause mortality, extends longevity [236], and reduces risk for cardiovascular mortality by 42–44%, compared to sedentary lifestyle [237]. The terms "physical activity" and "physical exercise" refer to body movements by the skeletal musculature and associated with the consumption of energy. Specifically, the term "physical training" indicates a regular, structured physical activity to improve and/or maintain physical fitness and well-being [238]. Exercise training elicits beneficial effects in a number of physiological adaptations, including maximal oxygen consumption (VO_{2max}), cardiac output, and maximal oxygen extraction, as well as maximal skeletal muscle blood flow capacity [239]. It is commonly recognized that physical training has beneficial effects on body composition and health, especially for weight loss [240], and also known as a nonpharmacological treatment of cardiovascular diseases [241].

5.1. Physical Exercise and Laminar Shear Stress. Regular exercises result in numerous health benefits, such as improving body composition and endothelial function and preventing insulin resistance, oxidative stress, and arterial hypertension [241]. One of the important mechanisms to improve endothelial function during exercising is the increased blood flow and shear stress which can improve vascular homeostasis by reducing the production of ROS and increasing the bioavailability of NO in the endothelium (discussed above) [242]. During exercising, repeated episodes of increased blood flow elicit an improvement in endothelial function and lead to the long-term benefits of regular exercise that prevent risk of cardiovascular diseases [243]. This mechanism is likely to involve chronic upregulation of NO production mediated by an enhanced expression of eNOS.

It is suggested that shear stress-mediated effects and consequent production and bioactivity of NO differ qualitatively and quantitatively according to the exercise involved [244]. Vascular laminar shear stress increases during exercise and is associated with a rapid upregulation of eNOS gene and protein expressions [245]. Varied durations of exercise training seem to influence the response of arteries to increase flow and shear stress [244]. It has been reported that improvement in NO-related vasodilation is observed in short- to medium-term training, whereas longer-term training is associated with arterial remodeling [244]. It is also important to note that laminar shear stress due to exercise has a predominant antioxidant effect and improves endothelial function, while oscillatory shear stress in hypertension is associated with opposite effects promoting oxidative stress and oxidative vasculature damage through a progressive increase in NADPH activity [246].

5.2. Physical Exercise, Arterial Pressure, and Oxidative Stress. Indeed, exercise is a double-edged sword for endothelial function [247]. Blood pressure distends arteries and causes stretching in vascular cells. Changes in pressure consequently generate circumferential stress (strain) in the compliant arteries. During exercise, because of increased heart rate and systolic pressure, expansion of arteries can induce cyclic circumferential strain on endothelial cells [248, 249]. Cyclic circumferential strain can also be increased by the relaxation of vascular smooth muscle, which induces vasodilation and stretching of the endothelium. The effects of cyclic circumferential strain are complex and variable. Increased exposure to cyclic circumferential strain has been shown to alter gene expressions in the endothelium [including eNOS and EDHF synthase (CYP450)] and promote the production of ROS, the expression of ICAM, selectin, and monocyte chemoattractant protein-1 (MCP-1) [250]. Indeed, chronic high blood pressure is associated with endothelial dysfunction and progression of atherosclerosis [251]. The major effect of increased cyclic circumferential strain on endothelial cells appears to be ROS-mediated proatherogenic [252]. The pattern of change in cyclic circumferential strain is relevant as transient increases in blood pressure and ROS production, which is associated with exercise bouts and may increase eNOS expression and other beneficial effects of exercise, whereas chronic increases in blood pressure may chronically elevate ROS, causing maladaptation [249]. Although it has been suggested that short-term exercise can cause oxidative stress by increasing LDL susceptibility to oxidation or vascular superoxide production [253], long-term and/or regular exercise has been shown to reduce oxidative stress and upregulate the expression of SOD [254] and other antioxidant defenses in human, which leads to positive arterial remodeling to normalize blood pressure and shear stress [255–258].

5.3. Physical Exercise and Arterial Remodeling. Arterial remodeling is the active process of structural alteration that occurs as a result of cell death, proliferation, and migration as well as changes in the extracellular matrix of a vessel and

is controlled by the crosstalk between endothelium and vascular smooth muscle cells [259]. Sensitivity to shear stress of endothelium is important in arterial remodeling and can activate signaling pathways in vascular smooth muscle cells [259]. Vasculature in hypertensive individuals undergoes accelerated vascular wall thickening and leads to degeneration and calcification of the vascular wall and vessel stiffening, while compensatory vessel wall enlargement is observed in atherosclerotic patients [260].

NO is an important endothelial regulator of flow- and pressure-induced arterial remodeling [260], while the congenital absence of eNOS causes adverse vascular remodeling [24, 261]. Upon long-term exercise training, NO-mediated structural adaptation occurs in the arteries, resulting in a chronic increase in vessel caliber, which structurally normalizes shear stress and cyclic circumferential strain (Figure 2). NO function is then returned towards baseline levels [262]. This process constitutes a long term and structural change mechanism for reducing shear stress, allowing NO bioactivity to return towards pretraining levels [244]. Moreover, positive arterial remodeling serves to enhance muscle performance by increasing the oxygen-exchange capacity and by increasing blood flow capacity. These adaptations potentially contribute to cardiovascular performance and health benefits in individuals who have long-term exercise training [262].

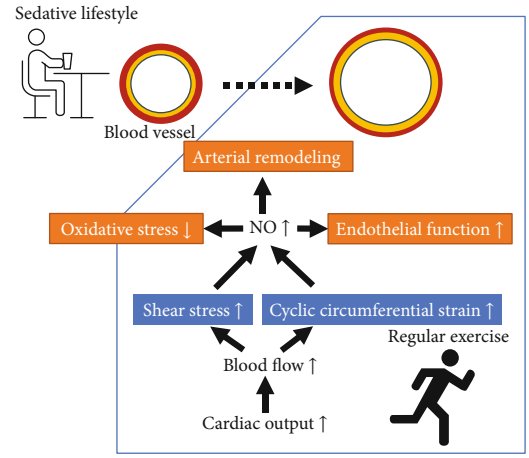

Figure 2: Regular exercise leads to arterial remodeling that contributes to cardiovascular performance and health benefits. During exercise, cardiac output and blood flow are increased, which generate shear stress and cyclic circumferential strain on arterial wall. Long period of exercise results in a long-term upregulation of eNOS. NO-mediated arterial remodeling results a chronic increase in vessel caliber, which structurally normalizes shear stress and cyclic circumferential strain

5.4. Physical Exercise in Animal Models. In healthy rats, a single resisted exercise session can improve endothelial function and NO synthesis in both the endothelium and the smooth muscle layer of mesenteric and promote insulin-induced vasodilation [263]. It is proposed that exercise stimulates factors that increase endothelial NO production including vascular distension, catecholamine release, and intermittent hypoxia. Exercise training in pigs [264] and hypertensive rats [265] has been shown to increase both gene and protein expression of eNOS. It is also suggested that the stimulation of NO production is dependent on the volume of exercise, and a greater demand of oxygen and nutrients is involved in the beneficial effects of exercise on the endothelium [263, 266]. Moreover, similar results are observed in hypertensive rats that single session of resisted exercise activates eNOS and promotes vasorelaxation [267]. Interestingly, expression of the prooxidant enzyme, NADPH oxidase, is reduced by exercise training in hypertensive rats, which may have a beneficial effect on the half-life of NO in the vascular wall [266]. These data reinforce that exercise can improve endothelial function, probably by stimulating NO production, even in hypertension. In diabetic rats, exercise training has been shown to normalize the diabetes-related endothelial dysfunction and improve insulin sensitivity [268, 269]. Moderate-intensity exercise reverses diabetes-related endothelial dysfunction independently of improvements in body weight or hyperglycemia in db/db mice. It is suggested that upregulations of eNOS and cytosolic Cu/Zn-SOD, but not MnSOD, play important roles in improving NO bioavailability, as well as in reversing endothelial dysfunction in diabetes via exercise [270]. In aging rats, exercising training increases sensitivity of blood vessel to shear stress and promotes acetylcholine-induced endothelial-dependent vasorelaxation [271], suggesting that exercising training is able to reverse age-related decline in vascular function.

In addition, long period of exercise can reduce the contractile response of the aorta to noradrenaline and increase the relaxation induced by acetylcholine in healthy rats [272–274], suggesting that the time of exercise exposure may also be critical to determine the beneficial effects of exercise on endothelial function. Chronic exercise training improves aortic endothelial and mitochondrial function through an adenosine monophosphate-activated protein kinase $\alpha 2$ (AMPK$\alpha 2$)-dependent manner in mice, which is also associated with increased mitochondrial antioxidative capacity (increased expression of MnSOD and catalase) [275]. These experimental studies suggest that physical exercise plays an important role in the prevention and treatment of endothelial dysfunction. However, additional studies are needed to establish the best kind, intensity, and duration of exercise to allow more efficient prescribing in clinical area.

5.5. Physical Exercise in Human Studies. In type 2 diabetic patients, lower-intensity exercise has physiological meaningful effects on endothelial function, while low to moderate intensity and aerobic exercises significantly increase flow-mediated vasodilation more than moderate- to high-intensity exercises and combined aerobic and resistance exercises, respectively [276]. Regular physical activity has been shown to promote the activities of antioxidant enzymes and stimulate glutathione levels in body fluids [277, 278]. In young prehypertensive patients, resistance training increases flow-mediated dilation and reduces blood pressure [279], as wells as improves resistance artery endothelial function and prooxidant/antioxidant balance [280]. In a recent randomized clinical trial, aerobic exercise training, resistance training, and combined training have also shown similar

beneficial in improving endothelial function but impacts on ambulatory blood pressure appear to be variable in middle-aged and older individuals with prehypertension or hypertension [281].

Aerobic exercise increases both gene and protein expression of eNOS in patients with coronary artery disease [245]. Six-month exercise training reduces arterial blood pressure and is associated with increased NO content (determined by plasma nitrite/nitrate levels) in hypertensive women [282]. However, biological sample acquisition from humans subjected to physical exercise is challenging, thereby the changes in NO production are predominantly evaluated based on the measurements of NO content in the exhaled air. Physical training is associated with an increase in NO content in the exhaled air [283], while some studies suggest a reduced NO content in the exhaled air after physical exercise [284–286]. This controversy is unsurprising due to the complexity of NO exchange and multisystemic nature of the physiological responses to physical exercise [249].Nevertheless, variations of NO content in the exhaled air may also depend on the levels of physical activity [284].

5.6. High-Intensity Exercise: Good or Bad? It is clinically important to select the appropriate intensity, duration, frequency, and kind of exercise, as high-intensity exercise can be harmful to human vessels [287]. In general, the guidelines for patients with mild-to-moderate essential hypertension recommend exercise at an intensity of around 50% of maximum oxygen consumption, such as walking, jogging, cycling, or swimming, for 30 minutes and 5 to 7 times per week [287–289]. In recent years, low-volume high-intensity exercise training has become advocated with data showing comparable benefits to traditional endurance-based training in skeletal muscle metabolic control and cardiovascular system function [290]. Aerobic training of high intensity, compared to the aerobic training of low intensity and controls, has been shown to improve endothelium function (flow-mediated vasodilatation) in patients with metabolic syndrome or diabetes [291]. Both continuous moderate-intensity aerobic exercise and high-intensity interval aerobic exercise can significantly improve endothelial function, in terms of flow-mediated dilation, the carotid femoral pulse wave velocity and the femoral dorsalis pedis pulse wave velocity in health men [292]. A recent randomized controlled, crossover study also suggests that short-duration maximal intensity exercise has comparable effects on endothelial function and oxidative stress with mild and moderate exercise [293].

On the other hand, collective evidence has suggested that production of ROS is greater than production of NO during high-intensity exercise, resulting in reduced endothelial function (Figure 3). For example, high-intensity exercise training has no beneficial effects on endothelial function in spontaneous hypertensive rat, but rather augments oxidative stress, resulting in eNOS uncoupling and ROS production and leading to further decrease in NO bioavailability and increase in ROS [294]. High-intensity exercise also increases the indices of oxidative stress, including plasma concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and serum concentration of ox-LDL, and decreases endothelium-dependent

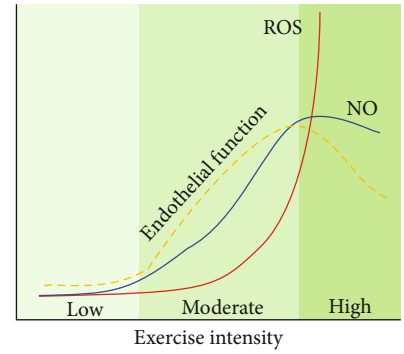

Figure 3: Schematic of the effect of different exercise intensities in vascular nitric oxide (NO) and reactive oxygen species (ROS) level and endothelial function. Low-intensity exercise may have minimal effect on NO and ROS production and physiological meaningful effects in endothelial function. During moderate-intensity exercise, production of NO is augmented while ROS production is increased in a slower rate, resulting in improvement of endothelial function. During high-intensity exercise, production of ROS is significantly greater that of NO, resulting in reduced endothelial function.

vasodilation in healthy men [295]. It is suggested that the massive increase in oxygen uptake that occurs in skeletal muscle during high-intensity exercise is associated with an increase in ROS [296]. Moreover, oxidation of tetrahydrobiopterin (BH_4) is suggested to be responsible in oxidative stress-induced eNOS uncoupling during high-intensity exercise. BH_4 deficiency is associated with the ROS production from dysfunctional eNOS; superoxide produced by uncoupled eNOS which also inactivates NO [297]. It has also been reported that the degradation of BH_4 by ROS is associated with the inhibition of eNOS [297, 298]. These findings suggest that BH_4 deficiency-induced eNOS dysfunction causes endothelial dysfunction in hypertension through promoting oxidative stress. It is possible that high-intensity exercise activates oxidative stress through exacerbation of BH_4 deficiency, as well as oxidation of BH_4. These data may suggest that high-intensity exercise increases oxidative stress in humans, which diminishes endothelium-dependent vasodilation.

Recently, high-intensity interval training (HIIT) has been proposed to have positive effects on metabolic profile and improves cardiovascular health [299–301] and used as an alternative to traditional endurance training to promote metabolic and cardiovascular health. The detailed mechanisms responsible for the beneficial effect of HIIT are not well known, but one proposed mechanism is that HIIT increases aerobic capacity and thus delays the onset of exhaustion [302]. The common formula of HIIT involves a 2 : 1 work-to-recovery ratio [303]. Interval running (30 s at VO_{2max} alternated with 30 s at 50% VO_{2max}) has been shown to provide a greater exercise training stimulus than continuous running to improve VO_{2max} [304]. In general, HIIT elicits greater aerobic capacity adaptations compared to chronic training in improving cardiovascular risk [303]. Recently, HIIT has been shown to improve arterial stiffness, cardiovascular health, and metabolic profiles in inactive individuals with obesity and type 2 diabetes [305–307]. Future studies are warranted to

investigate the effects and underlying mechanisms of HITT on endothelial function in a different pathophysiological condition, including aging, diabetes, and hypertension.

5.7. Exercise and Diet Combination. In order to maximize the beneficial effects of exercise on vascular function, the combination with a healthy diet is reasonable. Most current studies support that exercise is effective to ameliorate HFD-induced endothelial dysfunction and improve microvascular reactivity in young, healthy men [308], as well as to improve insulin action and reductions in glycemia and prevent endothelial dysfunction after high-sugar-food ingestion with endurance exercise performed on the previous day [80]. In addition, it is also suggested that the combination of supervised diet and exercise training is effective to improve vascular function and multiple adolescent obesity-related end points [309]. In addition, interval exercise combined with a low-calorie diet improves endothelial function in obese adult female [310]. Moreover, a recent study has demonstrated that aerobic exercise prior to a high-sugar meal has no improvement on endothelial function, blood glucose, insulin, ET-1 or NO concentrations, or insulin sensitivity in postmenopausal women [311]. Therefore, it is synergistic to improve vascular function when proper exercise is carried out in parallel with a healthy diet.

6. Conclusions

Healthy lifestyle and diet are important in reducing the risk for metabolic and cardiovascular diseases. A large body of evidence underlines the importance of proper diet and physical exercise in preventing oxidative stress and endothelial dysfunction, which are risk factors for cardiovascular diseases. Diet is an important source for antioxidants. In general, consumption of balanced diet with reduced amount of added sugar and saturated fat has been shown to reduce oxidative stress and promote endothelial function. However, it has been suggested that the indiscriminate use of antioxidants may even be harmful, since basal levels of ROS are imperative for certain cellular functions [312]. Overconsumption of certain nutrients as well as overintensive exercise may impede some essential cellular defense mechanisms. A fine balance between oxidative stress and antioxidants is important for normal function in the cells, and interfering with this balance may lead to unfavorable effects. While the best diet composition and exercise intensity may vary among individuals and physiological conditions, further detailed studies are needed. Thereby, before the ultimate problem is solved, a balanced diet and regular exercise are always helpful.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

AWCM wrote the initial draft of the manuscript. HL and NX critically reviewed and edited the manuscript. All authors agreed to its publication.

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Research Article

General, 21-Day Postoperative Rehabilitation Program Has Beneficial Effect on Oxidative Stress Markers in Patients after Total Hip or Knee Replacement

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Imbalance in prooxidant-antioxidant equilibrium plays an important role in the progression of osteoarthritis (OA). Postoperative rehabilitation significantly improves the functional activity of patients with OA. We aimed to assess the effect of the general 21-day postoperative rehabilitation on the oxidative stress markers in patients after total hip arthroplasty or knee replacement. Patients (n = 41) started individually designed postoperative rehabilitation ca. 90 days after endoprosthesis implantation. We used the six-minute walk test (6MWT) to quantify the changes in their exercise capacity. We analyzed the oxidative stress markers: total antioxidant capacity (TAC), total superoxide dismutase (SOD), Cu-Zn-superoxide dismutase (CuZnSOD) and ceruloplasmin (Cp) activity, malondialdehyde (MDA) and lipofuscin (LPS) concentration in patients serum to asses changes in the oxidative stress intensity. We found that after 21-days postoperative rehabilitation program: the average distance walked by patients increased by 69 m; TAC increased by 0.20 ± 0.14 mmol/l; both SOD isoforms activities increased by 1.6 (±1.7) and 1.72 (±1.5) NU/ml, respectively; but Cp activity decreased by 1.8 (0.7-3.7) mg/dl. Also, we observed lower concentrations of lipid peroxidation markers: by 19.6 ± 24.4 μmol/l for MDA and by 0.4 ± 0.5 RF for LPS. A 21-day postoperative rehabilitation program effectively reduces oxidative processes, which helps the patients after total hip or knee replacement in a successful recovery.

1. Introduction

Knee and hip osteoarthritis (OA) are frequent conditions in elder individuals, as osteoarthritis itself is one of the most common joint disorders in the world [1]. Pain and disability, two most frequent OA complications, are prevalent among middle-aged and elderly individuals [2]. Total hip arthroplasty and knee replacement performed in patients with end-stage arthritis become more frequent since the population is aging. The effective procedure relieves patients from pain, facilitates functional activities and promotes their return to the daily activities [3, 4]. The positive role of general rehabilitation in OA has been confirmed in numerous studies. Rehabilitation, regardless of its type, significantly reduces pain, increases range of motions and muscle strength, and reduces the use of medications [5–7]. By some clinical

practitioners, it is even adapted as preparation for the arthroplasty procedure [8, 9]. However, no scientific report specifies what type of rehabilitation therapy is most optimal for the patient's recovery process after total hip arthroplasty and knee replacement and no routine rehabilitation protocol has been developed as of yet.

Osteoarthritis is a multifactorial process of joint degeneration and various mechanisms may underlay its development [10]. One of them is the disruption of pro-oxidants and antioxidants equilibrium, which promotes cellular oxidative stress and leads eventually to OA progression [11]. During exacerbated physical exercises, oxygen uptake and energy demand increase rapidly. This intensifies mitochondrial energy metabolism, which enhances free radicals formation [12, 13]. The intensity of oxidative stress can be assessed using various general and specific indicators [14–16]. Total antioxidant capacity (TAC) shows the interaction between synergistic and antagonistic antioxidants. Its measurements are essential to correctly assess the results of other, more specific, oxidative stress markers [17]. Superoxide dismutase (SOD) along with its copper-zinc isoform (CuZnSOD) is one of the most important detoxifying enzymes and indicators of oxidative stress and reactive oxygen species increase [7]. Ceruloplasmin (Cp), mainly described as the acute phase protein and copper carrier to tissues, is also a prominent antioxidant scavenging reactive oxygen species and having pleiotropic effects on the body's antioxidative and oxidative metabolism [18]. When oxidative stress exceeds the compensatory capabilities of the cells and SOD fails to scavenge free radicals effectively, lipid peroxidation and cell damage occur. MDA is a product of this reaction and may be successfully used as an indicator of the severity of the oxidative processes in cells. Given these facts, malondialdehyde (MDA) and SOD are considered matching oxidative stress markers [12, 13]. Lipofuscin is another product of the unsaturated fatty acids oxidation. It is closely related to the natural aging processes and its accumulation in the body manifests as pigment deposits in skin, liver, kidneys or heart muscle [19]. Lipofuscin accumulation in organs may result from the imbalance between its formation and its degradation in lysosomes, and the formation process is directly related to an increased concentration of other oxidative stress markers [19].

It is known that rehabilitation significantly improves functional activity and quality of life of patients with osteoarthritis. However, it remains mostly unknown how rehabilitation affects biochemical processes of the body, especially antioxidative processes at the cellular level. This study aimed to assess the effect of a general 21-day rehabilitation program on the oxidative stress markers in patients after total hip arthroplasty or knee replacement.

2. Materials and Methods

2.1. Ethical Statement and Permissions. The study was carried out according to the Declaration of Helsinki and the protocol was fully approved by the Ethics Committee of the Medical University of Silesia in Katowice (N° KNW/002/KB1/106/17; 03.10.2017). Every participant of the study received the description of the protocol and was informed about its benefits and possible risks and returned the written informed consent before the study started.

2.2. Study Group. Forty-one patients after total hip (n =29; 71%) or knee (n =12; 29%) replacement, aged 61.0 ± 8.1 years, were included in the study. Twenty-two of them were men and 19 were women (54% and 46%, respectively) and on the initial examination day, they were, on average, 89.6 ± 9.7 days after the replacement surgery. Due to concomitant health conditions that occurred during the study, results of five patients were excluded from the oxidative stress markers analyses. Further details are presented in section 3.4.

Upon the arrival to the outpatients' clinic, the resting electrocardiogram (ECG) and blood pressure measurement were recorded, the body mass and height measurements were taken for each patient. Also in each case, the clinical interview was carried out to exclude patients with inflammatory disorders, infections, renal or hepatic insufficiency, active coronary artery disease, diabetes, heart failure, hormonal replacement therapy or supplementation with antioxidants that might have occurred or taken place 3 months before the study.

2.3. General Rehabilitation Program. All patients underwent a 21-day general rehabilitation program that started ca. 90 days after knee or hip endoprosthesis implantation. Rehabilitation sessions were conducted daily for 21 days, starting between 8 : 00 and 8 : 45 am. The main components of general rehabilitation program were physiotherapy, daily living activities training and patient's education on nutrition. The individual rehabilitation programs consisted of aerobic walking (30-45 min), strength training (20-30 min), rotor/bicycle training (30-45 min) and a cool-down phase (15 min). The patients were advised to continue the learned exercises and pro-health behaviour over the course of the day to keep their physical fitness and biochemical parameters at the beneficial level [20]. The programs were individually created for each patient, in regards to the choice of exercises (different strength and balance exercises) and training modalities (number and sets of repetitions as well as the duration of resting time), and then monitored in the rehabilitation by the responsible physiotherapist.

Osteoarthritis severely reduces the patient's exercise capacity. To quantify the exercise capacity, clinical practitioners use the 6-minute walk test (6MWT) [21]. In our study, each patient underwent the 6MWT twice: before starting and after completing the 21-day general rehabilitation program. The aim was to assess changes in functional exercise capacity and thus, to determine the effectiveness of the rehabilitation program [22, 23].

2.4. Samples Collection. Blood samples were collected before the initial and after the final rehabilitation session. Blood samples (5 mL) from ulnar vein were collected in the morning, at 8 : 00 AM, before breakfast. Blood was collected to the standard blood tubes: with EDTA (1.6 mg/ml EDTA-K_3; S-Monovette, SARSTEDT) and into tubes with a clot activator (S-Monovette, SARSTEDT). The samples for serum analysis were centrifuged at 4000 rpm for 10 minutes at 4°C,

and stored in −80°C. Plasma and serum samples were subsequently frozen and stored at −80°C until biochemical analyses could be performed.

2.4.1. Oxidative Stress Marker Analysis. The state of the antioxidant system was analyzed in the serum samples. The enzymatic antioxidant markers were assessed by measuring total superoxide dismutase (SOD), copper-zinc-superoxide dismutase (CuZnSOD) and ceruloplasmin (Cp) concentration. Non-enzymatic antioxidant systems were analyzed by assessing total antioxidant capacity (TAC), lipofuscin (LPS) and malondialdehyde (MDA) concentration.

2.4.2. Total Antioxidant Capacity (TAC). TAC was measured using a commercial kit (Randox Co., England). The 2,2'-azino-di-(3-ethylbenzothiazoline sulphonate) (ABTS) was incubated with a peroxidase (metmyoglobin) and hydrogen peroxide to produce the radical cation ABTS+, which has a relatively stable blue-green color and can be measured at 600 nm. The suppression of the color was compared to the standard for TAC measurement assays (Trolox). The results are expressed as Trolox equivalent (mmol/l). The inter- and intra-assay coefficients of variations (CV) were 1.1% and 3.8%, respectively.

2.4.3. Superoxide Dismutase (SOD) Activity (EC 1.15.1.1). SOD isoenzymes' activity was determined with the use of the spectrophotometric method by Oyanagui [23]. KCN was used as the inhibitor of the CuZnSOD isoenzyme. SOD activity was calculated against a blank probe (containing bidistilled water). Enzyme activity was expressed as nitrite units (NU) per mg of protein. One NU exhibits 50% inhibition of formation of nitrite ion under the method' s condition [24].

2.4.4. Ceruloplasmin (Cp) Concentration. Ceruloplasmin (Cp) concentration was measured using the p-phenylenediamine kinetic method by Richterich [25] and expressed in mg/dl after calibration with pure ceruloplasmin isolated from a healthy donor serum pool. The inter- and intra-assay coefficients of variations (CV) were 3.1% and 6.1%, respectively.

2.4.5. Malondialdehyde (MDA) Concentration. MDA concentration was measured using the spectrophotometric method (wavelengths: 552 nm for emission, 515 nm for excitation; Perkin Elmer LS45 spectrofluorimeter by Ohkawa et al. [26]. and standard curve prepared for 1,1,3,3-tetraethoxypropane - the product of malondialdehyde and thiobarbituric acid reaction. MDA concentration was expressed in (μmol/l).

2.4.6. Lipofuscin (LPS) Concentration. LPS concentration was determined according to Tsuchida et al. [27]. Serum was mixed with ethanol-ether (3 : 1, v/v), shaken and centrifuged. The intensity of fluorescence was determined using a PerkinElmer spectrophotometer LS45 at a wavelength of 345 nm (absorbance) and 430 nm (emission) in a dissolved solid. The values are expressed in relative lipid extract fluorescence (RF), where 100 RF corresponds to the fluorescence of 0.1 μg/ml quinidine sulphate in 0.1 N sulfuric acid. LPS concentrations are shown in RF. The inter- and intra-assay coefficients of variations (CV) were 2.8% and 9.7%, respectively.

Table 1: Endoprosthesis implantation frequency in the studied group according to surgery type and gender. Statistical significance was set at $p < 0.05$.

Surgery type	Men n = 22	Women n = 19	χ^2	p
Hip	18 (82%)	11 (58%)	2.81	0.093
Knee	4 (18%)	8 (42%)		

2.5. Statistical Analysis. Distribution of variables was evaluated by the Shapiro-Wilk test and quantile-quantile plot. The interval data were expressed as a mean value ± standard deviation (M ± SD) in the case of normal data distribution or as a median (lower – upper quartiles; Me (Q1-Q3)) in the case of skewed or non-normal data distribution. The t-Student's test for dependent variables or non-parametric Wilcoxon's test were used for data comparison. In the case of skewed data distribution, the logarithmic transformation was done before analysis. To assess the relationship between qualitative variables, the χ^2 test was used. Statistical significance was set at a $p < 0.05$ and all tests were two-tailed. Statistical analysis was performed using data analysis software system Statistica, version 13.3.0 (TIBCO Software Inc., USA).

3. Results

In our study groups we observed interesting statistical tendency. Hip arthritis was more frequent in male patients, while knee arthritis was more frequent in female patients, but those results were not statistically significant ($\chi 2 = 2.81$, $p = 0.093$, Table 1).

3.1. General Health Indicators. Biochemical and morphological characteristics of the blood of patients before and after the 21-day general rehabilitation program are presented in Table 2. Our results show that the individually designed general rehabilitation had positive effects on the patients' blood glucose and lipids concentrations: glucose, total cholesterol, LDL, and TG were significantly lower and HDL levels were significantly higher when compared to their initial levels. Also, we observed that CRP, platelets and haematocrit were lower after the rehabilitation what proves that general rehabilitation helps to reduce inflammation and prevents from clot formation.

3.2. Body Mass. The 21-day general rehabilitation program did not influence the patients' body mass. Their body mass in the pre-rehabilitation period was 86.1 (± 11.7) kg on average and was the same as in the post-rehabilitation period (84.4 ± 11.6 kg) ($p = 0.546$; Table 3).

3.3. Six-Minute Walk Test (6MWT). The 21-day general rehabilitation program improved patient's walking capacity by 69 m. The average distance walked by patients before rehabilitation program was 428.9 (± 46.8) m, whereas after rehabilitation they were able to cover the distance of 497.8

Table 2: Comparison of blood parameters of the patients with hip or knee endoprosthesis before and after a 21-day general rehabilitation program. The results (n =41) are presented as median (lower-upper quartile) or mean ± SD. Statistical significance was set at $p < 0.05$.

Parameter	Before	After	Δ = before-after	t	p
CRP (mg/l)	1.8 (1.3-2.3)	1.5 (1.1-2.0)	0.2 (0.1-0.3)	3.59	**<0.001**
Glucose (mmol/l)	5.2 ± 0.6	4.9 ± 0.6	0.2 ± 0.3	5.72	**<0.001**
ESR (mm/l)	9.0 (7.0-13.0)	9.0 (7.0-11.0)	—	1.87	0.069
Serum creatinine (μmol/l)	80.2 ± 10.3	81.9 ± 9.5	—	1.64	0.109
Platelets (K/μl)	226.5 ± 53.2	217.4 ± 35.1	9.1 ± 26.3	2.15	**< 0.05**
Haematocrit (%)	41.3 ± 3.3	40.2 ± 2.7	1.1 ± 2.0	3.37	**< 0.01**
RBC (M/μl)	4.6 ± 0.4	4.6 ± 0.4	—	0.06	0.953
HGB (g/dl)	13.7 ± 1.1	13.8 ± 0.9	—	1.83	0.074
WBC (K/μl)	6.7 ± 1.6	6.9 ± 1.2	—	1.22	0.230
Cholesterol (mg/dl)	230.6 ± 34.1	215.8 ± 29.9	14.9 ± 18.7	5.09	**<0.001**
HDL (mg/dl)	39.3 ± 4.1	40.6 ± 3.5	-1.3 ± 1.9	4.36	**<0.001**
TG (mg/dl)	154.9 (143.8-167.8)	145.9 (135.4-154.3)	11.6 (4.5-21.8)	3.05	**< 0.01**
LDL (mg/dl)	159.4 ± 31.5	145.8 ± 27.6	13.7 ± 18.7	4.66	**<0.001**

Abbreviations: CRP – C-reactive protein; ESR – erythrocyte sedimentation rate; HDL – high-density lipoprotein cholesterol; HGB – haemoglobin; LDL – low-density lipoprotein cholesterol; RBC – red blood cells; WBC – white blood cells; TG – triglycerides.

Table 3: Results of 6MWT and body mass of the patients (n =41) with hip or knee endoprosthesis before and after a 21-day general rehabilitation program. Statistical significance was set at $p < 0.05$.

General health indicator	Before	After	Δ = after-before	t	p
Body mass (kg)	86.1 ± 11.7	84.4 ± 11.6		0.607	0.546
6MWT (m)	428.9 ± 46.8	497.8 ± 54.2	68.9 ± 27.8	14.866	**<0.001**

Abbreviations: 6MWT – the 6-minute walk test.

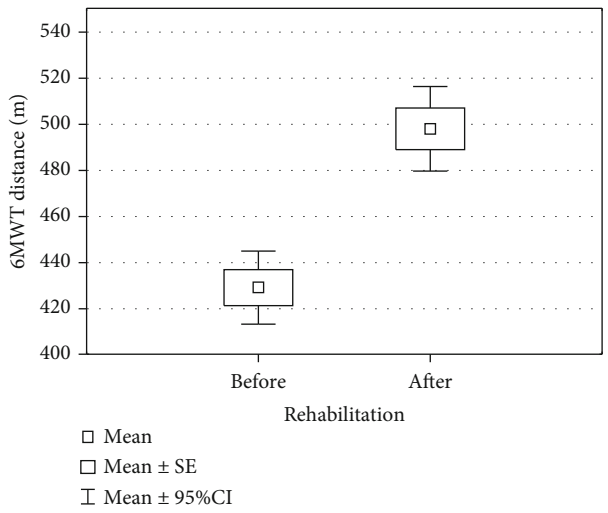

Figure 1: Results of the 6-minute walk test distance (6MWT) (m) of the patients (n =41) with hip or knee endoprosthesis before and after a 21-day general rehabilitation program. Symbols used in the plot: inner box – mean value, outer box – mean value ± standard error, whiskers – mean value ±95% confidence interval.

(± 54.2) m. The increase in the results of 6MWT was statistically significant (p <0.001) (Table 3, Figure 1).

3.4. Oxidative Stress Markers. Since five patients were excluded from the study due to concomitant health conditions, the results of oxidative stress markers were obtained for 36 patients: 18 men and 18 women, aged 58.8 ± 8 years. 24 of them were after total hip (66.6%) and 12 of them were after knee (33.3%) replacement surgery performed 89 ± 2 days earlier.

We observed an increase in total antioxidant capacity (TAC) by about 0.20 ± 0.14 mmol/l (95% PU: 0.15-0.25) (p <0.001; Table 4; Figure S1 in Supplementary Materials) and in the activities analyzed of superoxide dismutase (SOD) isoforms. Total SOD activity was on average 1.6 ±1.7 NU/ml (95% PU: 1.1-2.2) (p <0.001; Table 4; Figure S2 in Supplementary Materials) higher and, similarly, CuZnSOD activity was on average 1.7 ±1.5 NU/ml (95% PU: 1.2-2.2) (p <0.001; Table 4; Figure S3 in Supplementary Materials) higher in the serum of patients after 21-day rehabilitation program when compared to their activities before starting the program. On the contrary, a 21-day general rehabilitation reduced ceruloplasmin (Cp) levels in patients' serum by 1.8 (0.7-3.7) mg/dl ($p < 0.001$; Table 4; Figure S4 in Supplementary Materials).

We observed significant decrease, by 19.6 ± 24.4 μmol/l (95% PU: 11.4-27.9), in MDA concentration in blood of patients after a 21-day rehabilitation program (p <0.001; Table 4; Figure S5 in Supplementary Materials). A similar effect of the rehabilitation program we observed for LPS concentration in the blood of patients after hip or knee replacement: its concentration in the post-rehabilitation period decreased by 0.4 ± 0.5 RF units (95%PU: 0.2-0.5)

Table 4: Comparison of oxidative stress markers measured in the serum of patients ($n = 36$) with hip or knee endoprosthesis before and after a 21-day general rehabilitation program. Statistical significance was set at $p < 0.05$.

Oxidative stress marker	Before	After	Δ = before - after	t	p
TAC (mmol/l)	1.07 ± 0.15	1.27 ± 0.17	-0.20 ± 0.14	8.427	**<0.001**
SOD (NU/ml)	10.1 ± 2.1	11.7 ± 2.1	-1.6 ±1.7	5.760	**<0.001**
CuZnSOD (NU/ml)	7.25 ±1.79	8.97 ±1.70	-1.72 ±1.50	6.859	**<0.001**
Cp (mg/dl)	27.5 (24.2-31.8)	25.4 (22.0-29.6)	1.8 (0.7-3.7)	4.813	**<0.001**
MDA (μmol/l)	152.5 ± 27.9	132.9 ± 24.4	19.6 ± 24.4	4.823	**<0.001**
LPS (RF)	1.6 ± 0.4	1.3 ± 0.3	0.4 ± 0.5	4.14	**<0.001**

Abbreviations: Cp – ceruloplasmin; CuZnSOD – copper-zinc SOD; LPS (RF) – lipofuscin (radiofrequency); MDA – malondialdehyde; SOD – total superoxide dismutase; TAC – total antioxidative capacity.

when compared to the period before rehabilitation ($p < 0.001$; Table 4, Figure S6 in Supplementary Materials).

4. Discussion

In this study, we assessed the impact of postoperative rehabilitation on antioxidant stress markers in patients after hip or knee replacement surgery. We analyzed the total antioxidant capacity (TAC), the activity of total superoxide dismutase (SOD), Zn-Cu-superoxide dismutase (ZnCuSOD) and ceruloplasmin (Cp) concentration, as well as the concentration of malondialdehyde (MDA) and lipofuscin (LPS) – the oxidative stress markers – in patients subjected to general rehabilitation after knee or hip endoprosthesis implantation. To the best of our knowledge, this is the first study designed to compare the oxidative stress markers before and after the general rehabilitation cycle. Here, we report a significant increase in TAC level, SOD and ZnCuSOD activities and simultaneous reduction in Cp, LPS and MDA plasma levels after the 21-day general rehabilitation program. The physical rehabilitation not only improved all oxidative stress parameters but also positively affected patients' general health, as indicated by glucose and lipids profiles and inflammation and blood clotting parameters. It also significantly improved patients' physical efficiency and exercise capacity determined by the 6-minute walk test (6MWT). Although the walked distance in 6MWT has increased after the rehabilitation process, the patients' body mass remained the same, as expected.

4.1. Total Antioxidant Capacity (TAC). Osteoarthritis affects patients' antioxidative and oxidative metabolism by significantly impairing antioxidant defense [28]. A decrease in total antioxidant capacity results from enhanced oxidative stress, making patient's body more susceptible to damage caused by the increased amount of reactive oxygen species [29].

We found that the 21-day rehabilitation program conducted after the endoprosthesis implantation surgery significantly enhanced total antioxidant capacity. Our results coincide with those obtained by Porter et al. [30] analyzing the effects of long-term aerobic training on TAC of the human body [30]. The study design analysis showed that the type of physical exercises from this study corresponds to exercises included in our rehabilitation program. Therefore our results support findings of others, that, not intense but rather long and regular physical effort contributes to the improvement of redox balance [7, 31]. Stanek et al. [32] observed a significant increase in TAC in patients with ankylosing spondylitis that were subjected to a series of whole-body cryotherapy followed by a subsequent kinesiotherapy. In this case, the combination of whole-body cryotherapy and kinesiotherapy was more effective that the kinesiotherapy itself. The authors concluded that their approach to ankylosing spondylitis treatment helped to decrease the oxidative stress ocurring during the active phase of this chronic inflammatory rheumatic disease [32].

4.2. Total Superoxide Dismutase (SOD) and cu-Zn Superoxide Dismutase (CuZnSOD). In our study, 21-day general rehabilitation program resulted in increased superoxide dismutase (SOD) and its Cu-Zn isoform (CuZnSOD) activity. According to two independent studies, by Scott et al. [33] and Olszewska-Słonina et al. [34], total SOD activity significantly depletes in patients with osteoarthritis. The latter study, by Olszewska-Słonina et al. [34], proved positive impact of endoprosthesis surgery on total SOD plasma concentration, but the influence of postoperative rehabilitation or exercise was not considered in this study [34]. It was, however, the subject of numerous studies showing that SOD activity in skeletal muscles increases after the exercise [35–38]. Paans et al. [31] observed that the increase in total SOD activity is much more significant in terms of duration than the intensity of physical training, what was supported by Ha et al. [7]. This research, on hatha yoga effect on oxidative stress, showed that total SOD activity increased better in response to exercise duration rather than to exercise intensity [7]. All of the above-mentioned studies contain exercise element that agrees with our study design and the rehabilitation protocol used by us.

Our results show that the implementation of the rehabilitation program shortly after the surgery might lead to increase of total SOD activity. Due to the methodology of our research, we had limited insight into the postoperative period only, not into the dynamics of the change. Nevertheless, the 21-day general rehabilitation, tailored to the patient's abilities, designed and controlled by a physiotherapist, resulted in a significant increase both in total SOD and CuZnSOD activity, what was compatible with results obtained by other research groups.

4.3. Ceruloplasmin (Cp). In this study, we analyzed the ceruloplasmin concentration as an oxidative stress marker in patients who underwent endoprosthesis surgery and postoperative rehabilitation due to osteoarthritis. According to El-Barbary et al. [39], high levels of oxidative stress occurring in the course of rheumatoid arthritis and osteoarthritis may lead to observed elevated plasma Cp concentration [39]. On the contrary, Kudriavtseva et al. [40] reported a decrease in Cp levels in the course of osteoarthritis, but this could have resulted from extremely high oxidative stress caused by severe tissue damage, having lead even to the depletion of the selected antioxidant systems. In this case, the inefficient redox system could not match Cp production, which manifested as a decrease in its level [40]. Our results did not indicate the occurrence of a similar phenomenon in any of the patients. Therefore, the decreased level of Cp after the 21-day rehabilitation program may be a good indicator of oxidative stress reduction after the rehabilitation process. By looking at the changes in ceruloplasmin levels, we argue that rehabilitation has a positive impact on patient body redox status.

4.4. Malondialdehyde (MDA). During exacerbated physical exercises, oxygen uptake and energy demand increase rapidly, which leads to the intensification of mitochondrial energy metabolism and, as a result, promotes free radicals formation which, if not scavenged effectively by SOD, cause lipid peroxidation and its by-product, malondialdehyde (MDA), formation. One of the main mechanisms of osteoarthritis pathogenesis is oxidative stress promoted by enhanced free radical production and therefore, not surprisingly, increased MDA levels are observed in the serum of the patients suffering from this condition [12, 13]. Many studies have shown that high-intensity exercise increases oxidative stress and intensifies lipid peroxidation processes [41–43], which is mainly the result of muscle damage and fatigue induced by exercise [43] However, the effect of physical activity on lipid peroxidation intensity is not completely known as it may be affected by many variables such as exercise type, intensity and duration [44, 45]. According to Dixon et al. [46], it is the intensity of resistance exercise bout that plays the main role in oxidative stress generation [46]. Also, it was found that MDA concentration, a by-product of the polyunsaturated fatty acid oxidation, increased during aerobic exercise [42, 43, 47]. On the other hand, it was proved that long-term participation in exercise can strengthen antioxidant defense, change beneficially redox status and reduce lipid peroxidation intensity and therefore their products formation [48–50]. Ha et al. [7] proved that 16 weeks of hatha yoga training contributed to a statistically significant reduction in MDA concentration in blood serum [7]. Moreover, Vincent et al. [50] showed that six months of low-intensity resistance exercise training, following an acute bout of aerobic exercise, lowered levels of lipid peroxidation in the serum of studied patients [50]. Stanek et al. [51] reported that decrease in antioxidant status played an important role in the pathogenesis of ankylosing spondylitis [51].

This may be due to a fact, that long-term resistance training causes qualitative and quantitative changes in respiration processes of skeletal muscle mitochondria, increasing thus antioxidant capacity [30].

In our study, we observed that MDA, the lipid peroxidation by-product, levels dropped down significantly in convalescents after the 21-day general rehabilitation. We conclude that this is the effect of the study design: the patients underwent regular, daily low-intensity physical exercises, without prolonged or acute bouts of exercise, that were individually tailored to their abilities. This might have changed the respiration processes in skeletal muscles at the mitochondrial level, as discussed above, and have increased patients' antioxidant capacity, manifested also by elevated TAC levels we observed in our study.

4.5. Lipofuscin (LPS). Lipofuscin is a product of the unsaturated fatty acids oxidation and its formation is directly related to an increased concentration of oxidative stress markers. Free oxygen radicals damage mitochondria and other cellular organelles, leading eventually to the formation of non-biodegradable lipofuscin that is stored in lysosomes, hindering their phagocytic activity and ability to degrade not-efficient and damaged mitochondria. This phenomenon is inevitably associated with the aging process [52–54]. To the best of our knowledge, there are barely any studies linking lipofuscin concentration and osteoarthritis in humans. Moreover, this subject was never analyzed in the context of lipofuscin serum level and its changes after rehabilitation. In our study, lipofuscin concentration significantly decreased after the 21-day general rehabilitation program. We assume that initially high lipofuscin concentration was associated with high oxidative stress resulting both from previous joint damage and recent surgery. The rehabilitation program had a positive effect on reducing oxidative stress in our patients and contributed to the subsequent reduction of lipofuscin concentration, as expected from literature analysis [55].

5. Conclusions

We can conclude that the 21-day postoperative general rehabilitation program has a significant impact on balancing oxidative processes and significant reduction of oxidative stress markers in patients with hip or knee replacement. Individually tailored, systematic physical effort is a crucial element of the postoperative protocol, which helps patients to recover effectively after the surgery by improving the redox balance.

Data Availability

The original data are available after contact with the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Conceptualization, B.S.P., D.S., J.P.; Methodology, D.S., B.S.P., E.C.; Software, E.C.; Validation, D.S. and M.I.; Formal Analysis, D.S., E.R., E.C.; Investigation, B.S.P., D.S., J.P., J.J.,

M.I., A.D., W.K.; Resources, B.S.P., J.P.; Data Curation, D.S., E.C.; Writing – Original Draft Preparation, D.S., A.D., W.K.; Writing – Review & Editing, D.S., B.S.P., J.J., M.I.; Visualization, D.S.; Supervision, D.S., B.S.P.; Project Administration, B.S.P..; Funding Acquisition, B.S.P.

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Supplementary Materials

This article is accompnied by Supplementary Materials which contain Figures with visualized data of oxidative stress markers levels in the serum of patients (n = 36) with hip or knee endoprosthesis before and after a 21-day general rehabilitation program. *(Supplementary Materials)*

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Review Article

Role of the Platelets and Nitric Oxide Biotransformation in Ischemic Stroke: A Translative Review from Bench to Bedside

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Ischemic stroke remains the fifth cause of death, as reported worldwide annually. Endothelial dysfunction (ED) manifesting with lower nitric oxide (NO) bioavailability leads to increased vascular tone, inflammation, and platelet activation and remains among the major contributors to cardiovascular diseases (CVD). Moreover, temporal fluctuations in the NO bioavailability during ischemic stroke point to its key role in the cerebral blood flow (CBF) regulation, and some data suggest that they may be responsible for the maintenance of CBF within the ischemic penumbra in order to reduce infarct size. Several years ago, the inhibitory role of the platelet NO production on a thrombus formation has been discovered, which initiated the era of extensive studies on the platelet-derived nitric oxide (PDNO) as a platelet negative feedback regulator. Very recently, Radziwon-Balicka et al. discovered two subpopulations of human platelets, based on the expression of the endothelial nitric oxide synthase (eNOS-positive or eNOS-negative platelets, respectively). The e-NOS-negative ones fail to produce NO, which attenuates their cyclic guanosine monophosphate (cGMP) signaling pathway and—as result—promotes adhesion and aggregation while the e-NOS-positive ones limit thrombus formation. Asymmetric dimethylarginine (ADMA), a competitive NOS inhibitor, is an independent cardiovascular risk factor, and its expression alongside with the enzymes responsible for its synthesis and degradation was recently shown also in platelets. Overproduction of ADMA in this compartment may increase platelet activation and cause endothelial damage, additionally to that induced by its plasma pool. All the recent discoveries of diverse eNOS expression in platelets and its role in regulation of thrombus formation together with studies on the NOS inhibitors have opened a new chapter in translational medicine investigating the onset of acute cardiovascular events of ischemic origin. This translative review briefly summarizes the role of platelets and NO biotransformation in the pathogenesis and clinical course of ischemic stroke.

1. Ischemic Stroke: Its Burden and Classification

Cardiovascular disease (CVD) remains the main cause of morbidity and mortality, as reported worldwide annually. In spite of constant progress in diagnostic and therapeutic strategies, according to the recent data, there were estimated 72.72 million cases of CVD and 17.8 million CVD deaths in the world population. Stroke was the fifth cause of death globally with the morbidity reaching approximately 7.750 million and mortality 2.750 million in 2017 [1]. Ischemic stroke is the most common type of acute cerebrovascular event, responsible for 81% of all the stroke cases [2]. The thromboembolic event is a common denominator of all the subtypes of ischemic stroke. Large artery atherosclerosis (LAA) is the causative event in 17-34% of ischemic strokes and is characterized by activation of platelets along with thrombus formation on atherosclerotic plaque in extra/intracranial arteries (ruptured atherosclerotic plaque accompanied with a cascade of thromboinflammation). Small vessel occlusion/lacunar stroke (SVO) is diagnosed in 20.5-29.0% of cases, and it proceeds from lipohyalinosis (vessel wall thickening induced to the greatest extent by hypertension). Further ischemic stroke subtypes include cardioembolic

(16-25.6%) which is predominantly generated by the atrial fibrillation (AF), then a stroke of unusual/other etiology (1.7-6%) and of unknown/undetermined etiology (14.2-29%) [3–6] (Figure 1).

Each of the noncardioembolic stroke subtypes is characterized by partially different pathophysiology, recurrence rate, magnitude of positive response to antiplatelet therapy, and survival rate (being relatively better for SVO than LAA strokes) [5, 7, 8]. Despite of the heterogeneous origin of particular subtypes of ischemic stroke, there are some uniform/-common mechanisms, mostly related to increased activation of platelet-derived hemostasis. Hence, some common therapeutic strategies may reveal to be effective both in the treatment of an acute phase and in the primary and secondary stroke prevention.

2. Characteristics of the Cerebral Vascular Bed and Pathophysiology of Cerebral Ischemia-Reperfusion Injury

Cerebral arteries with their curvatures and bifurcations are characterized by a plaque-prone development anatomy. Contrary to the coronaries, carotid and cerebral vessels are subjected to high shear stress, which protects from atherosclerotic plaque enlargement but on the other hand also predisposes to intraplaque hemorrhage and plaque rupture [9]. Nevertheless, when hypercholesterolemia appears, endothelial dysfunction is promoted, limiting the positive action of physiological shear stress, and plaque formation is observed [10]. Some data suggest that low shear stress may change the expression of genes for inflammatory proteins leading to the origin of atherosclerosis-related inflammation [11, 12]. At high shear flow rates, as found in carotid/cerebral arteries or moderately stenosed vessels, the initial capture of circulating platelets to the endothelium is mediated by the von Willebrand factor (vWF) at the vascular wall without other stimulating factors [13]. It is suggested that the plasma level of vWF—to some extent—is a marker of endothelial cell damage and it predicts the onset and progression of atherosclerotic lesions in patients with hypertension. Hypotensive therapy, by non-drug-specific reducing endothelial damage and vWF expression, contributes to inhibition of both thrombus and atherosclerosis formation pointing thus at its protective role in the primary and secondary prevention of ischemic stroke [14].

3. Platelets as the Common Denominator of the Acute Ischemic Events and Pleiotropic Drug Target

Several years ago, Htun et al. have shown that patients with ischemic stroke or transient ischemic attack (TIA) were characterized by significantly increased P-selectin (CD62P) expression in platelets and circulating platelet-leukocyte aggregate concentration. Interestingly, other authors discovered that patients with the LAA infarction elicit higher platelet-leukocyte aggregate formation, when compared with the SVO group [15–17]. Differences in the CD62P concentration between stroke patients and controls returned to normal after 90 days of observation or gradually with implementation of antiplatelet treatment (stronger correlation with clopidogrel than with acetylsalicylic acid (ASA), but no association with warfarin treatment). Noteworthily, such treatment had no effect on normalization of the circulating platelet-leukocyte aggregate level in those patients [18, 19]. Initialization of thromboinflammation in ischemic stroke can be explained by the elevated platelet expression of the CD40 ligand (CD40L) in activated platelets, which, by triggering the expression of adhesive molecules, such as P-selectin, E-selectin, and ICAM-1, leads to formation of platelet-leukocyte aggregates [20, 21]. Moreover, Ishikawa et al. observed that, after induction of middle cerebral artery occlusion (MCAO) in the CD40-deficient rats, impaired platelet and leukocyte adhesion occurred leading to smaller brain infarct size in comparison to the control group [22]. Additionally, Jiang et al. demonstrated similar results in male rats treated with CD40 antagonist infusion before reperfusion of the occluded middle cerebral artery [23]. In human studies, high concentration of CD40 is associated with poor outcome at 3 months after ischemic stroke [15]. Moreover, leukocytes (especially regulatory T lymphocytes) have significant function in thromboinflammation during ischemic stroke by promoting ICAM-1 expression on platelets and endothelia which facilitates adhesion of granulocytes and platelets to the vessel wall [24, 25]. Hyperaggregable leukocytes, monocytes, and endothelia tend to activate platelets by platelet-activating factor secretion in the time of cerebral ischemia [26]. The PAF function is not only to activate adhesion of platelets and leukocytes (mostly neutrophils) to the damaged endothelium, but it also causes tissue edema through the increase of the vascular permeability in the peripheral tissues, increases secretion of granule-based

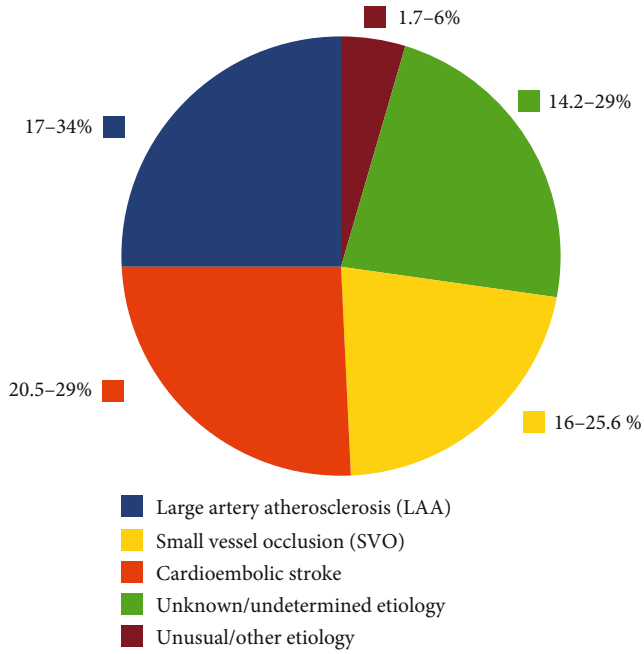

Figure 1: Incidence of each stroke subtype.

enzymes in platelets, and enhances superoxide and arachidonate metabolism in neutrophils generating neurotoxicity leading to brain damage after ischemic stroke [27, 28].

Platelet secretion of thromboxane A2, adenosine diphosphate (ADP), matrix metalloproteinase-9 (MMP-9), and other platelet-derived soluble mediators promote thrombus formation in a positive feedback loop [29]. ADP is one of the most prevalent platelet activators under physiological condition. It also plays a significant role in cardiovascular disease development. Puurunen et al. in the prospective study of Framingham population identified platelet hyperreactivity to ADP to be associated with myocardial infarction and ischemic stroke incidence [30]. What is more, persistent elevation of platelet aggregation in response to ADP at three months after ischemic stroke is connected with more than threefold increased recurrence of stroke. Interestingly, cross-incubation of control platelets with plasma from stroke patients resulted in activation of platelets measured by the raised basal platelet calcium level and release of serotonin from platelets. These results, accompanied with the study by Dougherty et al. suggesting that ASA and dipyridamole treatment have no effect on platelet hyperreactivity to ADP, suggest that the lower threshold of platelet activation in ischemic stroke patients may be predominantly associated with the presence of plasmatic factors rather than with platelet functional disturbances [19, 31]. Recently, an increasing number of studies suggest that nitric oxide deficiency and nitric oxide synthase inhibitors can be one of the factors responsible for greater platelet aggregation in ischemic stroke patients.

4. The Role of Nitric Oxide Synthase and of Nitric Oxide in Ischemic Stroke

Endothelial vasodilative dysfunction, identified by decreased NO bioavailability, is a well-known risk factor for ischemic stroke. Changes in the nitric oxide concentration during the course of cerebral infarction can also be used as an important prognostic tool for ischemic stroke outcome. To date, three major isoforms of the NOS are described in the literature: neuronal constructive (nNOS), inducible (iNOS) and endothelial constitutive (eNOS). Each catalyzes the reaction of NO production, and in the catalytic cycle, the Fe^{3+}+NO complex is the final intermediate from which in normal circumstances NO easily dissociates [32, 33]. However, nitric oxide overproduction autoinhibits the catalytic site of the NOS by reduction of iron to the stable Fe^{2+}+NO complex [34]. In the presence of oxygen, the enzymatic inactive Fe^{2+}+NO bond generates nitrate (reactive nitrogen species) and ferric ion, making the catalytic site of NOS again available for NO production. The described above oxygen dependency of NOS action plays a crucial negative role in ischemia and hypoperfusion [35]. Nitric oxide synthase produces not only NO and nitrates but also reactive oxygen species. Comparing to inducible NOS, eNOS and nNOS are responsible for higher production of superoxide, which is considered to be involved in atherosclerosis and recruitment of additional platelets to the sites of injury. On the other hand, iNOS and nNOS are more inclined than endothelial NOS to producing reactive nitrogen species (RNS) in which the undesirable role is to destabilize structure and function of proteins, leading to impaired catalytic activity of enzymes and even to cell apoptosis [36, 37].

After induction of middle cerebral artery occlusion, increased NO plasma concentration is observed for up to 30 minutes with its subsequent reduction in the following hours [38, 39]. After a gradual decrease, the level of NO and peroxynitrite (especially after reperfusion) increased again after 4 hours, reaching a maximum at 46 hours and lasting for up to seven days [40, 41]. The described above fluctuation of nitric oxide concentration is probably associated with different NOS subtype activities. The activity of eNOS and nNOS increases at the same time as nitric oxide concentration within the first minutes after induction of MCAO and significantly reduces thereafter [39]. The expression of iNOS is detected in the brain at 12-70 hours following cerebral ischemia and lasts up to 7 days, while the brain myeloperoxidase activity (a marker of neutrophil infiltration) is observed only after 4 hours, significantly increases at 22 h, and then decreases. These observations suggest that the initial increased level of NO after ischemia is connected with endothelial and neuronal nitric oxide production. While NO production by eNOS and nNOS slowly decreases, brain infiltration by neutrophils and their NO production by iNOS are responsible for the fluctuation of the NO bioavailability after ischemic stroke [40–42] Figure 2.

Dobrucki et al. observed lower concentration of NO before induction of ischemic stroke in spontaneously hypertensive rats (SHR) and higher concentration of O^{2-} release (connected with higher peroxynitrite production) after induction of middle cerebral artery occlusion leading to larger infarct size in SHR as compared to the control group [43]. Serrano-Ponz et al. found similar results in human studies. Those authors identified an increase in nitric oxide metabolites from day 1 to day 2 to be beneficial for the ischemic stroke patients as measured by the National Institutes of Health Stroke Scale (NIHSS) at day 7 and at 3 months and measured by the modified Rankin Scale at 3 months, while a steep increase of nitric oxide metabolite concentration from day 2 to day 7 was associated with a multiple increase in infarct volume [44]. According to Taffi et al., the high nitric oxide plasma level 30 days after cerebral infarct is associated with poor outcome in nonlacunar stroke, since a 10-unit increase in NO concentration predicts a 1-point reduction in the NIHSS score. Better outcome in patients with lacunar stroke is probably connected with higher concentration of NO in the first 24 hours after cerebral infarction and lower concentration of peroxynitrite [45].

At the molecular level and in animal model-based studies, during the first few hours after cerebral ischemia, nitric oxide production by eNOS is improving cerebral blood flow (CBF) within the ischemic penumbra (area of brain tissue surrounding the infarct that is at risk of infarction) in order to reduce infarct size and volume [46, 47]. It is documented that both eNOS-deficient mice and administration of eNOS inhibitors to rats provoke a decrease in absolute CBF in animals (up to 25–35% of the control level) [48, 49]. The activity of nNOS throughout 2 hours after reperfusion of MCAO is

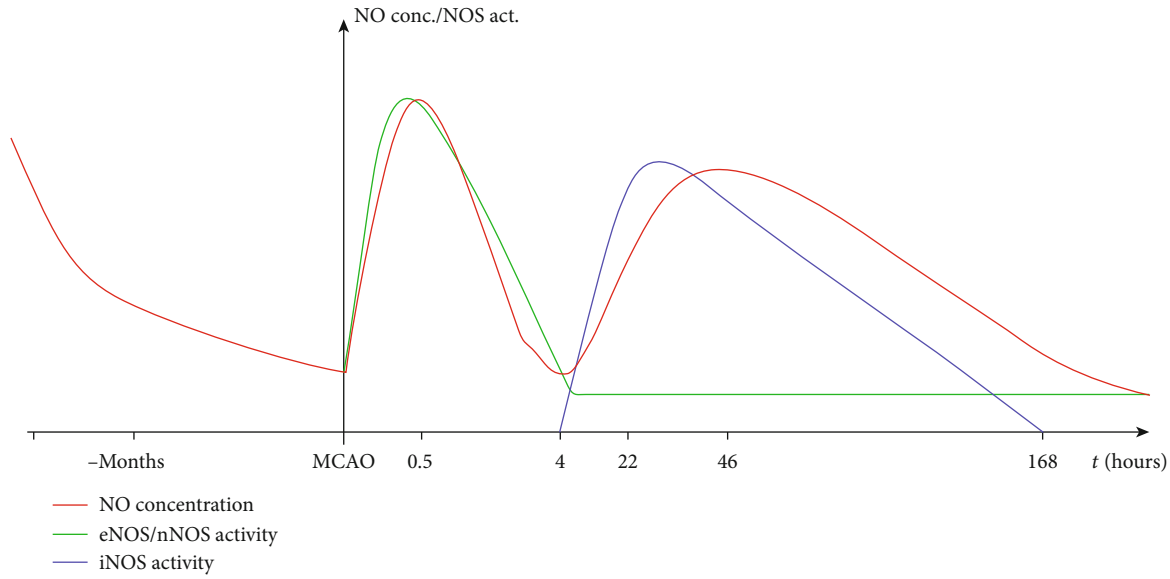

Figure 2: Concentration of nitric oxide and activation of NOS isoforms during the course of ischemic stroke.

also enhanced. However, in nNOS-deficient mice, CBF is significantly higher after reperfusion, which suggests the adverse effect of nNOS activation during the course of ischemic stroke [50]. Zeng et al. found that hypoxic or ischemic brain injury during early reperfusion is associated with the generation of NO from nNOS which activates the early c-Jun N-terminal kinase 1/2—a signaling pathway involved in neuronal death [51]. Stagliano et al. proved that immediate administration of a specific inhibitor for nNOS (3-bromo-7-nitroindazole) after induction of common carotid artery thrombosis in rats accelerated sensorimotor recovery [52]. While some of the authors postulate that nonspecific inhibitors of eNOS and nNOS (L-N^G-nitroarginine methyl ester (L-NAME)) reduce infarct size [53], others suggest that its biological function is partly dependent on simultaneous fluctuating N-methyl-D-aspartate (NMDA) concentration. NMDA in normal conditions is an activating neurotransmitter, but during ischemia, it is liberated from damaged neurons and has further neurotoxic activity. Globus et al. observed that brain lesions induced by NMDA was not affected by L-NAME administration. The reason for such a correlation is not fully understood. Activation of the NMDA receptor by neurotransmitters released from damaged neurons in the ischemic or penumbral area leads to NO overproduction and an increase in CBF in order to support the enhanced metabolic demand of the excited neurons. On the one hand, NO plays a role in the intracellular cascade of events leading to cell death following NMDA receptor activation; on the other hand, NO ensures adequate blood supply especially to the penumbral area. Probably, the final outcome of nitric oxide influence depends on the balance between these two processes [54, 55].

Nitric oxide produced by iNOS in the microglia (brain-based macrophages) may also lead to neuronal damage associated with the neurotoxicity mediated by NMDA receptors [56]. However, the main source of NO from iNOS during ischemic stroke originates from neutrophils. As mentioned before, infiltration of brain tissue by those phagocytes increases gradually during the first few days in the course of cerebral ischemia and reperfusion. Nitric oxide derived from neutrophils' iNOS is used in the inflammation process by peroxynitrite formation and in stimulation of neural apoptosis [57]. Garcia-Bonilla et al. discovered that after MCAO, iNOS-deficient mice engrafted with iNOS-positive bone marrow cells exhibited larger infarcts compared to iNOS-deficient mice autotransplanted with iNOS-deficient blood marrow cells. This study confirms that leukocytes play a significant role in the neuronal damage in ischemic stroke patients [58].

5. Pharmacological Approach: Nitric Oxide Donors and NOS Inhibitors

Some authors suggested that antiplatelet drugs affect nitric oxide biotransformation. According to Serebruany et al., modified-release dipyridamole and aspirin similarly increased primary diminished plasma eNOS activity in post-stroke patients in comparison to the control group [59]. In Gelosa et al.'s study, ticagrelor given in the early phase after permanent MCAO in rats significantly attenuated chemotaxis of leukocytes and reduced expression of iNOS [60]. Zhao et al. showed no association between acetylsalicylic acid (ASA), clopidogrel, or dipyridamole administration and NO metabolites together with cyclic guanosine monophosphate (cGMP) levels in patients with prior ischemic stroke and in the control group [61].

Each NOS subtype plays different roles during ischemic stroke which is demonstrated in diverse effects observed by use of particular NOS inhibitors. Pretreatment with statins or Rho-kinase inhibitors improve cerebral blood flow in the ischemic area and penumbra, decrease cerebral infarct volume, and improve neurological function after MCAO by increasing eNOS activation.in mice [62, 63] Nevertheless, inhibition of eNOS by L-N-(1-iminoethyl)ornithine (L-NIO) before MCAO elevates iNOS expression and exacerbates brain damage [64]. Aminoguanidine (iNOS inhibitor)

administration at 6 and 12 h after reperfusion in mice reduces NO concentration only in the penumbral region and lessens infarct size. Different iNOS inhibitors were studied by Armengou et al., in which N-(3-(aminomethyl)benzyl) acetamidine (1400W) administrated at the onset of ischemia and at 8-hour intervals for 3 days after MCAO resulted in a 55% reduction of infarct size, as measured 72 hours after induction of cerebral ischemia [65]. The protective role of iNOS inhibition in the thromboinflammation process is the most probably connected with decreased leukocyte activity. Matsuo et al. confirmed the essential role of neutrophils in ischemic stroke by documenting smaller infarct size in the neutropenic animals after reperfusion [66]. From a practical clinical perspective, edaravone, which directly enhances NO production, is recommended by Japanese guidelines for neuroprotection in ischemic stroke patients within 24 hours of onset [67]. This drug is commonly used in amyotrophic lateral sclerosis and exerts also a neuroprotective effect in reperfusion injury by reducing levels of superoxide increasing NO production and decreasing nNOS expression in cerebral neurons [68]. According to the Acute Infarction Study Group, administration of this substance < 72 h after ischemic stroke and through 14 days was connected with significant improvement in functional outcome evaluated by the modified Rankin Scale [69]. What is more, in Feng et al.'s systemic review on the edaravone influence on patients with acute ischemic stroke, the use of the drug was associated with neurological improvement in the intervention group compared with the control group (RR = 1.99) [70].

The plasma level of L-arginine (a substrate for NOS) is decreased in patients following the ischemic stroke and subsequently rises between 6 and 24 hours after the event. According to Armengou et al., plasma L-arginine concentrations are negatively correlated with the infarct volume and are significantly lower in patients with early neurologic deterioration as well as in those with poor outcome [65]. In Morikawa et al.'s study, administration of L-arginine 5 minutes after MCAO reduced infarct volume in rats measured 24 hours after vessel occlusion [47]. Lower levels of free radical production leading to smaller infarct size were also documented in Mason et al.'s and Zhao et al.'s animal studies of direct nitric oxide donor (diethanolamine nitric oxide and ZJM-289 novel NO-releasing derivative of 3-n-butylphthalide) administration during reperfusion [71, 72]. Although in human studies the transdermal glyceryl trinitrate (GTN) administration < 48 h and <5 days after cerebral ischemia did not improve functional outcome for ischemic stroke patients, in Woodhouse et al.'s analysis, transdermal GTN was safe and correlated with better functional outcome and with fewer deaths when administered within 6 hours of stroke onset. Significant beneficial effects were also achieved in disability (Barthel Index), quality of life, cognition, and mood [73–75]. Willmot et al. in their meta-analysis of preclinical studies confirmed the time-dependent effect of NO donors and L-arginine administration, specifying that early treatment (within 60 minutes) of ischemia was associated with the highest outcomes in comparison to neutral ones in those studies assessing treatment up to 48 hours following induction of ischemic stroke [76].

In human cells, NOS converts L-arginine to L-citrulline with a concomitant synthesis of NO, while asymmetric dimethylarginine (ADMA) is the most potent competitive inhibitor of this reaction. ADMA could play a crucial role in the CVD development as its higher plasma concentrations are significantly associated with cardiovascular risk factors, such as intima-media thickness of the carotid artery [77], hypertension [78], and diabetes mellitus types 1 and 2 [79, 80]. What is more, Ercan et al. showed that the plasma ADMA level measured during the first 24 hours in the group of patients after acute ischemic stroke was significantly higher than that in the control group [81]. Petrova et al. documented earlier reduction of plasma ADMA concentration in stroke patients after thrombolysis in comparison to the no-reperfusion group [82]. On the other hand, only symmetric dimethylarginine (with no effect on NO production) was a predictor of mortality in patients after acute ischemic stroke during 7.4 years of follow-up, while no correlation for ADMA was noted [83]. It seems that the intracellular compartment could be more significant in pathophysiology of CVD than its plasma level. Masuda et al. found the endothelial concentration of ADMA to be up to 10-fold higher than in plasma, while Yokoro et al. showed that protein arginine N-methyltransferase 1 (PRMT1) and dimethylarginine dimethylaminohydrolase-1 (DDAH-1)—enzymes responsible for the biotransformation of ADMA—are expressed also in erythrocytes, leukocytes, and platelets. Those authors also suggested that the side effect of protein methylation (a protective mechanism against highly reactive oxygen-derived free radicals) can lead to ADMA overproduction, which in consequence lowers cellular NO production, can cause endothelial damage, and can increase platelet activation and aggregation [84–87]. However, to date, there is no study conducted analyzing the association between nitric oxide biotransformation (including ADMA, PRMT1, and DDAH-1) and human platelets of ischemic stroke patients.

6. Platelet-Derived Nitric Oxide (PDNO)

A vast majority of studies documented the association between decreased endothelial NOS expression and clinical disorders predisposing to stroke, such as diabetes mellitus, atherosclerosis, hypertension, and cigarette smoking in patients [88–91]. Radomski et al. were the first authors to describe the inhibitory role of platelets' NO production on a thrombus formation. It has been found that L-arginine administration increases platelet NO formation leading to cGMP synthesis and protein kinase G (PKG) activation in thrombocytes' cytosol which in consequence inhibits thrombus formation. The described NO/cGMP/PKG pathway's antiaggregatory properties in platelets depend on provoking Ca^{2+} sequestration and inhibiting platelet degranulation [92] by

(1) refilling intraplatelet Ca^{2+} stores by promoting sarcoplasmic reticulum adenosine triphosphatase (ATPase), decreasing intracellular Ca^{2+} levels, and inhibiting influx of Ca^{2+} [93]

(2) inhibition of the inositol 1,4,5-trisphosphate-stimulated Ca^{2+} release from the sarcoplasmic reticulum [94]

(3) attenuating the TxA_2 receptor function by its phosphorylation [95]

(4) phosphorylation of vasodilator-stimulated phosphoprotein (VASP), which enables VASP binding to the platelet cytoskeleton leading to inhibition of proaggregatory glycoprotein IIb/IIIa (GPIIb/IIIa) activation [96]

Without PKG action, cGMP also prevents platelet activation by inhibition of phosphodiesterase type 3 which increases intracellular cyclic adenosine monophosphate (cAMP; potent antiaggregation factor) [97] and inhibits phosphoinositide 3-kinase leading to GPIIb/IIIa fibrinogen receptor inactivation [98]. Nitric oxide blocks thrombus formation also on cGMP-independent mechanisms. The nitrosylation of the N-ethylmaleimide-sensitive factor inhibits aggregation by down-regulating alpha granule secretion and GPIIb/IIIa activation [99, 100]. What is more, irreversible nitration of platelet proteins by peroxynitrites results in inhibition of platelet adhesion to fibrinogen and decreased aggregation [101, 102].

Moreover, many other authors also showed that upon activation, platelets produce NO which inhibits adhesion and aggregation [92, 103–106]. Williams et al. demonstrated that high shear stress alone is sufficient to increase NO production in platelets leading to reduction of thrombus generation under blood flow. Moreover, authors described that reduction in thrombus formation (at a shear rate of $1000\,s^{-1}$) was abolished in the presence of L-NAME (the NO inhibitor), while at venous levels of shear rate ($100\,s^{-1}$), this substance had no effect on platelet activation and aggregation [107]. Cozzi et al. showed that platelet deposition is inversely related to platelet NO production and that intracytoplasmic Ca^{2+} elevation triggers platelet NO formation. Those results can suggest that the increase in intraplatelet Ca^{2+} concentration enhances the NO production which, in turn, limits thrombus size [108].

Some of the authors suggest that the generation of nitric oxide by resting platelets is constant (and is not elevated by L-arginine administration) [92, 103, 109]. Li et al. showed that basal production of NO by platelets activates cGMP-dependent protein kinase G (PKG) and enhances vWF-induced activation of platelets, which promotes rather than inhibits thrombus formation [110]. Those results can lead to the conclusion that platelet responses to NO and cGMP are both pro- and antiaggregative. However, in a study by Radziwon-Balicka et al., incubation with L-arginine inhibited platelet aggregation (by generation of NO) regardless of the platelet-activating stimulus concentration [111]. As a result from these studies, nitric oxide may serve as a platelet negative feedback regulator alone and only additional reaction of nitric oxide with superoxide anion can promote enhanced thrombus formation [112, 113].

7. Expression of the Nitric Oxide Synthase in Platelets

For many years, there was controversy whether platelets express their own nitric oxide synthase producing PDNO. Some of the authors suggested that contamination platelet samples by leukocytes account for suspected platelet NO production [114]. However, in a study on platelet subpopulations, Radziwon-Balicka et al. achieved high purity of their isolations (<2 leukocytes/100 000 platelets) and still detected significant nitric oxide production. Salvemini and colleagues showed that leukocyte contamination of >1% can inhibit aggregation via a NO-dependent mechanism, while in Radziwon-Balicka et al.'s study, leukocyte contamination was less than 0.002%. Finally, the authors concluded that this low leukocyte concentration in samples cannot account for detected NO production in the analyzed probes [112, 115–117].

In human megakaryocytes, both endothelial and inducible nitric oxide synthase isoforms are detected [118]. But whether platelets have the capacity to synthesize iNOS remains uncertain [119]. There is an interesting hypothesis that iNOS detected in platelet sample could derivate from leukocyte contamination. However, in Radziwon-Balicka et al.'s study, in the leukocyte-free probe samples (<2 leukocytes/100 000 platelets) the iNOS-selective antagonist 1400W was unable to reverse the antiaggregating effect of L-arginine. According to this study, the presence of iNOS in platelets is improbable [112].

Some authors also postulated that platelets do not contain eNOS and that this NOS isoform exists only in endothelial cells. However, Radziwon-Balicka et al. ultimately confirmed the presence of eNOS in triton-resistant platelet caveolae by a more specific identification method (fluorescence-activated cell sorting, while others used mass spectrometry) [112, 114, 120]. Both in animal and human studies, the endothelial nitric oxide synthase isoform is proved to produce PDNO [112, 121, 122]. Freedman et al. demonstrated that bleeding time was significantly decreased in eNOS-deficient versus wild-type mice. What is more, the bleeding time in thrombocytopenic eNOS-deficient mice transfused with eNOS-deficient platelets was significantly decreased compared with the same breed of mice transfused with wild-type platelets [121]. Moreover, Morrell et al. showed that the infusion of platelets from eNOS-deficient mice to animals with normal expression of eNOS resulted in increased granule exocytosis and stimulation of aggregation [99]. Riba et al. documented that vWF connection with platelet Gp Ib not only stimulates adhesion and aggregation but also activates platelet eNOS (measured by the increase in cGMP formation) with the presence of ADP and TxA_2. Interaction between collagen and platelet glycoprotein VI (GPVI) receptor activated platelet eNOS (with costimulation by ADP and TXA_2) only partially [123, 124]. Freedman et al. in a different study showed that inhibition of platelet eNOS increased P-selectin expression on the platelet surface after stimulation with ADP [106]. P-selectin is essential for leukocyte-platelet complex formation, and inhibition of platelet eNOS enhances the formation of those aggregates (especially monocyte-platelet aggregates) [125, 126].

8. Subpopulations of Platelets

Initially, the diversity among platelet size and density was attributed to the platelet aging processes. The large-dense platelets were identified as young thrombocytes recently released into the streaming blood, whereas the small and

low-density ones were postulated to represent older subpopulation. Nevertheless, studies by the Thompson et al. and Penington et al. have demonstrated that platelet size heterogeneity depends rather on platelet production from the different three ploidy classes of megakaryocytes (differ in their organelle content concentration) [127, 128]. Large-dense platelets contain a greater amount of glycogen, orthophosphate, and ADP and are characterized by upregulated glycolysis, glycogenolysis, and protein synthesis than small and low-density ones [129]. Large-dense thrombocytes aggregate more (due to higher ADP release and lower ADPase activity), require higher amount of prostacyclin concentration to inhibit aggregation, and adhere stronger to collagen (due to higher expression of the membrane GPIa/IIa receptors) than small and low-density ones [130–132]. Simultaneously, other authors showed that small and low-density platelets have an enhanced intracellular Ca^{2+} response to thrombin, which provokes them to a greater aggregation in response to these stimuli. Moreover, small and low-density platelets comparing to large-dense ones contain lower levels of the phosphorylated form of vasodilator-stimulated proteins, which can be the consequence of their weaker response to antiaggregative NO stimulation [133–135]. Although large-dense thrombocytes have higher aggregation and adhesion ability, small and low-density platelets react greater on thrombogenic stimuli with lower autoinhibitory response to NO.

In Kiliçli-Camur et al.'s study, high mean platelet volume (MPV) (associated with platelets' large and dense subpopulation) was increased during acute myocardial infarction and in the first subsequent weeks. What is more, patients with coronary artery disease (CAD) and elevated MPV had greater risk of acute myocardial infarct in comparison to those with a lower MPV, regardless of the extent of the coronary lesions [136]. Many studies also connected high MPV with ischemic stroke. In Butterworth and Bath's study, MPV was significantly higher in the ischemic stroke group than in the controls. Additionally, in stroke subgroup analysis, MPV was associated with cortical stroke but not with lacunar stroke [137]. Moreover, in Özkan et al.'s study, high MPV was associated with acute ischemic stroke only in patients with noncardioembolic stroke (with sinus rhythm and without heart failure or left atrial enlargement) [138]. More importantly, high MPV predicts also the risk of a second stroke up to 4 years before the acute event (11% increase of the relative risk of stroke for each femtoliter of MPV increase) and unfavorable outcome after cerebral infarction (death or dependency at 3 months follow-up) [137, 139]. These results underline the influence of platelets in CVD development and additionally suggest that MPV could be another risk factor for CVD development and progression.

9. Clinical Importance of PDNO and Expression of eNOS in Platelet Subpopulation

Radziwon-Balicka et al. identified in humans the thrombocyte subpopulations based on the presence of endothelial nitric oxide synthase (eNOS-positive or eNOS-negative platelets). Thrombocytes that are eNOS-negative constitute about 20% of total human platelet population and fail to produce NO, which attenuates their cGMP signaling pathway and—as result—promotes adhesion and formation of larger aggregates. The authors postulate that the role of e-NOS-negative platelets in thrombogenesis is probably to initiate adhesion and aggregation (the seed platelet hypothesis), while e-NOS-positive ones limit thrombus formation through NO production [112]. In the presence of vascular injury, eNOS-negative thrombocytes are the first to adhere to exposed collagen and/or to the von Willebrand factor. Thanks to the absence of endogenous NO generation, a quicker activation of integrin αIIbβ3 appears, alongside with stabilization of initial rolling and adhesion of platelets [140]. Further recruitment of eNOS-positive platelets to a site of injury and formation of a greater aggregate is supported by matrix metalloproteinase secreted by eNOS-negative thrombocytes. Following aggregation, the eNOS-positive platelets form the bulk of a thrombus due to their greater thromboxane generation in comparison to the eNOS-negative thrombocytes. Finally, the limitation of aggregate size is achieved through nitric oxide generation by eNOS-positive platelets, when their number in the thrombus overbalances eNOS-negative ones [112].

Some data suggest that platelet-derived nitric oxide (PDNO) might be connected with development of cardiovascular disorders, including ischemic stroke [91, 141]. Ikeda et al. showed a negative correlation between PDNO and age, mean arterial pressure, total cholesterol, and LDL-cholesterol level. What is more, the PDNO release was also significantly decreased in long-term smokers [142]. Queen et al. has demonstrated that platelet nitric oxide synthase activity at baseline was lower in diabetic patients than in control subjects, while the platelet nitric oxide generation stimulated by beta-adrenoceptors attenuated in the course of diabetes [143]. Another study postulates that lower PDNO production was an independent predictor for acute coronary syndrome with odds ratio reaching 4.0 [144]. Laufs et al. showed the influence of PDNO on the course of ischemic stroke pointing out that statin-related improvement in the outcome is mediated by the increase in the eNOS expression in the thrombocytes and aorta [145]. Therefore, changes in the eNOS-negative to eNOS-positive platelet ratio might result in modification of the risk and outcome of acute ischemic cardiovascular events such as ischemic stroke or acute coronary syndrome [112].

On the other hand, there is still some controversy about association between nitric oxide and ischemic stroke. Platelets are characterized by the expression of several activation pathways. Noteworthily, Taka et al. showed that the NO donor and the NO synthase inhibitor did not affect shear-induced platelet reactivity or vasodilatation in stroke-prone spontaneously hypertensive rats [146]. Interestingly, Lafrati et al. found that eNOS-deficient animals showed a prolongation of time to occlusion, which was explained by the compensatory mechanism. Although eNOS-deficient mice had increased platelet recruitment, simultaneously they had also enhanced fibrinolysis due to lack of NO-dependent inhibition of Weibel-Palade body release (containing tissue plasminogen activator) from the endothelium [147]. What is

more, results from other studies show that a cumulative effect of NO on ischemic stroke could cause harm; as in animals treated at reperfusion with the nonselective NOS inhibitor, the infarct volume was significantly almost twofold decreased [148]. Manickam et al. suggests also that inhibition of peroxynitrite and other ROS production by superoxide dismutase rather than nitric oxide itself protects against ischemia/reperfusion injury in the brain [149]. Hence, the Janus-faced action of NO in stroke requires further precise studies.

10. Prevention and Treatment of Ischemic Noncardioembolic Stroke: A Translational Focus on Platelets in the Shade of Current Guidelines and Trials in Cardiovascular Medicine and Neurology from Bench to Bedside

Specific therapeutic strategy for ischemic stroke is thrombolytic therapy (alteplase treatment), in which efficacy has been clearly shown especially when performed within the therapeutic time frame (up to 4.5 hours from the onset of stroke symptoms and in particular cases, if the risk-benefit ratio approves its implementation, within 6 hours) [150]. Additionally, every patient eligible for mechanical thrombectomy (complementary treatment option to alteplase infusion) should have previous thrombolysis performed (depending on inclusion/exclusion criteria). According to the American Heart Association/American Stroke Association (ASA/AHA) 2019 guidelines, intravenous aspirin should not be administered within 90 minutes after the start of i.v. alteplase treatment because it increases risk of symptomatic intracranial hemorrhage more than twofold without any positive effect on functional outcome within 3 months of observation. The safety and efficacy of i.v. glycoprotein IIb/IIIa inhibitors administered after alteplase infusion or thrombectomy is uncertain [151–154].

Intravenous administration of tirofiban is the most commonly used antiplatelet therapy following rescue angioplasty with or without stenting after myocardial infarction. In acute ischemic stroke, tirofiban has been reported to facilitate further recanalization if primary mechanical thrombectomy failed and the highest benefit was achieved in LAA ischemic stroke subtype. Thus, tirofiban can be an interesting adjuvant therapy after unsuccessful thrombolysis/thrombectomy [155–157]. However, recent guidelines for the early management of patients with acute ischemic stroke recommends consideration of antiplatelet/antithrombotic therapy < 24 hours after treatment with i.v. alteplase only if the patient has concomitant conditions for which such treatment given in the absence of i.v. alteplase is known to provide substantial benefit or withholding such treatment is known to cause substantial risk. This recommendation is based only on a single-center retrospective analysis, which found no increased risk of hemorrhage with early initiation of antiplatelet or anticoagulant therapy after i.v. alteplase or endovascular treatment compared with initiation > 24 hours after ischemic stroke [154, 158]. Those recommendations prevent clinicians from wide usage of tirofiban in ischemic stroke patients after unsuccessful thrombolysis.

Approximately 8% of patients with ischemic stroke are admitted to the hospital in the time window allowing thrombolysis procedure [159]. For the remaining, about 90% of the only available current treatment option is secondary prevention of ischemic stroke. According to AHA/ASA 2019 guidelines for stroke prevention, administration of acetylsalicylic acid (160-300 mg/24 h) is recommended in patients with acute ischemic stroke within 24 to 48 hours after onset of disease or >24 hours after alteplase treatment with lifetime continuation of such antiplatelet treatment [154]. In Chen et al.'s meta-analysis, early use of ASA (<48 h) in acute ischemic stroke decreased the risk of recurrent stroke or death in a hospital with a nonsignificant increase in hemorrhagic stroke or hemorrhagic transformation of the original infarct [160]. In animal studies, the high-dose ASA therapy in temporary induced ischemia significantly reduced infarct size compared to placebo, in humans corresponding dosage would account for 19 grams of ASA with probably unfavorable benefit/risk ratio (higher hemorrhage risk) [161]. What is more, patients with diagnosed minor noncardioembolic ischemic stroke (NIHSS score ≤ 3) or at high-risk transient ischemic attack (TIA) ($ABCD_2$ (Age, Blood Pressure, Clinical Features, Duration, Diabetes) score ≥ 4) who did not receive thrombolysis should be treated with dual antiplatelet therapy (ASA and clopidogrel) started within 24 hours after symptom onset and continued for 21 days [162]. Finally, ASA alone is significantly reducing the 6-week risk of recurrent ischemic stroke by about 60% and disabling or fatal ischemic stroke by about 70% (with the greatest benefit in patients with TIA or minor stroke) [163]. However, according to the CAST study, the number needed to treat for ASA to prevent one stroke within one year is 100 patients [164].

Acetylsalicylic acid is a drug of choice in the secondary prevention of ischemic stroke. In case of intolerance, it can be replaced by clopidogrel 75 mg daily according to 2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases [165, 166]. The meta-analysis by Paciaroni et al. even postulates clopidogrel to be a better choice in the secondary prevention of ischemic stroke due to the significant risk reduction for major adverse cardiovascular and cerebrovascular events, any ischemic or hemorrhagic stroke, and recurrent ischemic stroke in patients who received clopidogrel versus ASA. The risk of bleeding was also lower for clopidogrel in comparison to acetylsalicylic acid [167]. However, 2019 updated guidelines for the early management of patients with acute ischemic stroke suggest that increasing the dose of acetylsalicylic acid or switching to an alternative antiplatelet agent in patients who have a noncardioembolic ischemic stroke while taking ASA is still not well established [154]. There are only few indications for dual ASA and clopidogrel therapy mainly due to high risk of life-threatening hemorrhages. Dual antiplatelet therapy is indicated in minor noncardioembolic ischemic stroke or with high-risk TIA (as described before), after myocardial infarct or after carotid artery stenting [154, 166, 168]. The benefit of single antiplatelet therapy for preventing stroke in asymptomatic patients with carotid artery stenosis > 50% is not proven to be beneficial in randomized control trials. However, optimal medical treatment with acetylsalicylic acid or clopidogrel is recommended for the majority of those

Table 1: Effect of different antiplatelet drug treatments on outcome in ischemic stroke.

	Mechanism of action	Primary prevention of ischemic stroke		Acute phase of ischemic stroke		Secondary prevention of ischemic stroke	
		Animal studies	Human studies	Animal studies	Human studies	Animal studies	Human studies
Acetylsalicylic acid	Cyclooxygenase inhibitor	Beneficial [182]	Neutral (beneficial after artery stenting) [166]	Beneficial [161]	Beneficial in TIA and minor stroke (NIHSS ≤ 3) [183]	Beneficial [184]	Beneficial [154]
Clopidogrel	Inhibitor of P2Y12 receptor	Beneficial [185]	Neutral (beneficial after artery stenting) [166]	Beneficial [186]	Beneficial in TIA and minor stroke (NIHSS ≤ 3) [183]	Beneficial [185]	Beneficial [154]
Prasugrel	Inhibitor of P2Y12 receptor	Beneficial [187]	Neutral (beneficial after ACS) [188]	Beneficial [186]	Harmful [154]	Beneficial [189]	Harmful [190]
Ticagrelor	Inhibitor of P2Y12 receptor	Beneficial [191]	Neutral (better prevention with higher hemorrhage incidence) [192]	Beneficial [191]	Harmful [154]	No data found	Neutral (better prevention with higher hemorrhage incidence) [193]
Cangrelor	Inhibitor of P2Y12 receptor	Neutral [188]	No data found	Beneficial [186]	Harmful [154]	No data found	Beneficial in stroke prevention in the perioperative period [194]
Vorapaxar	PAR-1 antagonist	No data found	Harmful [195]	No data found	Harmful [169]	No data found	Harmful/neutral [196, 197]
Tirofiban	GPIIb/IIIa blocker	Beneficial (group effect) [198]	No data found [199] (neutral/harmful in the second-generation GPIIb/IIIa blockers)	Beneficial [172]	Beneficial [157]	Beneficial [198]	Uncertain [200]
Abciximab	GPIIb/IIIa blocker	Beneficial (group effect) [198]	No data found [199] (neutral/harmful in the second-generation GPIIb/IIIa blockers)	Beneficial [201]	Uncertain [154]	Beneficial [198]	Harmful [199]
Eptifibatide	GPIIb/IIIa blocker	Beneficial (group effect) [198]	No data found [199] (neutral/harmful in the second-generation GPIIb/IIIa blockers)	Beneficial [201]	Beneficial [151]	Beneficial [198]	Uncertain [200]
Anfibatide	GPIb blocker	Beneficial [202]	No data found	Beneficial [172]	No data found	No data found	No data found
Caplacizumab	Anti-vWF antibody, blocker of platelet GPI-vWF adhesion	Beneficial [173]	No data found	Beneficial [173]	No data found	No data found	No data found
ADAMTS13	Recombinant human enzyme transforming vWF to smaller, less active forms	Beneficial [175]	No data found	Beneficial [203]	No data found	No data found	No data found
rHA-infestin-4	XIIa inhibitor	Beneficial [176]	No data found	Beneficial [204]	No data found	No data found	No data found
Revacept	Competitive blocker of platelet	Beneficial [177]	Beneficial [205]	Beneficial [206]	No data available (ongoing study of	No data found	No data found

Table 1: Continued.

	Mechanism of action	Primary prevention of ischemic stroke		Acute phase of ischemic stroke		Secondary prevention of ischemic stroke	
		Animal studies	Human studies	Animal studies	Human studies	Animal studies	Human studies
	GPVI adhesion to vWF				patients with stable coronary artery disease undergoing elective PCI)		
F-0401	Dihydropyridine calcium antagonist with PAF antagonistic action	Beneficial [179]	No data found	Beneficial [207]	Beneficial (in the study of human astrocytoma and neuroblastoma cells) [207]	No data found	No data found
BN 50739	PAF antagonist	Beneficial [180]	No data found	Beneficial [209]	No data found	No data found	No data found
Inclacumab	P-selectin neutralizing antibody	No data found	No data found	No data found	No data available (beneficial in non-ST-segment elevation myocardial infarction) [181]	No data found	No data found

patients in the primary prevention of ischemic stroke to reduce the risk of stroke and other cardiovascular events, as these patients are also at twice the risk for myocardial infarct. In symptomatic extracranial carotid stenosis, antiplatelet monotherapy is always recommended [166]. Newer drugs from the same class as clopidogrel (inhibitors of P2Y12 receptor: prasugrel, ticagrelor, and cangrelor) are not beneficial in the acute phase of ischemic stroke. Vorapaxar, protease-activated receptor-1 antagonist (PAR-1), treatment during acute myocardial infarct is proved to be beneficial in clinical tests; its use in acute ischemic stroke is harmful leading to a greater hemorrhagic transformation [154, 169]. Another antiplatelet drug, abciximab (glycoprotein IIb/IIIa inhibitor) as medical treatment for the secondary prevention of ischemic stroke, is potentially harmful and should not be used, while efficacy of eptifibatide is not well established yet [154, 170].

Even though the main function of antiplatelet agents is to inhibit platelet-platelet aggregation, in case of penumbral protection, platelet-endothelium adhesion and platelet-leukocyte aggregation are similarly important. Anfibatide by inhibiting adhesive properties of platelets (blocker of platelet glycoprotein receptor Ib) significantly reduces infarct size, increases the number of intact neuronal cells, and improves neurobehavioral function by reducing postischemic blood-brain barrier damage, leukocyte migration, and microthrombus formation [171, 172]. Caplacizumab (humanized anti-vWF antibody) by binding to vWF inhibits platelet adhesion to the vessel wall (platelet GPIb—vascular vWF interaction blockade). This drug in Momi et al.'s study both prevented middle carotid artery thrombosis and reduced brain damage without provoking hemorrhage by inducing reperfusion when given before or up to 15 minutes after complete artery occlusion. Tirofiban (GPIIb/IIIa blocker) prevented thrombosis but did not induce reperfusion and caused striking brain hemorrhage [173, 174]. The activity of vWF is regulated by a disintegrin-like and metalloprotease with thrombospondin type I repeats-13 (ADAMTS13) that transforms vWF to smaller, less active forms. According to Zhao et al., infusion of a high dose of recombinant human ADAMTS13 into a wild-type mouse immediately before reperfusion reduces infarct size and improves functional outcome without producing cerebral hemorrhage pointing thus at ADAMTS13 to be a useful potential therapeutic target in ischemic stroke [175]. Another potential drug connected with GPIb interactions is specific factor XIIa inhibitor rHA-infestin-4. This substance completely inhibits occlusive arterial thrombus formation in mice and rats while leaving hemostasis fully intact [176]. The revacept (GPVI-Fc fusion protein) blocks competitively binding of vWF to collagen and GPVI-mediated platelet adhesion. Goebel et al. showed that this medication prevents thrombus formation after endothelial injury and, if applied immediately before reperfusion in mice with ischemic stroke, significantly improves functional outcome and decreases cerebral infarct size [177]. de Brito Toscano et al. showed that a platelet-activating factor (PAF) receptor-deficient mouse had a smaller brain-infarcted area in comparison to the control group [178]. Furthermore, pretreatment with F-0401, dihydropyridine calcium antagonist with PAF antagonistic action, prevents the occurrence of brain edema, disruption of the blood-brain barrier, and neuronal damage caused by cerebral ischemia [179]. Pretreatment with PAF antagonist (BN 50739) before induction of focal cortical lesions in anesthetized rats improved the penumbral cerebral blood flow and reduced edema and the progression of neuronal damage [180]. Inclacumab (a potent and selective P-selectin-neutralizing antibody) appears to reduce myocardial damage after percutaneous coronary intervention (PCI) in patients with non-ST-segment elevation myocardial infarction. However, there is no study conducted analyzing effects of inclacumab on ischemic stroke [181] (Table 1).

Despite the numerous antiplatelet drugs implemented in clinical practice and large body of evidence for their effectiveness in particular clinical scenarios, the precise and disease-

dedicated therapy remains a difficult task for clinicians. Further studies, based more and more on translational medicine, are required to combine pathophysiological knowledge and the basics of pharmacotherapy with data from epidemiological clinical trials in order to formulate optimal recommendations.

11. Clinical Implications and Future Directions

In summary, there is a large body of evidence on the important role of nitric oxide in the pathophysiology of thrombogenesis in patients at high and very high cardiovascular risk. However, there are scarcely no studies separating the contribution of endothelial and platelet-derived NO in the onset and clinical course of particular cardiovascular events of atherothrombotic origin. The study discovering the eNOS-negative and eNOS-positive subpopulations of platelets constitutes a milestone that may change the paradigm stating that decreased endothelial NO bioavailability and endothelial dysfunction itself may promote the onset of acute ischemic events. What is more, the quantitative analysis accompanied with the verification of some activation-related platelet features might become a useful tool as a prognostic biomarker for ischemic stroke and thromboinflammation. Furthermore, the role of ADMA in platelets, which elevated the plasma level, is a well-known cardiovascular risk factor and requires further studies. A detailed analysis of the platelet ADMA biotransformation (including its synthesis with PRMT, transmembrane translocation with CAT, and degradation by the DDAH) should provide some new important data on this issue. Future experimental studies using the selective iNOS and nNOS inhibitors or antiplatelet agents blocking the GPI and GPVI receptors in the management of brain ischemia-reperfusion injury are required to clarify the Janus-faced action of nitric oxide in stroke.

Conflicts of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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Research Article

Evaluation of Oxidative Stress in Patients with Difficult-to-Heal Skin Wounds Treated with Hyperbaric Oxygen

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Objective. To determine the concentration of thiobarbituric acid reactive substances (TBARS) in erythrocytes and blood plasma, and the activities of selected antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in erythrocytes in patients receiving hyperbaric oxygen (HBO) treatment due to difficult-to-heal "skin wounds". *Material and Methods*. Indices of oxidative stress were assessed in venous blood taken from 23 patients three times: immediately before HBO procedure, approx. 5 minutes after leaving the hyperbaric chamber, and after 25 HBO procedures. Moreover, selected blood counts were measured in the collected material two times: prior to treatment and after 25 HBO procedures. *Results*. A statistically significant positive correlation between the CAT activity and the TBARS concentration in the erythrocytes of patients was found before treatment in the hyperbaric chamber ($r = 0.394$; $P \leq 0.05$). No statistically significant changes in the TBARS concentration in erythrocytes and blood plasma were observed both after the first HBO procedure and after 25 procedures. No statistically significant changes in the activities of CAT, SOD, and GPx were noted. Platelet count decreased by 18.7% ($P \leq 0.05$) after 25 HBO procedures. Granulocyte count decreased by approx. 21% ($P \leq 0.05$), and granulocyte percentage by 11.8% ($P \leq 0.01$). In turn, the percentage of lymphocytes and monocytes increased after the treatment by 16.6% ($P < 0.05$) and 16.4% ($P < 0.05$), respectively. *Conclusions*. Exposure to HBO due to difficult-to-heal skin wounds does not significantly affect the levels of oxidative stress in the peripheral blood of patients and, from the point of view of oxidation–reduction processes, appears to be a safe therapeutic method for the treatment of chronic wounds.

1. Introduction

Chronic wounds are defined as wounds which require more than 8 weeks to heal despite optimal local treatment [1]. In industrialised countries, approx. 1–1.5% of the population is at risk of developing a chronic wound [2]. These wounds do not undergo the normal phases of wound healing in an orderly and timely manner [3]. Despite differences, chronic wounds have some common characteristics, e.g., increased levels of growth factors that can control cell migration, enzyme expression, and differentiation, proinflammatory cytokines that can regulate cell activity and functions [4], as well as reactive oxygen species (ROS) that have both defensive and signalling roles [3]. In local wound treatment, the TIME strategy developed by the European Wound Management Association is used, which contributes to the stimulation of natural healing mechanisms and includes: T—tissue debridement, I—infection and inflammation control, M—moisture balance, and E—epidermisation stimulation [5]. More advanced therapies, such as topical platelet-derived growth factor (PDGF), negative-pressure wound therapy, or bioengineered cell-containing therapies [6], are used to treat wounds that do not improve over a few weeks.

Chronic wound therapy may also include hyperbaric oxygen (HBO) use. Among the benefits of this form of therapy are increased oxygenation of hypoxic and ischaemic

tissues, improved blood circulation, reduced oedema, and accelerated healing. HBO also has a bactericidal and bacteriostatic effect [7–9]. During the procedure, the patient is placed in a hyperbaric chamber and provided 100% oxygen for breathing [10], which increases the partial pressure of oxygen in the lungs, increases plasma oxygen concentration compared with normal conditions, and significantly increases the oxygen diffusion radius from capillaries to the surrounding tissues [11]. Hyperoxic condition has been shown to temporarily increase intracellular ROS and reactive nitrogen species (RNS) levels, which may promote certain processes associated with wound healing, such as proliferation and wound remodeling [12, 13]. Recently, hydrogels which rapidly generate molecular oxygen up to hyperoxic levels have been employed in wound treatment [13].

Generation of ROS, including free oxygen radicals, can lead to both beneficial and adverse effects, which depends on their concentration and intracellular location [14]. ROS are generated as natural by-products of metabolism, and their primary source in cells is the respiratory chain. In the process of cellular respiration, some oxygen naturally undergoes incomplete reduction, which leads to the formation of ROS [15]. Breathing 100% oxygen increases ROS generation in the body.

Thiobarbituric acid reactive substances (TBARS), the most prevalent substrate of which is malondialdehyde (MDA), secondary lipid peroxidation product, are one of the markers of oxidative stress. It has been demonstrated that an increase in their concentration may be indicative of oxidative damage to cell membranes [16]. Among the body's defences against excessive ROS generation are antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) [17].

Cellular and molecular wound healing mechanisms are very complex, and their disturbances have not been fully understood. Moreover, it is not clearly known how hyperbaric oxygen affects the systemic oxidant–antioxidant equilibrium in the process of wound healing. Few studies looking at this problem have been conducted, and sometimes conflicting results have been obtained. Therefore, it seems reasonable to undertake research in this area. The aim of the study was to evaluate the TBARS concentration in the erythrocytes and blood plasma, as well as the activity of selected antioxidant enzymes: CAT, SOD, and GPX, in the erythrocytes of people with difficult-to-heal skin wounds undergoing HBO treatment. Moreover, selected peripheral blood counts were determined.

2. Materials and Methods

2.1. Patient Population and Study Design. The study included 23 patients (mean age 45.7 ± 16.3 years) of the Mazovian Centre for Hyperbaric Therapy and Wound Treatment in Warsaw Poland, undergoing HBO treatment due to the occurrence of difficult-to-heal skin wounds following mechanical injuries (in traffic accidents and other circumstances).

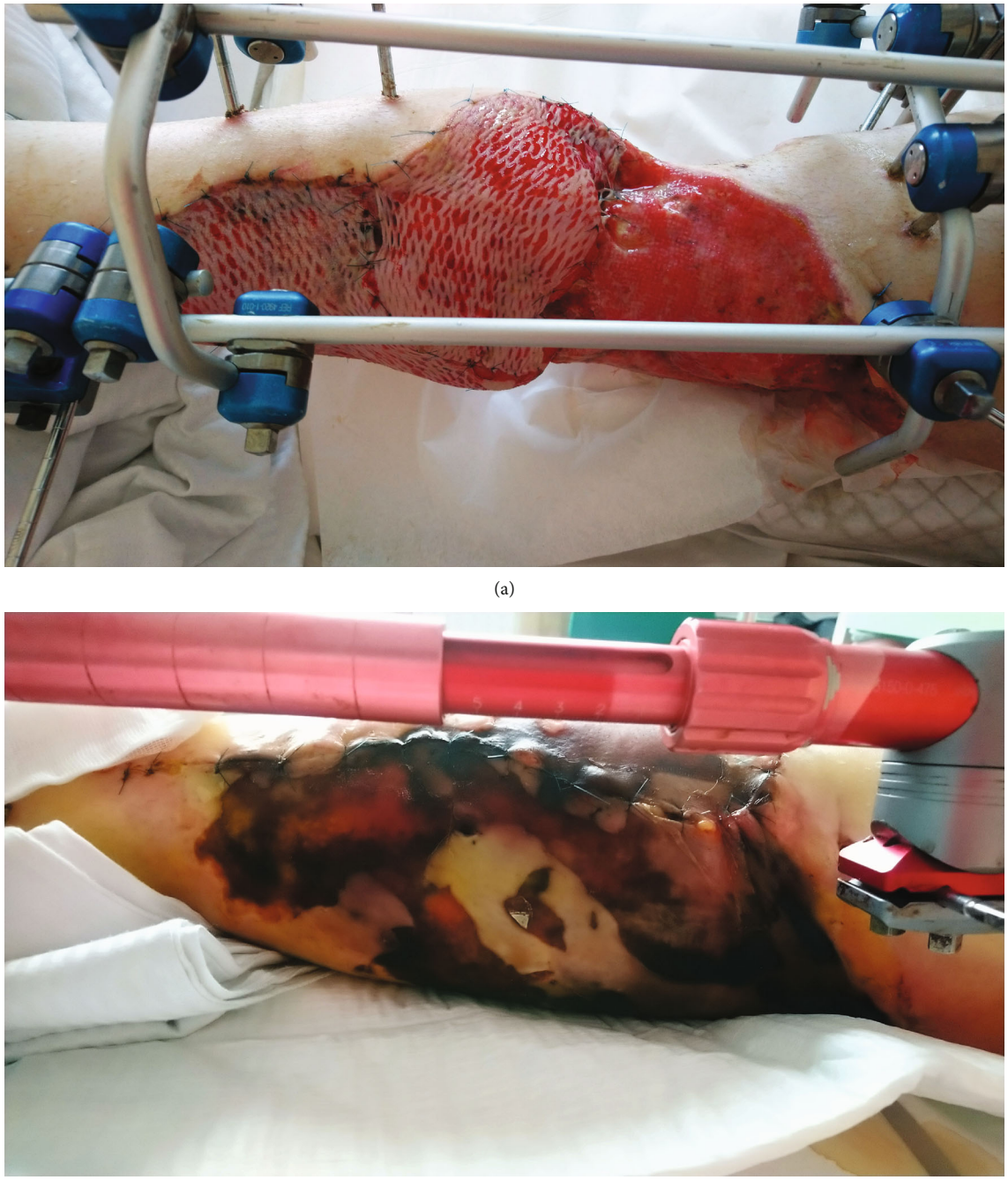
People with health problems characterised by proven oxidative stress were excluded from the study (cardiovascular disease, atherosclerosis, hypertension, but particularly diabetes mellitus). The patients were asked to abstain from drinking alcohol, smoking, and taking vitamins and other preparations that could affect the oxidant–antioxidant equilibrium during the experiment. The patients provided their written consent for the participation in the experiment. The study was approved by the Bioethics Committee of Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Poland (approval no.: KB 260/2016).

The patients underwent 25 HBO therapy procedures in the Haux Starmed 220 hyperbaric chamber which created reproducible environmental conditions (temperature, pressure, humidity, and ability to breathe pure oxygen for the same amount of time). The pressure inside the chamber during the procedure was 0.25 MPa, and the patients breathed 100% oxygen in three 20-minute cycles separated by two 5-minute intervals during which they breathed the air filling the chamber. Moreover, two 10-minute compression and decompression periods were included. During the experiment, local treatment in all patients was the same and was not changed. Ready-to-use hydrocolloid dressings were used on wounds. Following the HBO procedures, healing and reduction of wound size (Figures 1(a) and 1(b)) were observed in the patients.

2.2. Biochemical Analysis. Blood for biochemical analyses was collected from the basilic vein at three time points: before the first HBO procedure, approx. 5 min after the first procedure, and after a series of 25 procedures. The concentration of TBARS was determined in erythrocytes and blood plasma. The activities of the main three antioxidant enzymes, CAT, SOD, and GPx, were determined in erythrocytes. Moreover, prior to the first HBO procedure and after 25 procedures, selected peripheral blood counts were determined using the Orphee - Mythic 22AL haematology analyser. All biochemical analyses were conducted in a laboratory at the Department of Medical Biology and Biochemistry of the Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Poland.

2.2.1. TBARS Concentration Measurement. The TBARS concentration was measured using the method by Buege and Aust [18] as modified by Esterbauer and Cheeseman [19]. Lipid peroxidation products were identified using thiobarbituric acid (TBA). The main lipid peroxidation product that reacts with thiobarbituric acid is MDA; therefore, for the sake of simplicity, the levels of TBARS were expressed as the concentration of MDA. The MDA concentration in erythrocytes was expressed in nmol MDA/g Hb, and that in blood plasma was expressed in nmol MDA/mL of plasma.

2.2.2. CAT Activity Measurement. The CAT activity was determined by measuring the decrease in the absorbance of a solution of hydrogen peroxide (H_2O_2) decomposed by this enzyme. The decrease in the absorbance value is directly proportional to the reduction of the H_2O_2 concentration in the solution [20]. The CAT activity was expressed in IU/g Hb.



(a)

(b)

Figure 1: Lower leg wound was caused by a communication accident (a) before treatment in the hyperbaric chamber, (b) after 25 procedures in the hyperbaric chamber.

2.2.3. SOD Activity Measurement. Determination of the SOD activity was based on the inhibition of adrenaline autoxidation to adrenochrome in alkaline conditions. To measure the SOD activity, a previously obtained haemolysate after removal of haemoglobin with a chloroform–ethanol mixture was used. Centrifugation generated two layers: the upper layer containing the enzyme and lower layer containing denatured haemoglobin and chloroform [21]. The SOD activity was determined by continuous recording of the reaction using a reaction kinetics programme on a Varian spectrophotometer and expressed in U/g Hb.

2.2.4. GPx Activity Measurement. The GPx activity was determined at 20°C using a method based on the decomposition of hydrogen peroxide by the enzyme with the concurrent oxidation of reduced glutathione [22]. The results were expressed in U/g Hb.

2.3. Statistical Analysis. The study results were presented as means with standard deviation (SD) values. To assess the normal distribution of the data, the Kolmogorov–Smirnov test was used. Statistical analysis of the oxidative stress parameters was performed using analysis of variance (ANOVA, Bonferroni post hoc test) (*STATISTICA v.* 9.1). Student's *t*-test for variables in paired measurements was used to compare mean values calculated for the blood counts. Dependencies between the analysed parameters were assessed using correlation matrices. A statistical hypothesis of the significance of correlation coefficients (r) was tested. Differences at significance level $P \leq 0.05$ were presumed as statistically significant. Results close to but higher than $P = 0.05$ may indicate certain tendencies and can be an inspiration for further research. In this study, such results are presented as being at the level of statistical tendency. The threshold of statistical tendency was established at $P = 0.09$.

3. Results

3.1. TBARS Level. No statistically significant changes in the TBARS concentration in erythrocytes and blood plasma were observed (Table 1). However, there was a certain tendency to change. The plasma TBARS concentration after 25 procedures decreased by 10.5% compared to that measured before the first procedure ($P > 0.05$).

3.2. Antioxidant Enzyme Activities. No statistically significant changes in the CAT, SOD, and GPx activities in the erythrocytes of the study participants were determined (Table 1). However, a tendency to change was observed in the erythrocyte CAT activity. The activity of this enzyme in erythrocytes after the first HBO procedure decreased by 3% compared with that before the procedure ($P > 0.05$).

A statistically significant positive correlation between the CAT activity and the TBARS concentration in the erythrocytes of patients was found prior to treatment in the hyperbaric chamber ($r = 0.394$; $P \leq 0.05$) (Figure 2).

3.3. Blood Counts. A statistically significant decrease in platelet count by 18.7% ($P \leq 0.05$) was observed in peripheral blood after 25 procedures in hyperbaric chamber (Table 2). Moreover, granulocyte count decreased by approx. 21% ($P \leq 0.05$), and granulocyte percentage by 11.8% ($P \leq 0.01$). In turn, the percentage of lymphocytes and monocytes increased by 16.6% ($P < 0.05$) and 16.4% ($P < 0.05$), respectively, after treatment completion. No statistically significant changes were observed for the remaining peripheral blood counts.

4. Discussion

Oxygen plays a key role in wound healing. This role has been demonstrated in processes such as oxidative killing of microorganisms, collagen synthesis, angiogenesis, and neovascularisation [23]. It has been proven that a higher oxygen supply can increase the capacity of epithelial cells for mitotic divisions [24]. Chronic wounds are often characterised by excessive ROS generation due to the absence of antioxidants, such as vitamins E, C, and A. The level of antioxidants has been proven to decrease with age, resulting in a delayed healing response in older people [23]. The precise role of oxygen in wound healing under hyperbaric oxygen conditions has not yet been clearly defined.

In the presented study, no statistically significant changes in the TBARS concentration were observed in both erythrocytes and blood plasma, which suggests that the use of HBO does not significantly affect the intensity of lipid peroxidation measured in the peripheral blood of patients with chronic wounds. Hyperbaric oxygen therapy increases oxygenation of tissues [25], but despite the increased generation of ROS directly caused by the increased amount of oxygen reaching the cells, there was no change in the oxidant–antioxidant equilibrium. Only a tendency of TBARS to decrease in blood plasma after 25 HBO procedures was seen.

A lack of increase of oxidative stress after HBO procedures was also demonstrated by Corcoran et al. [26] in patients with osteonecrosis by measuring plasma concentrations of F_2-isoprostanes and isofurans. A statistically significant decrease in plasma MDA was reported by Sureda et al. [27] in patients with chronic wounds after 20 HBO procedures. The authors also reported a significantly lower level of this secondary lipid peroxidation product one month after the wound had healed compared with the initial concentration. In turn, in the ulcer tissue of patients with diabetic foot ulcer, an increased MDA concentration was demonstrated on the 14th day of HBO treatment compared with that in the control group—patients on standard treatment not including HBO [28]. In other studies, increased MDA was observed in the blood plasma of patients with diabetic foot only after the first HBO procedure compared with the concentration measured before it, whereas after completion of 15 HBO procedures, the level of this lipid peroxidation product was unchanged vs. baseline. In turn, the levels of 8-isoprostane and advanced oxidation protein products (AOPPs) increased in a statistically significant manner vs. baseline only after completion of 15 HBO procedures [29]. In our earlier study in patients with different conditions after hyperbaric oxygen therapy, there were no statistically significant changes in the TBARS concentration in both blood plasma and erythrocytes [30].

However, there are publications which demonstrate that long-term exposure to high oxygen concentrations, in the form of HBO, induces generation of ROS which results in cell damage. Alleva et al. [31] demonstrated a beneficial effect of α-lipoic acid in patients with chronic wounds treated with HBO. The authors showed that supplementation of this antioxidant reduces oxidation of lipids and DNA caused by oxygen exposure, which can promote the beneficial effects of HBO treatment. Increased lipid peroxidation after HBO procedures has been suggested by some studies conducted in animals. For example, Giulivi et al. [32] showed an increase in the MDA concentration in the lungs after HBO procedures in rats, while in another study, Liu et al. [33] recorded an increased TBARS level in the cell membrane of erythrocytes of female rats with experimentally induced diabetes. It appears that further studies are necessary for a clear identification of the effect of HBO on the lipid peroxidation process.

Table 1: The concentration of thiobarbituric acid reactive substances (TBARS) in erythrocytes and blood plasma, and the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were determined in patients with difficult-to-heal skin wounds receiving hyperbaric oxygen (HBO) treatment.

Determined parameter HBO	Before procedure	After 1st HBO procedure	After 25th HBO procedure
TBARS er. (nmol MDA/g Hb)	25.36 ± 7.94	23.87 ± 7.57	25.11 ± 7.89
TBARS pl. (nmol MDA/mL)	0.57 ± 0.15	0.55 ± 0.15	0.51 ± 0.14
CAT (10^4IU/g Hb)	64.06 ± 10.47	62.14 ± 11.90	65.51 ± 6.59
SOD (U/g Hb)	626.49 ± 85.04	732.31 ± 88.74	732.31 ± 82.57
GPx (U/g Hb)	8.58 ± 4.10	7.40 ± 4.63	7.08 ± 4.00

TBARS er.: thiobarbituric acid reactive substances in erythrocytes; TBARS pl.: thiobarbituric acid reactive substances in plasma; CAT: catalase; SOD: superoxide dismutase, GPx: glutathione peroxidase. Results are expressed as Mean ± SD. No statistically significant differences.

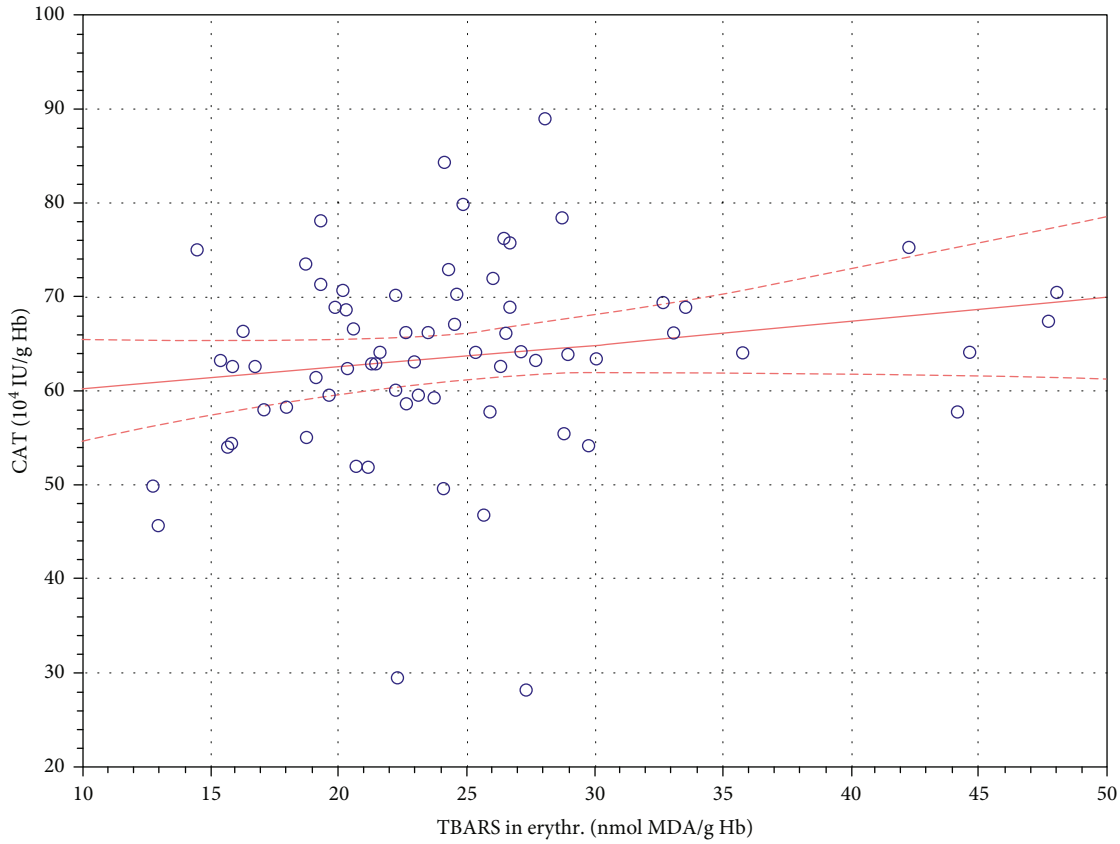


Figure 2: Linear regression of the catalase (CAT) activity versus the thiobarbituric acid reactive substances (TBARS) concentration in the erythrocytes of patients with difficult-to-heal skin wounds before initiation of treatment in a hyperbaric chamber ($r = 0.394$; $P \leq 0.05$).

Prior to the HBO treatment in the hyperbaric chamber, we found in patients a statistically significant positive correlation between the CAT activity and the TBARS concentration in erythrocytes ($r = 0.394$; $P \leq 0.05$), which could indicate a disturbance in the oxidant–antioxidant mechanisms in the course of healing of difficult wounds. There were no statistically significant changes in the activity of the investigated antioxidant enzymes in erythrocytes, which confirms a lack of a significant effect of hyperbaric oxygen on the course of oxidation–reduction processes. The study only showed a certain tendency to decrease the catalase activity after the first HBO procedure (Table 1). It seems that the repeatability of HBO procedures can increase the tolerance of mechanisms which determine the oxidant–antioxidant equilibrium to increased ROS generation. Results similar to those presented in this study were obtained by Sureda et al. [27] who reported no statistically significant changes in the CAT, SOD, GPx, as well as glutathione reductase activities in the erythrocytes of patients with chronic wounds.

In turn, a statistically significant reduction in the CAT activity in erythrocytes after an HBO procedure was shown in our previous study in which patients with different conditions had received HBO treatment no more than three times in their lives at the time of the experiment. However, no

Table 2: Selected peripheral blood counts of patients with difficult-to-heal wounds treated with hyperbaric oxygen (HBO).

Determined parameter	Before procedure	After 25th HBO	Normal range procedure
HGB (g/dl)	13.94 ± 1.99	13.65 ± 1.99	11.0–17.0
HCT (%)	38.24 ± 5.06	40.48 ± 12.21	35.0–55.0
RBC ($10^6/\mu$l)	4.24 ± 0.46	4.09 ± 0.53	4.0–6.20
PLT ($10^3/\mu$l)	368.26 ± 136.51	299.33 ± 130.57*	150–400
WBC ($10^3/\mu$l)	8.21 ± 3.09	7.05 ± 1.89	4.0–12.0
LYM (%)	27.88 ± 8.37	32.5 ± 7.76*	25.0–50.0
MON (%)	7.06 ± 1.7	8.22 ± 1.8*	2.0–10.0
GRA (%)	62.29 ± 9.43	54.91 ± 6.99**	50.0–80.0
EOS (%)	2.88 ± 1.72	3.38 ± 2.25	0.0–5.0
BAS (%)	0.44 ± 0.21	0.45 ± 0.17	0.0–2.0
LYM ($10^3/\mu$l)	2.1 ± 0.55	2.28 ± 0.37	1.0–5.0
MON ($10^3/\mu$l)	0.56 ± 0.21	0.6 ± 0.18	0.1–1.0
GRA ($10^3/\mu$l)	5.16 ± 2.61	4.08 ± 1.27*	2.0–8.0
EOS ($10^3/\mu$l)	0.22 ± 0.16	0.26 ± 0.19	0.0–0.40
BAS ($10^3/\mu$l)	0.0095 ± 0.03	0.0048 ± 0.2	0.0–0.2
MCHC (g/dl)	35.12 ± 2.96	33.77 ± 6.9	31.0–35.50
MCH (pg)	32.99 ± 4.52	33.46 ± 2.84	26.0–34.0
MCV (fl)	92.64 ± 7.96	90.75 ± 15.08	80.0–100.0
RDW (%)	15.33 ± 1.36	15.31 ± 1.21	10.0–16.0
MPV (fl)	8.38 ± 0.92	8.7 ± 1.03	7.0–10.5
PCT (%)	0.31 ± 0.1	0.27 ± 0.1	0.20–0.50
PDW (%)	13.59 ± 1.09	13.91 ± 1.23	10.0–18.0

HGB: haemoglobin; HCT: haematocrit; RBC: erythrocytes; PLT: platelets; WBC: leukocytes; LYM: lymphocytes; MON: monocytes; GRA: granulocytes; EOS: eosinophils; BAS: basophils; MCHC: mean haemoglobin concentration in red blood cells; MCH: mean haemoglobin mass in red blood cells; MCV: mean red blood cell volume; RDW: red blood cell distribution width; MPV: mean platelet volume; PCT: plateletcrit; PDW: platelet distribution width. Values are expressed as means ± standard deviations of the means. *Statistically significant difference compared with parameters measured before the HBO therapy (*$P \leq 0.05$; **$P \leq 0.01$).

statistically significant changes in the CAT activity were observed in the erythrocytes of people treated with HBO multiple times prior to the experiment, as well as no significant changes in the SOD activity were found in the erythrocytes of all study participants, regardless of the number of prior procedures in the hyperbaric chamber. Also, there were no changes in the GPx activity in erythrocytes following a procedure in the hyperbaric chamber, but the activity of this enzyme prior to treatment in patients who had previously used hyperbaric chamber many times was significantly lower than in patients who had received HBO treatment no more than three times [30]. In patients with sudden sensorineural hearing loss, a decrease in the CAT activity in erythrocytes was observed after the first procedure in the hyperbaric chamber, the SOD activity in erythrocytes decreased after the 14th procedure, and the GPx activity increased after the 14th procedure [34].

In a study by other authors, a statistically significant increase in the CAT activity in the blood plasma of patients with chronic wounds was demonstrated after both the first and the fifth day of HBO treatment compared with the activities determined before each of these procedures [27]. After the 20th procedure, there was an increase in the CAT activity as well, but it was not statistically significant. However, the authors did not identify any statistically significant changes in the SOD activity in blood plasma. Ma et al. [28], on the 14th day of their experiment, showed higher CAT and SOD activities in ulceration specimens derived from patients treated with HBO (twice daily for 90 minutes at 2.5 atm for 2 weeks) due to skin wound ulcers of the foot caused by diabetes, compared with such activities in people treated conventionally without the use of the hyperbaric chamber. At the same time, a significant improvement was observed in the healing of foot wounds following HBO treatment. In turn, studies in rats proved that repeated treatment with HBO for 1 hour increases the expression of the CAT gene and activity of the enzyme in the cardiac muscle after ischaemia [35].

Blood counts revealed a statistically significant decrease in the number of platelets in patients after 25 procedures in hyperbaric chamber (Table 2). Other statistically significant changes in blood counts were related to the number and percentage of granulocytes which decreased after completion of the HBO procedures. Conversely, the percentages of lymphocytes and monocytes increased significantly. No statistically significant changes were observed for the remaining peripheral blood counts. Concurrently, all investigated blood count values were within their respective normal ranges. A statistically insignificant reduction of the platelet count in patients after HBO procedures was reported by Gunes and Aktas [36]. The findings described by these authors, similarly to ours presented here, included no statistically significant effect of HBO treatment on haemoglobin, haematocrit, red blood cells, mean corpuscular volume, mean corpuscular haemoglobin, red blood cell distribution width, mean corpuscular haemoglobin concentration, platelet distribution width, and mean platelet volume. Studies by other authors showed a statistically insignificant reduction of haematocrit and erythrocyte counts after 20 HBO procedures in patients with various pathologies associated with hypoxia [37].

The statistically significant decrease in platelet count observed in the present study may be associated with a certain degree of normalisation of coagulation processes under increased oxygenation of tissues during exposure to HBO. In a study of healthy lowland volunteers who remained under hypoxic conditions at the height of 5200 m above sea level, researchers showed an increase in platelet count and fibrinogen concentration on the 7th day of the experiment as a result of acclimatisation [38]. However, during a prolonged stay at a high altitude, the platelet count in healthy individuals was observed to decrease after 3 and 13 months compared with the values observed at sea level [39]. Changes in the number and/or percentage of leukocytes demonstrated in this study may result from an anti-inflammatory effect of

hyperbaric oxygen treatment. Grimberg-Peters et al. [40], among other groups, showed a supportive effect of HBO in an analysis of the effect of such a treatment aimed at reducing inflammation on the activity of neutrophils isolated from severely injured patients (days 1–2 after trauma). In turn, a statistically significant decrease in the number of leukocytes after therapy combined with HBO compared with baseline was observed by Irawan et al. [41] in patients with a diabetic foot ulcer.

This study has some limitations. It could include more parameters, which characterize the redox equilibrium, and concern the injured tissue/tissues. However, the first issue is always depended on study funding, while the second one, in the case of such wounds, in such patients, raises ethical questions. However, first of all, this study included a small group of patients; therefore, the obtained results should be considered as preliminary to further studies in that area.

5. Conclusions

Analysis of the obtained results shows that the effect of hyperbaric oxygen on the oxidation–reduction processes is not clear. However, the lack of significant changes suggests that HBO therapy does not increase systemic oxidative stress and, from the point of view of maintaining the oxidant–antioxidant equilibrium, it seems to be a safe therapeutic method for the treatment of chronic wounds.

Data Availability

The study data used to support the findings of this study are included within the article.

Disclosure

The study was conducted as part of regular employment of the authors by the Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Toruń, Poland.

Conflicts of Interest

The authors declare that there are no conflicts of interests.

Authors' Contributions

Jarosław Paprocki designed the study, participated in conducting the assays, interpreted the results, and wrote the manuscript. Marta Pawłowska was involved in conducting the assays of the parameters determined. Paweł Sutkowy was involved in the statistical analysis of the results. Jacek Piechocki participated in the selection of patients and provided medical care during the hyperbaric oxygen procedures. Alina Woźniak revised the manuscript critically for important intellectual content and approved the final version. All authors have read and approved the final manuscript.

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Review Article

Novel Molecular Mechanisms of Pulmonary Hypertension: A Search for Biomarkers and Novel Drug Targets—From Bench to Bed Site

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Pulmonary hypertension (PH) is defined as increased mean pulmonary artery pressure (mPAP) above 25 mmHg, measured at rest by right heart catheterization. The exact global prevalence of PH is difficult to estimate, mainly due to the complex aetiology, and its spread may be underestimated. To date, numerous studies on the aetiology and pathophysiology of PH at molecular level were conducted. Simultaneously, some clinical studies have shown potential usefulness of well-known and widely recognized cardiovascular biomarkers, but their potential clinical usefulness in diagnosis and management of PH is poor due to their low specificity accompanied with numerous other cardiovascular comorbidities of PH subjects. On the other hand, a large body of basic research-based studies provides us with novel molecular pathomechanisms, biomarkers, and drug targets, according to the evidence-based medicine principles. Unfortunately, the simple implementation of these results to clinical practice is impossible due to a large heterogeneity of the PH pathophysiology, where the clinical symptoms constitute only a common denominator and a final result of numerous crosstalking metabolic pathways. Therefore, future studies, based mostly on translational medicine, are needed in order to both organize better the pathophysiological classification of various forms of PH and define precisely the optimal diagnostic markers and therapeutic targets in particular forms of PH. This review paper summarizes the current state of the art regarding the molecular background of PH with respect to its current classification. Novel therapeutic strategies and potential biomarkers are discussed with respect to their limitations in use in common clinical practice.

1. Introduction

Pulmonary hypertension (PH) is defined as increased mean pulmonary arterial pressure (mPAP) above 25 mmHg, measured at rest by right heart catheterization [1]. The exact global prevalence of the disease is difficult to estimate mainly due to the complex aetiology, and its spread may be significantly underestimated.

Based on the hemodynamic parameters assessed during right heart catheterization (especially DPG (diastolic pressure gradient) and PVR (pulmonary vascular resistance)), PH was divided into pre- and postcapillary PH. Postcapillary PH occurs as isolated or combined pre- and postcapillary PH. Additionally, taking under consideration clinical assessment, pathophysiology, pathological similarities, and treatment approaches, the PH patients were categorized into 5 groups with concurrent subgroups (Table 1) [2, 3].

It is currently assumed that prevalence of PH is around 0.3% in general population, although some studies estimate it to 6.6% [4, 5]. Pulmonary hypertension is more common in women than in men (1.8 : 1.0), and the incidence increases with age.

Pulmonary hypertension is characterized by a complex aetiology. The pathophysiological mechanisms leading to increased pressure in the pulmonary vessels are primarily connected with vascular remodelling. They can be caused by primary dysfunctions of endothelial cells (ECs) or smooth muscles accompanied by proliferative disorders, oxidative damage, abnormal angiogenesis, or capillary leak. Vascular remodelling can also occur secondarily to vascular overload

Table 1: Comprehensive clinical classification of pulmonary hypertension (updated from Simonneau et al. [3]).

1. Pulmonary arterial hypertension (PAH)	1.1. Idiopathic	
	1.2. Heritable	1.2.1. BMPR2
		1.2.2. ALK1, ENG, SMAD9, CAV1, KCNK3
		1.2.3. Unknown
	1.3. Drug and toxin induced	
	1.4. Associated with the following:	1.4.1. Connective tissue diseases
		1.4.2. Human immunodeficiency virus (HIV) infection
		1.4.3. Portal hypertension
		1.4.4. Congenital heart diseases
		1.4.5. Schistosomiasis
1′. Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)	1′.1. Idiopathic	
	1′.2. Heritable	1′.2.1. EIF2AK4 mutation
		1′.2.2. Other mutations
	1′.3. Drug, toxin, and radiation induced	
	1′.4. Connective tissue diseases	
	1′.5. Human immunodeficiency virus (HIV) infection	
1″. Persistent pulmonary hypertension of the newborn (PPHN)		
2. Pulmonary hypertension due to left heart disease	2.1. Left ventricular systolic dysfunction	
	2.2. Left ventricular diastolic dysfunction	
	2.3. Valvular disease	
	2.4. Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies	
3. Pulmonary hypertension due to lung disease and/or hypoxia	3.1. Chronic obstructive pulmonary disease	
	3.2. Interstitial lung disease	
	3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern	
	3.4. Sleep-disordered breathing	
	3.5. Alveolar hypoventilation disorders	
	3.6. Chronic exposure to high altitude	
	3.7. Developmental abnormalities	
4. Chronic thromboembolic pulmonary hypertension (CTEPH)		
5. Pulmonary hypertension with unclear multifactorial mechanisms	5.1. Hematologic disorders: chronic haemolytic anaemia, myeloproliferative disorders, splenectomy	
	5.2. Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis (LAM)	
	5.3. Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders	
	5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis, segmental PH	

BMPR2 = bone morphogenetic protein receptor type 2; EIF2AK4 = eukaryotic translation initiation factor 2 alpha kinase 4.

associated with a retrograde passive transmission of elevated venous pressure (i.e., in left-sided heart diseases), mechanical narrowing of pulmonary arteries by embolic material, impaired immune processes, and hypoxia-associated vasoconstriction. An important role is also played by the Euler-Liljestrand reflex, in which the presence of alveolar hypoxia causes vasoconstriction and blood redistribution to better oxygenize parts of the pulmonary vascular. Such condition, augmented by the imbalance between vasoconstricting and vasodilating factors, leads to a cascade of abnormalities that exacerbate each other (a vicious circle). Numerous signalling pathways and signalling molecules participate in these phenomena, which finally leads to functional and structural changes in the pulmonary vessels [2].

This review summarizes the state of the art regarding the knowledge on the pathomechanisms of PH. We also discuss some data from multiomic studies that could provide new biomarkers reflecting the burden of the pulmonary vascular remodelling and defining some novel drug targets.

2. Genetic Mutations as a Cause of Familial Pulmonary Arterial Hypertension (PAH)

2.1. TGF-β Family Protein Signalling Pathway Dysfunction in PAH. Familial pulmonary hypertension occurs in approximately 3.2% of patients with pulmonary hypertension [6]. The signalling pathway has an important role in the regulation of physiological changes in the pulmonary vascular endothelium, transmitted through the receptor proteins of the TGF-β family, which modulate the transcription and translation of numerous genes responsible, among others, for regulating the inflammatory response, cell proliferation, and differentiation. Disruption of this pathway may cause abnormal vascular remodelling, exacerbate atherosclerosis, and affect myocardial fibrosis.

Factors from the TGF-β signalling family regulate cell metabolism by transducing the signal through the transmembrane complex. This structure consists of two type II receptors and two type I receptors with serine-threonine kinase activity. In mammals, seven types of receptor I (ALK1-7), five types of receptor II (including but not limited to BMPR2), and two types of receptor III (endoglin and beta-glycan) have been characterized. As a result of receptor stimulation by an appropriate ligand, phosphorylation of intracellular substrates occurs and signal is transmitted to the cell nucleus through receptor-regulated SMAD proteins (R-SMAD, which include SMAD1-3, SMAD5, and SMAD8). They form a complex with the SMAD4 protein and thus transfer the signal inside the cell's nucleus. This pathway is known as the canonical BMP signalling.

In the pathogenesis of pulmonary hypertension, the main abnormalities are connected with mutations altering the properties of ALK1 and ALK5, BMPR2 and endoglin receptors, and SMAD8 signalling proteins (they are responsible for inhibiting signalling by R-SMAD) [7–10].

A series of studies report that the loss of signal mediated by SMAD1/5/8 plays an important role in the pulmonary vascular remodelling and the pathogenesis of PAH. Functional studies on BMPR2 mutations have shown that BMPR2 mutations disrupted or downregulated SMAD1/5/8 signalling.

Shintani et al. identified a nonsense mutation in the SMAD8 gene (also known as SMAD9) which introduces a premature stop codon into exon 2 and results in a truncated protein, not phosphorylated by the constitutively active ALK3 and ALK1 proteins. As a result, the interaction with SMAD4 is impossible, the signal is not transmitted into the nucleus, and the transcription of the target genes is not downregulated [11].

Yang et al. [12] revealed that BMP4 caused either inhibition or stimulation of PASMC cell proliferation, depending on cell origin. Thus, normal PASMCs isolated from the proximal pulmonary artery were inhibited, whereas normal PASMCs isolated from peripheral arteries were stimulated to proliferate in the presence of BMP4. It was demonstrated that BMP4 causes phosphorylation of SMAD1/6/7 and activates the canonical BMP signalling and, at the same time, the SMAD-independent p38MAPK and ERK1/2 pathways.

In the further part of the said study, Yang et al. [12] pointed out a predominant role of SMAD1-dependent growth suppression and indicated the proproliferative p38MAPK- and ERK1/2-dependent pathways in PASMCs in response to BMP4 activation. Using the p38 and ERK1/2 inhibitors, the authors prove that these proteins have a significant role in distal vessel PASMC proliferation. In other studies, it was revealed that the presence of a mutant BMPR2 receptor led to constitutive activation of p38MAPK even in the absence of ligand stimulation [13]. Moreover, it was indicated that in cultured cells from BMPR2-mutated patients, dysfunction of SMAD1 leads to an antiproliferation defect which may be amplified by p38MAPK inhibition.

These observations may indicate potential new targets in the therapy of FPAH patients, using agents that could inhibit the abnormal proliferation in PASMCs. Disruption of the p38MAPK signalling pathway may be a new goal of antiproliferative strategies to prevent or reverse the remodelling associated with the BMPR2 mutation.

Gore et al. showed that the expression of TGF-β family proteins is significantly higher in lung tissue and plasma of patients suffering from idiopathic pulmonary hypertension. In addition, higher concentration of TGF-β mRNA was determined in pulmonary vascular smooth muscle. They also demonstrated that endothelial cells treated with serum, where previously other ECs stimulated by TGF-β were grown, resulted in increased proliferation rate of the former. It confirms that previously postulated vascular muscle proliferation is dependent on factors secreted by endothelial cells, which is further intensified by TGF-β stimulation. The increase in muscle proliferation by TGF-β was abolished when the antiendoglin antibodies were added [7].

Noteworthily, significant amounts of endoglin and ALK1 encoding mRNA and the proteins themselves were determined in ECs. Other studies have shown that endoglin (CD105) plays a key role in maintaining the balance of signal pathways transmitted by the ALK1 and ALK5 receptors. Thus, it regulates vascular epithelial proliferation and its presence is shown on the surface of dividing endothelial cells in vivo [14].

However, the role of the TGF-β/ALK pathway in the pathogenesis of pulmonary hypertension is not clearly defined. Similar to the research of Gore et al., the ALK1 overexpression in studies, in the animal model of monocrotaline-induced pulmonary hypertension, was shown by Ramos et al. [15] However, a study conducted on a similar model by Zakrzewicz et al. showed a decrease in expression of the TGF-β pathway proteins [16]. The concentration of other circulating ligands, such as activins and growth and differentiation factors (GDFs), which also interact through the TGF-β receptor family, is also increased in PAH, which probably stimulates growth cells, and thus contributes to the remodelling of pulmonary vessels and is an additional pathomechanism for the development of pulmonary hypertension [10]. However, the importance of these processes requires further study.

Mutations in the genes encoding the TGF-β family signalling pathways have also been shown to cause pulmonary hypertension associated with Osler-Weber-Rendu disease. Most of them were detected only in individual patients and relate to mutations in ENG genes (encoding endoglin) 61% and ACVRL1 (encoding receptor-like activin kinase type 1

(ALK1)) 37% [17]. Other mutations were also revealed in MADH4 genes (encoding SMAD4) and genes localized on the 5q and 7p chromosomes [18, 19].

2.2. BMPR2 Mutation in PAH. The best-known cause of hereditary pulmonary hypertension is a mutation in the bone morphogenetic protein receptor type 2 (BMPR2) gene, which belongs to the TGF-β superfamily. It is detected in 55% of patients with familial pulmonary arterial hypertension (PAH) and in at least 26% of patients with idiopathic pulmonary hypertension [20, 21]. Initially, it was found that this protein is responsible for regulating bone and cartilage growth and differentiation, hence its name. Further studies have shown that it also regulates the proliferation, apoptosis, differentiation, and migration of pulmonary artery cells. As a result, excessive proliferation in pulmonary muscle cells (PMCs) occurs, which results in overload and subsequent right ventricular failure with the simultaneous development of pulmonary hypertension [22]. BMPR2 gene mutation has been shown to present haploinsufficiency, so one remaining functional copy of the gene does not allow to produce competent protein and results in disruption of biological function and reduction of apoptotic response of pulmonary smooth muscle cells compared to controls [21, 23].

It is estimated that about 15-20% of patients with the BMPR2 gene mutation will develop pulmonary hypertension [24]. Girerd et al. pointed out that patients with the BMPR2 mutation are significantly younger at the moment of diagnosis compared to patients in which no such mutation was found (38.53 ± 12.38 vs. 45.78 ± 11.32 years, $P < 0.001$) [25, 26]. It was indicated that, as in the entire group of PH, also in BMPR2 mutant population, women dominate (female : male ratio 2.4 : 1) [26]. It is postulated that the difference may be due to effect of oestrogen on vascular cells. It results in abnormal cell division and vasoreactivity inhibition. In vitro, oestrogens increase the proliferation of epithelial smooth muscle cells, which is inhibited by antioestrogen drugs, e.g., tamoxifen.

It is worth noting that the most important enzyme regulating oestrogen metabolism is cytochrome P450 isoform 1B, which is highly expressed in oestrogen-dependent tissues, as well as in lung tissue, where it can be responsible for regulating oestrogen balance. West et al. revealed lower transcript concentration of CYP1B1 in women with pulmonary hypertension. This may lead to higher local oestrogen levels and an increased risk of PAH [27]. In addition, lower enzyme levels may also result in oestrogen transformation, by other enzymes, into more potent compounds with higher PH inducing feature [28].

BMPR2 mutation carriers have been shown to be characterized by higher mean pulmonary artery pressure (mPAP) and pulmonary vascular resistance (PVR), are less likely to receive a positive response in an acute hemodynamic test, and more often exacerbate a fatal disease or require lung transplantation. However, no significant differences in the survival were found [25]. In the other studies, Austin et al. did not reveal the same statistical significance in the marked hemodynamic parameters in 169 healthy patients with a mutation in the BMPR2 gene [29].

Austin et al. pointed out also that the BMPR2 missense mutation was linked with significantly worse outcomes and the disease has a more severe clinical course (age at the time of diagnosis, shorter time to lung transplant or death), compared to the group with nonsense BPMR2 mutation. Such correlation was not confirmed by Girerd et al. in other study [26, 29].

Noteworthily, some papers highlight that the BMPR2 mutant men present worse prognosis and higher mortality than female mutation carriers; however, further research studies are needed to confirm this observation [26, 30].

2.3. Caveolin-1 Mutation in PAH. Caveolin-1 is a scaffolding protein that is encoded by the *CAV1* gene and constitutes a part of the plasma membrane contributing to formation of caveola subtype of specialized microdomains known as lipid rafts. They are abundant of many receptors on the surface and are the point where many signalling cascades start. It has been shown that caveolin-1 interacts with, e.g., G proteins, TGF-β receptor 1, endothelial nitric oxide synthase (eNOS), and nitric oxide synthase 2A [31–33].

Zhao et al. [34, 35] used genetically changed mice with CAV1 deletion to verify the role of caveolin in the pathogenesis of PAH. They reported that mutated mice have presented increased activity of eNOS, which leads to vascular remodelling and PH phenotype. It was proven that according to induced oxidative stress, superoxide and nitric oxide (NO) react to form peroxynitrite, which in turn causes increased protein kinase G (PKG) tyrosine nitration and disrupts its appropriate function resulting in PAH development. Interestingly, high levels of NO *per se* in CAV1–/– mice do not cause PH since production of superoxide is critically important [36].

Interestingly, the same study also proved that CAV1–/– mice were protected from developing the changes in pulmonary arteries while being treated with superoxide scavenger, with NOS inhibitor, or by adenovirus-mediated restoration of PKG activity in CAV1–/– lungs. Furthermore, double-knockout mice, deficient in CAV1 and eNOS (Nos3), also did not present the PAH phenotype [35].

In another study, Courchamp et al. found that in CAV1–/– mice, a lack of caveolae causes severe pathomorphological defects in the alveolar septum. They proved that normal double-layered alveolar architecture was replaced by multilayered, unorganized tissue. In addition, uncontrolled endothelial cell proliferation and fibrosis have been observed [36].

2.4. KCNK3 Mutation in PAH. The KCNK3 gene encodes an outward K+ channel that is sensitive to changes in extracellular PH and is inhibited by extracellular acidification. Accordingly, it is also called Twik-related acid-sensitive K+ channel (TASK1). The primary role of KCNK3 channels is to control the resting membrane potential in many cell types, including human PMCs. They take part in relaxing the arteries through the action of smooth muscle cells [37].

It was reported that KCNK3 expression and function are incomplete in patients with idiopathic hereditary pulmonary arterial hypertension and in the PAH rat model induced by

monocrotaline. To confirm that defective potassium channels lead to PAH phenotype, the KCNK3-selective KCNK3 blocker was used. After the long-term blockade, they demonstrated distal artery neomuscularization and increased proliferation of PAECs, PASMCs, and inflammation level. Therefore, the important role of KCNK3 in downregulating cell proliferation and modulation of pulmonary arterial tone is postulated [38].

Ma et al. carried out the whole-exome sequencing in a family with multiple members suffering from pulmonary arterial hypertension without identifiable mutations known to be associated with PAH and revealed five novel mutations in KCNK3 genes which resulted in a loss of function and reduction in potassium current which was confirmed electrophysiologically. They proved that channel function can be restored to a variable extent using the phospholipase A2 inhibitor (ONO-RS-082). Moreover, administration of prostacyclin analogue, treprostinil, or cAMP analogue, 8-bromo-cAMP, also causes KCNK3 activation. Similar conclusions were made in other studies where KCNK3 activation reduces the development of PH in the monocrotaline rat model. This approach modifying the receptor may constitute an interesting therapeutic strategy for human PAH. These studies highlight the significant progress made in using genetic testing in PAH patient care [39].

2.5. EIF2AK4 Mutation in Pulmonary Veno-occlusive Disease (PVOD) and/or Pulmonary Capillary Hemangiomatosis (PCH). The eukaryotic translation initiation factor 2 alpha kinase 4 (EIF2AK4) is a serine-threonine kinase that phosphorylates the alpha subunit of eukaryotic translation initiation factor 2 alpha (eIF2α) and changes gene expression in response to cellular stress. To date, EIF2AK4 mutations were identified in all familial PVOD/PCH genetically tested individuals and in 20-25% sporadic cases [40, 41].

EIF2AK4 regulates angiogenesis by altering gene expression in response to stress factors, mainly amino acid deficiency. It also participates in the cellular response caused by viral infection, glucose deficiency, or UV exposure. In response to hypoxia, EIF2AK4 reduces cell proliferation, thereby protecting against hypoxia-induced remodelling [42].

Chaveroux et al. evaluated the role of EIF2AK4 in maintaining homeostasis in oxidative stress. The authors exposed EIF2AK4–/– mice to imbalanced diet, partially depleted for leucine, and evaluated levels of oxidized proteins in mouse livers. They indicated increased oxidative stress, one of the well-established factors in PH pathogenesis proving thus that EIF2AK4 activity is necessary to resist oxidative stress caused by leucine deficiency [43].

Eichstaedt et al. studied the role of EIF2AK4 mutation as a potential reason of incomplete penetrance in the family with BMPR2 mutations. They conducted Sanger sequencing in all living members of the family burdened with HPAH caused by the BMPR2 mutation. They were looking for other mutations that could affect incomplete penetrance and reported that all family members who were suffering from pulmonary hypertension (confirmed with right heart catheterization) had an additional mutation in the EIF2AK4 gene. The results may support the hypothesis of a second hit model where a single mutation is linked with low penetrance; however, two independent mutations lead to a synergistic effect resulting in full-blown disease [44]. The occurrence of several mutations in genes related to PAH may be more frequent than originally thought.

Moreover, animal studies point out that the EIF2AK4/eIF2α/ATF4 pathway leads to induction of the TRB3 gene transcription in response to a leucine-deficient diet. TRB3 is considered to be responsible for regulating the BMP signalling pathway through modulation of the Smurf1 protein level by changing its ubiquitination and degradation. The decrease in TRB3 concentration in the case of the EIF2AK4 mutation may therefore lead to imbalance of Smurf1 ubiquitination. It may eventually interfere with TGF-β family transmission and be another reason exacerbating pulmonary hypertension development in the case of BMPR2 haploinsufficiency [45].

What is more, in the Barrios-Rodiles et al. study, the authors analyzed the dynamic protein-protein interaction networks in the TGF-β pathway and found EIF2AK4 to interact with SMAD4, SMAD1, ALK1, and endoglin (ENG) [46].

However, further research is necessary to determine the importance of these interactions in the pathogenesis of pulmonary hypertension.

The availability of molecular genetic diagnosis has opened up a new field for patient care, including genetic counselling. However, in spite of multidirectional genetic tests, to date, no clear therapeutic approach has been defined for patients with PAH. A large group of patients, despite the optimal currently available therapy, finally undergoes lung transplantation.

3. Epigenetic Modifications in Pulmonary Hypertension

The analysis of PAH patients' families indicates that the occurrence of the disease and its severity is modulated by additional factors besides the genetic mutations themselves. The epigenetic modifications could explain the nature of the PAH clinical course.

3.1. DNA Methylation. To date, the best studied epigenetic modification is the DNA methylation. As it is well known, the level of the gene expression is dependent on the promoter methylation degree. The greater the degree of promoter methylation, the stronger the transcription repression of a given gene. The addition of the methyl groups is catalyzed by DNMT (DNA methyltransferase) enzymes (including DNMT1, DNMT3A, and DNMT3B). There are at least two mechanisms, by which methylation blocks gene expression. In the first, the addition of a methyl group changes the spatial structure of the DNA sequence and prevents the transcription factor from attaching. In the second mechanism, methylation leads to the attachment of specific proteins, which prevents transcription factors from accessing chromatin. Both processes lead to transcription blockage [47].

Napoli et al. indicated that the BMPR2 promoter is significantly more methylated in patients with HPAH compared to controls. This epigenetic modification leads to PAH phenotype even in heterozygous mutant patients. It is

known along with the Gimelbrant et al. study that only one of the alleles—maternal or paternal—is expressed in the cell. That is why the hypermethylation of wild allele in heterozygous proband may cause null mutation and, as a result, change the penetration of PAH in affected families leading to the early onset of the disease. Using drugs that change the methylation level of the BMPR promoter may be a potential therapeutic strategy for HPAH [47–49].

The other already-found hypermethylated gene in a patient with PAH is ABCA1. It is responsible for coding the proteins that transport cholesterol and phospholipids across the cell membrane to form the high-density lipoprotein (HDL). In addition to this, it was revealed that evaluation of ABCA1 methylation may become a useful biomarker to separate patient at risk of PAH [47].

3.2. Modifications of MicroRNA (miRNA). The other dysfunctional mechanisms in PAH are microRNA (miRNA) deviations. miRNAs are small noncoding RNAs that work by regulating gene expression at the posttranscriptional level. The entire miRNA group has been shown to regulate over 60% of genes encoding proteins in a human cell. As a part of the RISC complex, miRNA causes inhibition of messenger RNA (mRNA) translation or regulates its degradation [50]. Studies show that dysregulation of miRNAs is well tolerated in normal tissues, but it can influence the function of the tissues experiencing pathological conditions. Changes in the concentration of miRNA molecules have been shown in patients with PAH. The study revealed that decreased expression of miR-124 causes an increased level of polypyrimidine tract-binding protein 1 (PTBP1, also known as hnRNPI), which results in elevated pyruvate kinase (PKM) isoform ratio. The increased PKM2/PKM1 ratio enables anaerobic catabolism, metabolic reprogramming, and enhanced proliferative capacity [50].

Interestingly, it has been proved that pharmacological manipulation of PKM2 activity with miR-124 overexpression or PTBP1 knockdown abolishes the imbalance between the proliferating and eliminated fibroblasts in the pulmonary vascular bed [51].

Despite the numerous studies taking under consideration the miRNA role, a small part of the complex network of gene expression-related relationships has been known to date. Unfortunately, most of these studies did not bring conclusive results that could be used in a new diagnostic and therapeutic approach in patients with PH.

3.3. Protein Acetylation and Deacetylation. Subsequent posttranscriptional modification includes histone acetylation and deacetylation. The process is catalyzed by HAT (histone acetyltransferases) and HDAC (histone deacetylases) which modify lysine residues within the N-terminal tail. In consequence of acetylation, the chromatin forms more relaxed structure and promotes gene transcription.

Sirtuin 1 (SIRT1) is one of the HDAC, which via histone deacetylation represses gene expression. The SIRT1 failure was reported in PAH patients' PASMCs, which results in an acetylation/deacetylation imbalance. It augments the PASMC proliferation and leads to vascular remodelling and increased vascular resistance in the pulmonary bed. It was proven, in knockout mice, that deficiency in SIRT1 gene aggravates chronic hypoxia-induced pulmonary hypertension [52].

To reverse this process, the SIRT1 activator resveratrol was used in PAH rats exposed to hypoxia. Interestingly, as a result, the retarded PASMC proliferation and increased apoptosis were noted [53].

Sirtuin 1 also alters mitochondrial metabolism by activating peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1a), which is a master regulator of mitochondrial biogenesis. It is also an important gluconeogenetic and glycolytic enzyme. SIRT1 deacetylates HIF-1, which represses HIF-1-dependent glycolytic enzymes (i.e., LDH, PFK-1, PGK-1) and glucose transporting proteins (GLUT1, GLUT3). Under anaerobic conditions, the NAD+ concentration (SIRT1 cofactor) decreases, which is followed by the SIRT1 reduction and HIF-1α activation [54].

3.4. Changes in Phosphoproteome-Kinase Activity

3.4.1. The Src Family of Kinases. The Src Family of Kinases (SrcFKs) constitutes a group of redox-sensitive tyrosine kinases expressed in vascular smooth muscle cells (VSMCs). They contain reactive cysteine residues at physiological pH, and a shift in cellular redox state results in cysteine oxidation, activating SrcFK to stimulate tyrosine phosphorylation. ROS-induced SrcFK activation is postulated to regulate VSMC proliferation and apoptosis [55]. In response to chronic hypoxia, SrcFKs stabilize HIF-1α and HIF-2α and stimulate prosurvival transcription factors [56]. SrcFKs and their downstream effectors like STAT3 might represent novel therapeutic targets for PAH management.

3.4.2. The Rho-Associated Protein Kinase (ROCK) Pathway (Figure 1). Rho-associated protein kinase (ROCK) belongs to the AGC (PKA/PKG/PKC) family of serine-threonine kinases and acts as the effector of the G protein Ras homolog family member A (RhoA). It is well established that ROCK participates in regulating vasoconstriction, cellular proliferation, apoptosis, and migration by exerting multidirectional influence on many signalling pathways [57]. It has been shown that Rho kinase concentration and activity are increased in blood cells and pulmonary arteries of patients with PAH compared to controls [58]. It is in line with previous findings from PH-induced animal models. Furthermore, the study by Do et al. [58] revealed a correlation between ROCK activation and PH severity (measured by mPAP) or disease duration. These relations were demonstrated for IPAH, CTD-PAH, and CHD-PAH, but not for CTEPH. Nevertheless, a similar correlation was not observed with respect to cardiac index, right atrial pressure, or plasma BNP concentration [58–61]. Many studies show that the RhoA/ROCK signalling pathway activation leads to vasoconstriction via the Ca^{2+}-dependent and Ca^{2+}-independent mechanisms. RhoA stimulates ROCK to phosphorylate myosin light chain kinase (MLCK) leading to its subsequent activation. As a result, it leads to accumulation of phosphorylated myosin light chain (P-MLC) and subsequent interaction with actin

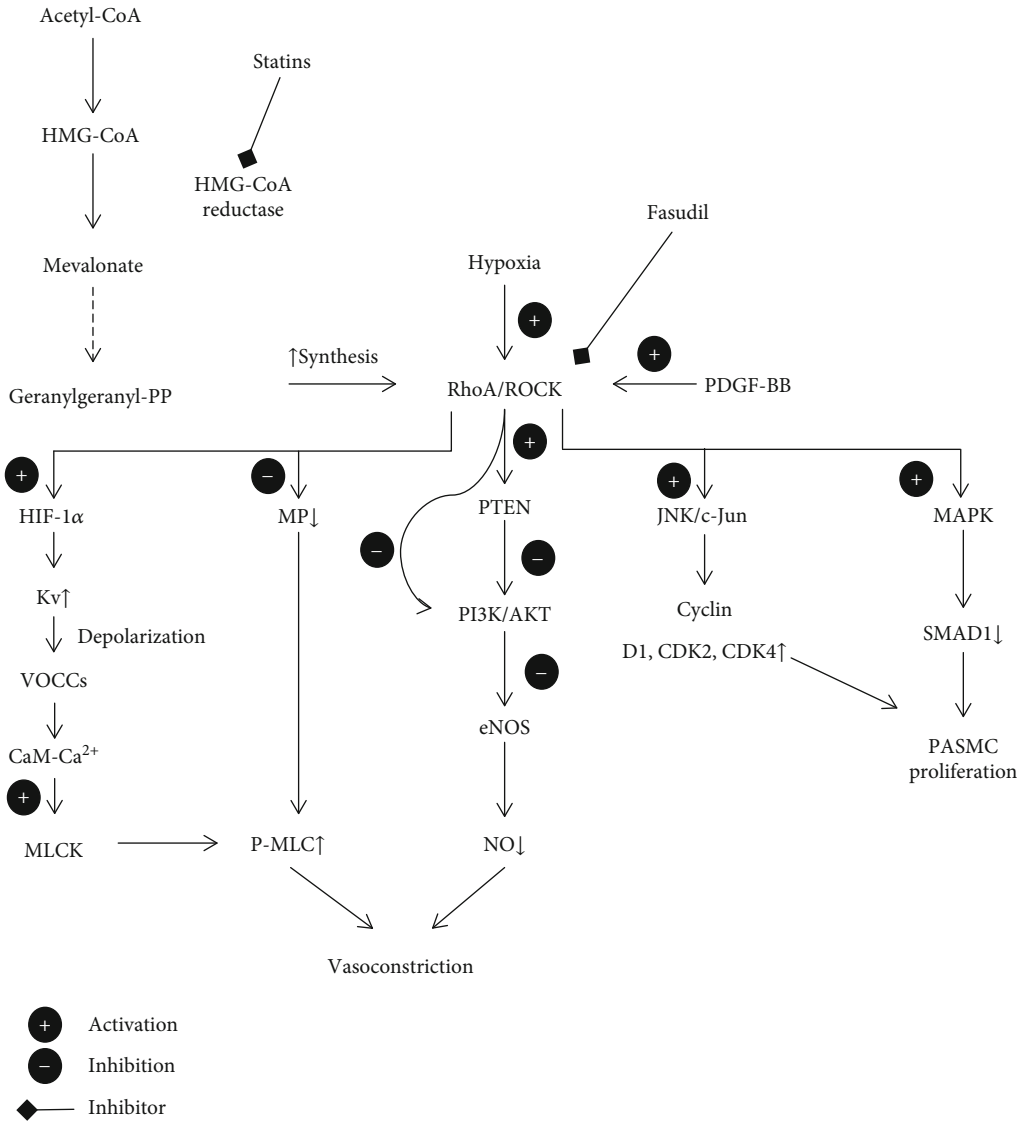

Figure 1: RhoA/ROCK signalling pathway in PH pathogenesis. CaM: calmodulin; eNOS: endothelial nitric oxide synthase; Kv: voltage-gated K+ channels; MAPK: mitogen-activated protein kinase; MLCK: myosin light chain kinase; MP: myosin phosphatase; NO: nitric oxide; PASMC: pulmonary arterial smooth muscle cell; PDGF-BB: Platelet-Derived Growth Factor BB; P-MLC: phosphorylated myosin light chain; PTEN: phosphatase and tensin homolog; RhoA: G protein Ras homolog family member A; ROCK: Rho-associated protein kinase; VOCCs: voltage-operated Ca^{2+} channels. (+) activation, (−) inhibition, and (←) inhibitor.

resulting in smooth muscle cell contraction in a Ca^{2+}-independent manner. This process may also affect left ventricular (LV) relaxation and LV filling pressure [62].

Indirectly, the RhoA signalling pathway may also potentiate pulmonary bed vasoconstriction by activating HIF-1α in hypoxic conditions. It induces the voltage-gated K+ (Kv) channel expression in PASMCs and leads to depolarization and activation of voltage-operated Ca^{2+} channels (VOCCs). Intracellular Ca^{2+} binds with calmodulin (CaM) and stimulus myosin light chain kinase (MLCK) and induces smooth muscle cell contraction in Ca^{2+}-dependent mechanism [61, 63–67].

Wei et al. [68] pointed out that the Rho signalling pathway enhances PAH by stimulating PASMC proliferation via disrupting BMP2/SMAD1 signal transduction. The intact signalling pathway contains BMP2-mediated phosphorylation of SMAD1 at the C-terminal (Ser463/465) and its subsequent translocation into the nucleus. However, it was proved that this process may be interrupt by SMAD1 phosphorylation at the linker region (Ser206) by mitogen-activated protein kinase (MAPK, MEK/ERK) which is one of the downstream signalling molecules of the RhoA/Rho kinase pathway [69]. This alternative SMAD1 modification leads to its ubiquitination and degradation and enhances PASMC proliferation. In the same study [70], ROCK and ERK1/2 inhibitors restored BMP2/SMAD1 function and reduced proliferation level.

It is well known that RhoA is activated by Platelet-Derived Growth Factor BB (PDGF-BB). Tang et al. [69] proved that cells stimulated by PDGF-BB via ROCK and

subsequent JNK/c-Jun pathway increase cell cycle protein translation (cyclin D1, CDK2, and CDK4) and promote G0/G1 to S phase passage. It is the other way how the RhoA/ROCK signalling pathway induces PASMC proliferation.

The other dysfunction caused by RhoA that contributes to PH endothelial dysfunction and potentiates disease progression is the decrease in nitric oxide bioavailability. ROCK inhibits eNOS expression via eNOS mRNA destabilization [71]. Additionally, ROCK decreases activity of the PI3K/AKT pathway directly or by PTEN phosphorylation, which in turn leads to reduced eNOS activity and nitric oxide production. It was pointed out that blockage of the RhoA/ROCK signalling pathway is able to restore NO pool [72]. It was proved by using mutant endothelial cells deficient in RhoA. Furthermore, using statins that block HMG-CoA reductase and inhibit geranylgeranyl pyrophosphate production which is necessary for RhoA synthesis presented a similar effect [73]. According to Absi et al., simvastatin may promote vasodilation also by inhibition of Ca^{2+} influx through the L-type of Ca^{2+} channels [74].

4. Metabolism Alterations in Pulmonary Hypertension

The up-to-date known mutations and epigenetic gene modifications found in patients with pulmonary hypertension do not provide a complete answer regarding the causes of the disease. This points at the role of regulating pulmonary vascular function affecting further levels of signalling cascades.

4.1. Energetic Metabolism in PH. Searching for other factors modifying the course of PAH, the deviations in the PAEC metabolism aroused scientists' interest. It was shown that these cells reprogrammed their metabolism by replacing energy acquisition in the Krebs cycle with glycolysis. This phenomenon, known as the Warburg effect, was mostly known as characteristic for autonomic cancer cells. These mutated cells, even in the presence of sufficient oxygen supply, prefer anaerobic respiration to support high proliferation. What is more, the cells are less likely to be exposed to reactive oxygen species. This leads to the "metabolic theory" of PH which indicates dysfunctions in cellular and mitochondrial metabolism as the cause of the disease (Figure 2).

The main product of the glycolysis is a pyruvate. Subsequently, this acid in aerobic conditions is metabolized by PDH (pyruvate dehydrogenase) into acetyl-CoA and enters the tricarboxylic acid cycle (TCA cycle). It was proved that PDH activity is reduced in PAH PAECs and the pyruvate is mostly reduced by lactate dehydrogenase A (LDHA) into lactate. It is in line with the results of few studies, where the increased level of lactate was noted in human plasma and rats' lungs and hearts [75–77]. Additionally, increased transcription and translation of LDHA was proved in the lungs and hearts of MCT-induced PAH rats [75]. The increased level of lactate leads to acidosis which further impairs right ventricle function.

The second metabolic deviation confirmed in PAH cells is increased fatty acid metabolism. Chen et al. pointed out higher levels of fatty acids, L-carnitine, acetyl-L-carnitine, and several long-chain acylcarnitines compared to controls. It may explain partially the decreased activity of PDH. Along with the Randle effect, fatty acid oxidation inhibits glycolysis and glucose transport via pyruvate dehydrogenase and phosphofructokinase suppression. Interestingly, knockout mice deficient in malonyl-coenzyme A gene with reduced fatty acid oxidation do not present pulmonary vasoconstriction and pulmonary arterial hypertension in hypoxic environment [75, 78].

The third metabolic change concerns the glutaminolysis. Normally, this process does not occur in the myocardium or is residual, but it was proved that it is aggravated in hypertrophic hearts. During glutaminolysis, the glutamine is transformed into α-ketoglutarate which enters the Krebs cycle. This pathway provides both energy and substrates for the synthesis of nucleic acids, proteins, and lipids, thus compounds necessary for proliferation. The level of glutamine concentration was pointed out as a biomarker of right ventricle hypertrophy which was confirmed in a mouse model [77, 79, 80].

The other important alterations relate to abnormal mitochondrial function. Commonly, mitochondria are considered to be responsible just for generating cells' supply of adenosine triphosphate (ATP); however, their functions are considerably more complex. In PASMCs, they are also oxygen sensors responding to decreased oxygen supply with hypoxic pulmonary vasoconstriction (HPV). It redirects blood to better oxygenated lung areas, while maintaining the appropriate ventilation : perfusion ratio. However, hypoxia reduces mitochondrial reactive oxygen species (ROS) and hydrogen peroxide production. It changes the cell's redox signalling pathways and disrupts metabolic balance. Subsequently, it begins pathological activation of transcription factors like cMyc, forkhead transcription factor, and hypoxia-inducible factor (HIF-1α). Finally, it ends with pyruvate dehydrogenase inhibition and metabolic shift in accordance with the Warburg effect [79].

Additionally, some recent studies revealed that PAH patients' mitochondria in pulmonary artery smooth muscle cells are twice more fragmented, when compared to controls. This phenomenon was observed in hypoxia- or monocrotaline-induced PAH rat cells as well. To explain, it should be noted that mitochondria are continuously dividing and joining together which is called fusion and fission and known as mitochondrial dynamics. The more excessive mitochondrial fragmentation is chiefly upregulated by dynamin-related protein 1 (DRP1) which is activated by the cyclin B1/CDK1 which reflects HIF-1α stimulation. Extended HIF-1α activation and associated mitochondrial fission promote glycolytic shift, proproliferative phenotype, and right ventricle myocyte energy ATP synthesis deviation. What is more, mammalian mitochondria play a key role in initiating apoptosis by releasing various cytotoxic proteins which starts different signal pathways leading to cell death. All the abnormalities that take place in mitochondrial dynamic in PAH PASMC impair apoptosis and aggravate vascular anomalies [81–83].

4.2. Regulation of Oxidative Stress: The HIF-1α Upregulation in Pulmonary Hypertension. The HIF protein is a

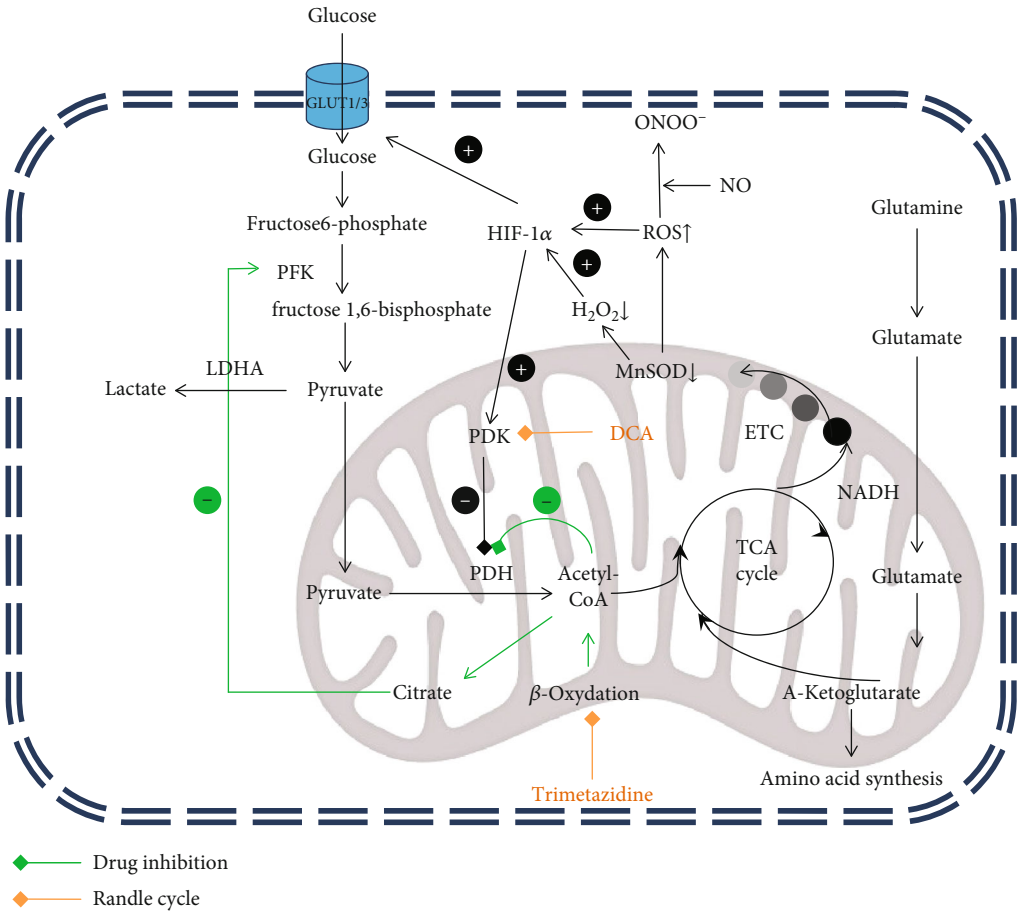

Figure 2: Metabolism alteration in pulmonary hypertension. DCA: dichloroacetate; ETC: electron transport chain; HIF-1α: hypoxia-inducible factor; LDHA: lactate dehydrogenase A; MnSOD: mitochondrial superoxide dismutase; PDH: pyruvate dehydrogenase; PFK-6: phosphofructo-1-kinase; ROS: reactive oxygen species; TCA cycle: tricarboxylic acid cycle. Orange arrow: drug inhibition; green arrow: Randle cycle.

heterodimer of the HIF-1α or HIF-2α and HIF-1β subunits. It is a main transcriptional regulator in a process of cells' adaptation activated in response to hypoxia. Under aerobic conditions, HIF-1α is not activated because of hydroxylation promoted by hydrolases requiring the presence of oxygen. The HIF-1 is then ubiquitinated which is followed by the proteasome proteolysis. However, in hypoxia, the HIF-prolyl hydroxylases (HPHs) are blocked, which leads to stabilization of the HIF-1α subunit with a subsequent increase in its cellular concentration. This in turn results in transcriptional activation of over 40 metabolism-regulating genes [84].

HIF-induced transcriptional activity is aimed at restoring the optimal oxygen supply by modifying genes, whose products regulate vascular tone and stimulate angiogenesis and erythropoiesis.

Several studies have shown the twofold higher HIF-1α expression in epithelial cells of patients with IPAH when compared to the control group. The levels of this protein were higher in the cells of patients with IPAH even under normoxia. Its higher expression in plexiform lesions was also shown, which indicates the role of HIF-1α in promoting the proliferative vasculopathy of IPAH [85].

Higher concentration of plasma HIF-1α may be explained by "pseudohypoxic" environment found in PAH mitochondria. The reason for that is the mitochondrial superoxide dismutase (MnSOD) dysfunction found in PH. MnSOD is normally responsible for scavenging reactive oxygen species (ROS) and converting them into hydrogen peroxide removed further by catalase or glutathione peroxidase (GPx). The decreased level and activity of MnSOD2 pointed out in PAH (mostly according to epigenetic silencing) resulted in reduced hydrogen peroxide (H_2O_2) production, which creates hypoxia-like status that activates HIF-1α [86]. Furthermore, reactive oxygen species have been shown to stabilize the HIF-1α and increase its concentration.

This hypothesis is in line with studies in which the overexpression of human extracellular superoxide dismutase (EC-SOD) in rats with monocrotaline- (MCT-) induced pulmonary hypertension significantly reduces pulmonary hypertension development and SMC proliferation [87, 88].

Reactive oxygen species also affect nitric oxide (NO) metabolism. It is synthesized by ECs and is one of the key factors regulating pulmonary vascular reactivity, which affects also SMC proliferation and migration. Furthermore, it is well known that the bioavailability of nitric oxide is lower in

patients with PH, and the disturbance in that signalling pathway is one of the components leading to the development of PH [89].

Increased oxidative stress and ROS concentration contribute to the severity of pulmonary hypertension by potentiating superoxide anion with nitric oxide reaction. It results in peroxynitrite anion formation. As a result, the pool of available nitric oxide is reduced. This is consistent with observations that the pulmonary pool as well as the total body NO is lower in IPAH patients, as compared with healthy controls. Indeed, studies showed that in EC cells in which the SODs were knocked down by RNA interference, the NO production was decreased [31].

HIF-1α induces also metabolic changes mentioned above by upregulating PDK expression, which results in pyruvate dehydrogenase kinase inhibition leading to glycolytic shift. HIF-1α also enhances the Warburg effect by activating glycolysis genes, GLUT1 and GLUT3 transporters, and by regulating the cellular pH [86]. Moreover, HIF-1α is also capable of inhibiting the hypoxia-induced cell death by limiting hypoxia-induced p53 phosphorylation. It also increases the rate of cell proliferation by induction of differentiation inhibitors. This results in the expansion of less differentiated cells with a higher mitotic potential. Their spread is further facilitated by increased expression of extracellular enzymes [84, 90].

5. Novel Biomarkers in Pulmonary Hypertension Diagnosis: Results from the Genomic, Transcriptomic, Proteomic, and Metabolomic Studies

As long as we are not able to treat pulmonary hypertension successfully, studies were also focused on identifying the new risk factors and risk assessment methods, so as to more accurately specify the group of patients requiring intensive supervision.

One of the most common risk assessment methods used to predict survival in patients with pulmonary arterial hypertension is the Registry Risk Score for Pulmonary Arterial Hypertension scale (REVEAL scale). It provides a risk assessment tool but requires using a variety of testing procedures including invasive diagnostic methods [91]. For this reason, the new noninvasive biomarkers assessing the severity and predicting a more advanced course of the disease which will be able to distinguish patients requiring more intensive management strategies are also sought.

5.1. Biomarkers Already Used in Clinical Practice. One of the paramount plasma biomarkers well established in PH are brain natriuretic peptide (BNP) and N-terminal brain natriuretic prohormone BNP (NT-proBNP). Simpson et al. were focused on finding the correlation among NT-proBNP, the plasma concentration of soluble suppression of tumorigenicity 2 (sST2) protein, and the disease severity or survival rate in group 1—the PAH patients. Both markers revealed to be useful as predictors for future mortality and disease severity. The primary sensitivity of NT-proBNP assessed in follow-up was greater than that of BNP. Contrary to Simpson et al., Cavagna and colleagues consider BNP as more specific (90% vs. 87%) and sensitive (60% vs. 45%) [92, 93]. BNP appears to be more specific in distinguishing PH patients, as far as it is less susceptible to elevation caused by comorbidity of renal dysfunction frequent in this population. On the other hand, NT-proBNP seems to be superior to BNP as a mortality predictor [94].

The usefulness of implementing the NT-proBNP to evaluate the risk of PH was also confirmed in the DETECT study. The plasma level of NT-proBNP is one of the components in the DETECT algorithm, which identify the subgroup of patients with systemic sclerosis at risk of PH that should be referred for echocardiography and subsequently in some cases for right heart catheterization (RHC) [95]. Furthermore, incorporating the sST2 and NT-proBNP data into the REVEAL scale improved its effectiveness [92]. In line with Simpson et al., Mirna et al. confirmed sST2 to be an effective general PH biomarker irrespective of PH subgroup [96]. At this point, it is worth noting that sST2 may be also elevated in other disease such as heart failure, myocardial infarction, or acute aorta dissection [97–100].

The second marker incorporated to the DETECT algorithm is uric acid (UA) level. It was confirmed that patients with PH have elevated level of UA which was connected with increased odds of PAH diagnosis at RHC in these patients. A UA level > 6.2 mg/dL was linked with fourfold higher chance of PAH diagnosis. One UA serum unit (also within normal limits) was found to raise mortality risk in PAH by 14%. It was also associated with disease severity. As with BNP level, combining UA with other noninvasive biomarkers increased diagnostic possibilities working synergistically. Furthermore, after incorporating vasodilator therapy, the UA level drops, which was associated with a reduction in total pulmonary resistance and improved survival [101–103].

The answer regarding pathophysiology of UA level and PAH patients' outcomes was partially characterized in the hypoxia-induced PH rat model, where increased xanthine oxidoreductase (XOR) activity was linked with hypoxic exposure, right ventricle hypertrophy, and pulmonary vascular remodelling. Additionally, UA decreases the NO and cGMP production in PAECs by activating arginase and promoting its attachment with L-arginine [101, 104].

Some other studies show a similar prognostic usefulness present in cardiac troponin (cTnT) or high-sensitivity troponin (hsTnT) wherein the elevated level was in line with worse prognosis and increased risk of hemodynamic destabilization. The troponin value over 30 pg/mL was strongly correlated with death within 12 months. Heresi et al. indicated higher hsTnT than cTnT sensitivity and proved that hsTnT is more precise in WHO functional class ≥ 2 patient identification than NT-proBNP or H-FABP. An elevated hsTnT level also corresponded with systolic RVC dysfunction, worse echocardiographic parameters (larger right atrial area), and worse 6 min walk distance (6-MWD) results [105–107].

5.2. Biomarkers Being in the Study Phase and Capable of Showing Some Clinical Utility. Simultaneously, Mirna et al. indicated heart-type fatty acid binding protein (H-FABP),

soluble urokinase-type plasminogen activator receptor (suPAR), and growth differentiation factor-15 (GDF-15) as more specific molecules. Different levels of all pointed-out biomarkers are characteristic for postcapillary PH (group 2), while H-FABP may be also increased in pulmonary hypertension that is associated with lung disease and/or hypoxia (group 3). This would make these indicators useful for determining the causes of the disease [96].

The group of new biomarkers that may be used to distinguish a patient with COPD with comorbidity of secondary PH includes inflammatory indexes such as platelet-to-lymphocyte ratio (PLR), neutrophil-to-lymphocyte ratio (NLR), and systemic immune-inflammation index (SII). Zuo et al. proved that all these indexes are significantly higher in a patient with acute exacerbation and PH compared to the control group just with acute exacerbation of COPD. NLR was characterized by higher discriminative ability than PLR and SII. It is also mentioned to be a systemic inflammation indicator associated with the worse prognosis [108].

Osteopontin (OPN), a small glycoprotein, may also be useful to evaluate PH progression. Its elevated level is considered to be connected with myocardial dysfunction and also acts as modulating factor of proliferation and remodelling in pulmonary bed cells. It is an additional risk factor of mortality in PAH. Simultaneous elevated levels of NT-proBNP and OPN in patients with PAH were associated with an 11-fold higher risk of death compared to patients with normal biomarker results [109, 110].

According to the metabolic shift described above, the composition of components essential to sustain altered metabolic pathways is different in PH patients. The elevated level of TCA and glycolysis intermediates as well as nucleosides and ketone bodies was found. After comparing diabetes mellitus, heart failure, and PAH patients' metabolome, Rafikov et al. distinguished eleven metabolites that may be useful in patient screening which are considered to be PAH metabolic fingerprint. As they noticed in their studies, the unique metabolic biomarkers that they characterized are specific for PAH patients and additional metabolic profiling should be performed for patients from other WHO PH groups [111].

Evaluating specific circular RNA (circRNA) was also described as an effective mechanism for predicting the IPAH. Circular RNA is a group of noncoding RNAs that was primarily considered to be a "junk" genetic material. A characteristic feature is back-splicing while two sites of RNA are linked together forming the final molecule [112]. Zhang et al. found that the circRNA (circ_0068481) level is significantly higher in patients with IPAH. As far as it has circular structures, it is more stable and resistant from nuclease degradation which makes it a better serum biomarker. The circ_0068481 was shown to have high sensitivity and specificity (respectively, 74.39% and 98.78%) in distinguishing PAH patients. A more elevated level was found in case of comorbidity of IPAH and right ventricular failure and in a patient who eventually died in a short time because of IPAH. It suggests circ_0068481 as a biomarker of poor outcome [113]. Additionally, the role of circRNA as a biomarker of CTEPH was also postulated. It was revealed that some circRNA may have an important role in pathogenesis via interaction with specific miRNAs [114].

Arvidsson et al. revealed that extracellular matrix (ECM) whose role in the pathogenesis of PH was confirmed could also play an important role in diagnosing patients. ECM takes part in vascular remodelling, leads to wall stiffness, and favours proliferation in PH. It was proved that the MMP-7 level is significantly higher compared to that in the healthy control group and lower in PAH than that in other WHO PH groups, which indicates in turn that MMP-7 may be a useful tool in allocating patients in each subgroup. The MMP-7 sensitivity and specificity were estimated to be 58.7% and 83.3%, respectively, in distinguishing patients with PAH from all patients with dyspnoea. In the same study, the metalloproteinase-2, metalloproteinase-7, metalloproteinase-9, and metalloproteinase-12 levels were also increased in PAH samples but the meaning of these results needs further investigation [115].

Noteworthily, there are also several other biomarkers including circulating angiogenic modulatory factors (VEGFR1, CRP, endostatin, or PCEB-ACE) or inflammatory markers (galectin-3, GDF-15) which were related to the pathophysiology of pulmonary hypertension. However, due to the relatively low specificity, in our opinion, they cannot find the usefulness in clinical practice.

6. Novel Drug Targets Modifying the Energetic Metabolism Alterations and Limiting the Posttranslational Modifications

6.1. Targeting Energetic Metabolism. As the metabolic mechanisms underlying PAH were explored, numerous studies were initiated to restore physiological signalling pathways. To recover the PDH function, dichloroacetate (DCA), a PDK inhibitor, was used. DCA is a pyruvate analogue that increases mitochondrial-dependent apoptosis in abnormal pulmonary artery cells not affecting other vessels and normal cells. It increases cytochrome c level and hydrogen peroxide (H_2O_2) production. H_2O_2 higher concentration is a result of PDH and electron transport chain restoration made by DCA. Cytochrome c and H_2O_2 lead to potassium voltage-dependent (Kv) channel activation and Kv1.5 expression upregulation. The increased outflow of potassium ions and cytochrome c interaction with caspase 3 promote DCA-dependent apoptosis. The other way that H_2O_2 may promote apoptosis is enhancing the likelihood of mitochondrial permeability transition pore (MTP) opening. MTPs increase mitochondrial membrane permeability and lead to mitochondrial swelling, which results in membrane injury and cytochrome c upconcentration [82, 116, 117].

It was proved in preclinical studies that oral DCA improves myocyte contractility, right ventricular function, and cardiac electrical remodelling. Its activity is limited to abnormal or proliferating PASMC which minimalizes the toxicity. DCA was used in patients with glioblastoma multiforme in a long-term clinical study, and now, the usefulness of DCA treatment is being checked in PAH [78, 82, 116, 117].

The second drug with potentially beneficial effects in PAH therapy is trimetazidine—a selective inhibitor of fatty acid beta-oxidation. It reduces activity of the mitochondrial enzyme long-chain 3-ketoacyl coenzyme A thiolase (3-KAT). Blocking thiolase, in line with the Randle cycle and reciprocal relationship between fatty acid oxidation and glucose oxidation, results in increased glucose oxidation. Additionally, trimetazidine restores intracellular phosphocreatine resources, thereby optimizing cell metabolism, and acts cytoprotectively. The trimetazidine effect on cardiomyocytes is to prevent adenosine triphosphate (ATP) deficiency, to reduce cellular sodium and calcium accumulation, and in this way to decrease the intracellular acidosis. Trimetazidine has also been shown to have beneficial effects on remodelling and improving left ventricular ejection fraction [118, 119].

The other promising target for drugs that may be used in PAH treatment is mitochondrial fission inhibition. By decreasing the DRP1-dependent signalling pathway with mitochondrial division inhibitor 1 (Mdivi-1), the PASMC proliferation rate is reduced and right ventricle myocyte energy ATP synthesis is restored. Mdivi-1 causes cell cycle arrest in the G2/M mitotic phase. The antiproliferative effect of Mdivi-1 was already proved in monocrotaline- and hypoxia-induced pulmonary hypertension in human PAH PASMCs and in rodent models [79, 81, 82].

6.2. Targeting Oxidative Stress and Inflammation. Luo et al. characterized the new CD146-HIF-1α signalling pathway which might become an interesting therapeutic target. CD146 is a cell adhesion molecule found in the vascular wall that plays a role in PASMC differentiation, migration, and proliferation. It is found in the crosstalk of signalling pathways such as hypoxic, PDGF-BB/PDGFRβ and the Notch one. All of them are widely recognized as relevant in PAH pathogenesis and are pointed to be promising avenues in PAH treatment. Disruption of the CD146-HIF-1α axis leads to limiting PAH progression but does not reverse the dysfunctions completely [120].

Other studies indicate the possibility of using doxycycline, a matrix metalloproteinase and angiogenesis inhibitor. Additionally, interferon 2α might be used as a proliferation and collagen synthesis inhibitor and a macrophage and immune system modulator [121].

6.3. Therapeutic Implications of MicroRNA in Pulmonary Hypertension. Since numerous studies show that dysregulation of miRNAs in the pulmonary vasculature results in abnormalities in gene expression and contributes to the pathogenesis of PAH, the restoration of miRNA expression to physiological levels may constitute an interesting approach for managing pulmonary hypertension, especially PAH. This may be obtained using the anti-miRNA (anti-miR) oligonucleotide-based and miRNA mimic-based approaches. Nevertheless, the administration of miRNA should be targeted to the specific vascular cells (endothelial cells, vascular smooth muscle cells, and fibroblasts), in order to minimize any off-target effects on other cells. Moreover, the dose of anti-miR or miRNA mimics delivered should be considered carefully in order to avoid the off-target effects, which requires developing the vascular cell-specific delivery methods along with techniques providing regulated miRNA release [122].

Interestingly, a recent study by Chen et al. [123] demonstrates that dysregulation of the dynamin-related protein 1 adapter proteins and mitochondrial dynamic protein of 49 and 51 kDa (MiD49 and MiD51) increases mitotic mitochondrial fission and promotes pulmonary arterial hypertension. What is more, the authors have shown that silencing MiDs causes cell cycle arrest through an ERK1/2- and CDK4-dependent mechanism, decreases cell proliferation rate, and increases apoptosis. In an experimental animal model, nebulizing miR-34a-3p or siMiDs resulted in regression of pulmonary hypertension which could constitute a novel therapeutic strategy, based on a molecularly defined new drug target.

6.4. Kinase Activity as a Therapeutic Target in Pulmonary Hypertension. Kinases hold a promise to become potential drug targets in pulmonary hypertension.

The Rho kinase activation in PAH sensitizes to calcium resulting in greater vasoconstriction which is not reversed by conventional vasodilators and likely contributes to vascular stiffening [124]. Rho kinase inhibitors, such as fasudil, have been studied in human PAH cohorts [125], and the results suggest that they might be effective and safe in PAH patients with right ventricular failure [126, 127]. Fasudil was recently also demonstrated to be effective in the short-term treatment of subjects with pulmonary hypertension resulting from left ventricular failure with preserved ejection fraction (HFpEF) [62]. Although the elimination half-life of a drug is short, recent studies are guided to extend its working time. Although fasudil appears to be a promising drug in PH treatment, further trials are needed to evaluate the long-term effects of its use.

Considering the role of SrcFK in promoting ROS-induced vascular remodelling, targeting SrcFK holds some therapeutic promise in PAH [128]. Imatinib, a tyrosine kinase inhibitor which is commonly used in the therapy of chronic myeloid leukaemia (CML) due to its inhibitory action on the chimeric BCR-ABL tyrosine kinase, is also a nonspecific inhibitor of the PDGF receptor and may reverse PH, as demonstrated in an experimental model [129]. Significant improvement in 6-MWD in patients receiving imatinib was found in the randomized double-blind placebo-controlled IMPRES trial. In this study, imatinib improved exercise capacity and hemodynamics in patients with advanced PAH, but serious adverse events and study drug discontinuations were common [130]. Interestingly, dasatinib, another BCR-ABL and PDGF receptor blocker was, demonstrated to increase PAH development when used in patients with CML [131].

6.5. Modification of Protein Nitration/S-Nitrosylation as a Therapeutic Approach in PAH Management. Preventing some posttranslational modifications, as it results from the proteomic studies, may also limit the progression of pulmonary hypertension [132]. The nitration of tyrosine to 3-

nitrotyrosine is an oxidative modification of tyrosine by nitric oxide and is associated with many diseases, and targeting of protein kinase G- (PKG-) I represents another potential therapeutic strategy for pulmonary hypertension [133]. What is more, some data suggests that inactivation of the RhoA by nitric oxide occurs via S-nitrosylation, which suggests that posttranslational modification and inactivation of RhoA by S-nitrosylation constitute a signalling mechanism that contributes to the regulation of vascular SMC proliferation [134].

7. Conclusions

To date, numerous studies on the aetiology and pathophysiology of pulmonary hypertension at molecular level were conducted. Simultaneously, some clinical studies have shown potential usefulness of some well-known and widely recognized cardiovascular biomarkers, such as BNP, NT-proBNP, hsTnT, sST2, osteopontin, or serum uric acid, in diagnosing and prognosing the outcome of pulmonary hypertension. The enthusiasm from their potential clinical usefulness in diagnosis and management of pulmonary hypertension is cooled down by their low specificity accompanied with numerous cardiovascular comorbidities of PH subjects. On the other hand, a large body of basic research studies provides us with novel molecular pathomechanisms, biomarkers, and drug targets, according to the evidence-based medicine principles. Unfortunately, the simple implementation of these results to clinical practice is impossible due to a large heterogeneity of the pathophysiology of PH, where the clinical symptoms constitute only a common denominator and a final result of numerous crosstalking metabolic pathways.

Therefore, future studies, based mostly on translational medicine, are needed in order to both organize better the pathophysiological classification of various forms of pulmonary hypertension and define precisely the optimal diagnostic markers and therapeutic targets in particular cases of PH.

Conflicts of Interest

The author(s) declare(s) that they have no conflicts of interest.

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Research Article

The Inhibition of P-Selectin Reduced Severe Acute Lung Injury in Immunocompromised Mice

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In an immunocompetent host, excess infiltration of immune cells in the lung is a key factor in infection-induced severe acute lung injury. Kidney transplant patients are immunocompromised by the use of immunosuppressive drugs. Immune cell infiltration in the lung in a renal transplant recipient suffering from pulmonary infection is significantly less than that in an immunocompetent host; however, the extent of lung injury in renal transplant patients is more serious than that in immunocompetent hosts. Therefore, we explored the role of platelet activation in a Klebsiella pneumoniae-induced lung injury model with P-selectin gene knockout mice or wild-type mice. Our study suggested that the inhibition of platelets reduced severe acute lung injury and increased survival after acute lung infection in mice. In addition, P-selectin expression on the surface of platelets in mice increased after administration of immunosuppressive drugs, and the extent of lung injury induced by infection decreased in P-selectin gene knockout mice. In conclusion, p-selectin plays a key role in severe acute lung injury in immunocompromised mice by reducing platelet activation and inflammatory processes.

1. Introduction

Renal transplantation is the best treatment for end-stage renal disease. Due to the use of immunosuppressive drugs, the immunity of kidney transplant recipients is obviously impaired, which easily induces postoperative infection, especially pulmonary infection [1, 2]. Approximately 10-20% of patients suffer from pulmonary infection after kidney transplantation [3]. Severe acute lung injury caused by infection is the main cause of early death [4]. At present, there is no effective treatment for severe acute lung injury.

When the body is infected, the immune system is activated and defends against infection through the following processes. First, macrophages in the alveoli eradicate pathogens, produce chemokines, and induce circulating polymorphonuclear leukocytes (PMNs) to accumulate in pulmonary microvessels [5]. Second, the binding of selectin and its ligand mediates the interaction between PMNs, platelets and vascular endothelial cells, which induces the PMNs to adhere to the vascular intima [5]. Third, activated PMNs migrate through the blood vessel wall to the lung tissue, produce inflammatory mediators, and attract more immune cells to aggregate in the lung; moreover, activated PMNs release active substances to eradicate pathogens [6]. Previous studies have suggested that excessive PMN infiltration in the lung is a key factor leading to severe acute lung injury [7–10]. However, continuous use of immunosuppressive drugs after renal transplantation reduces the immunity of patients. When pulmonary infection occurs, PMN infiltration in the lung in a renal transplant recipient is significantly less than that in an immunocompetent host; however, the extent of lung injury in renal transplant patients is more serious than that in immunocompetent hosts. Therefore, we hypothesized that other factors play an important role in severe acute lung injury induced by pulmonary infection after renal transplantation.

Numerous studies have shown that platelets are related to the inflammation [11–13]. Platelets participate in inflammation and release inflammatory factors to increase vascular permeability. Furthermore, platelets participate in inflammation by mediating PMN infiltration in the lung [14–17]. We hypothesized that immunosuppressive drugs significantly reduce PMN infiltration in the lung after renal transplantation, but platelets induce PMNs to adhere to pulmonary vascular endothelial cells, aggregate and activate in the lung, and release a large number of active factors, leading to severe acute lung injury.

P-selectin, also called granule membrane protein 140, antigen CD62, or platelet activation dependent granule-external membrane protein (PADGEM), is a 140 kD adhesion molecule that mediates the interaction of stimulated endothelial cells or platelets to leukocytes in the vascular surface [18]. Mayadas et.al confirmed that the combination of P-selectin and its ligand PSGL-1 mediates the adhesion of platelets to vascular endothelial cells and promotes platelet release and aggregation [19]. Moreover, the adhesion of platelets to the vascular endothelium releases platelet activating factor and other inflammatory mediators, resulting in increased permeability of the air-blood barrier [20]. Therefore, p-selectin may play an important role in lung injury after kidney transplantation.

At present, the role of platelets in severe acute lung injury is incompletely understood. In the present study, we aimed to explore the effects of platelet P-selectin on severe acute lung injury in immunocompromised mice.

2. Materials and Methods

2.1. Animals. Wild-type male C57BL/6 mice (20-25 g) were purchased from the Center for Animal Experiments of Wuhan University (Wuhan, China). P-selectin gene knockout mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in a temperature- and humidity-controlled (40%) animal room at 25°C with a 12 h light/dark cycle and free access to food and water. All experimental procedures were approved by the ethical committee of Wuhan University. The mice were anesthetized intraperitoneally with 40 mg/kg sodium pentobarbital. Wild-type male C57BL/6 mice were used to establish the immunocompromised host (ICH) model and Klebsiella pneumoniae-induced lung injury (KPN) model. The mice were randomly assigned to the control, ICH, KPN, KPN + ICH, and KPN + ICH + clopidogrel (Clop) groups. The KPN + ICH + clopidogrel group (clopidogrel, 1.25 mg/kg body weight, dissolved in normal saline) was intraperitoneally administered once a day for three days prior to KPN modeling, and an equal volume of saline was intraperitoneally administered at the same frequency for the KPN + ICH group.

2.2. Immunocompromised Host (ICH) Model. To establish the animal model of ICH, eighteen C57BL/6 mice were divided into three groups (6 mice in each group): the control group, FK506 (tacrolimus) group and FK506 + DXM (dexamethasone) group. FK506 group mice received daily intraperitoneal injections of tacrolimus (0.3 mg/kg/d; Astellas Ireland Co., Ltd) for seven consecutive days. FK506 + DXM group mice received daily intraperitoneal injections of tacrolimus (0.3 mg/kg/d) and dexamethasone (50 mg/kg/d; Huazhong Pharmaceutical Co., Ltd) for seven consecutive days. Mice receiving normal saline injection were used as the control group. Seven days later, the mice were anesthetized and sacrificed, and blood, thymus tissues and spleen tissues were collected.

2.3. Klebsiella Pneumoniae Induced-Lung Injury (KPN) Model. The mice were intratracheally challenged with Klebsiella pneumoniae (0.05 ml; Cat. no. CMCC46114; National Center for Medical Culture Collections) at concentrations of 2×10^8 CFU/ml, 6×10^8 CFU/ml, 2×10^9 CFU/ml or 6×10^9 CFU/ml. Twenty-four hours after challenge with Klebsiella pneumoniae, the mice were anesthetized and sacrificed by inferior vena cava puncture and exsanguination. Blood and lung tissue samples were collected.

2.4. Hematoxylin and Eosin (H&E) Staining. Sections ($4 - \mu$m thickness)were serially cut to perform the morphometric analysis of lung tissues. These sections were stained with hematoxylin for 15 min and eosin for 5 min at room temperature to perform histological analysis. Histological examination was performed under a light microscope (magnification: ×400; Olympus BX43; Olympus Corporation). In each tissue sample, five random areas were scored, and the mean value was calculated by the modified scoring system described by Hasan et.al [21] and XiaoLi Wang et.al [22]. The histology score was the sum of the following four parameters: size of alveolar spaces, thickness of alveolar septa, alveolar fibrin deposition and neutrophil infiltration (0: absent and appears normal; 1: light; 2: moderate; 3: strong; and 4: intense; total score is 16).

2.5. Wet/Dry Weight Ratio of the Lung. The change in the ratio of wet/dry weight was used as an indicator of lung edema formation. At 24 hours after Klebsiella pneumoniae or normal saline administration, the mice were anesthetized and sacrificed. Blood and lung tissue samples were collected. Gauze was used to dry the blood cells and water on the surface of the lung tissue. Then, the lung tissues were weighed and dried in an oven at 65°C for 24 h to obtain the lung wet/dry (W/D) ratio.

2.6. Systemic Platelet and Leukocyte Counts. Blood samples were collected from the mice 24 hours after Klebsiella pneumoniae or normal saline administration. Platelet and leukocyte counts were performed in ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood using a hematology analyzer (Sysmex XN-350; Sysmex Corporation, Kobe, Japan).

2.7. Elisa. Serum samples were warmed to room temperature. Serum levels of P-selectin (Cat. no. SEA569Mu; CLOUD-CLONE CORP), TNF-α (Cat. no. 88-7324; Thermo Fisher Scientific), IL-6 (Cat. no. 88-7064; Thermo Fisher Scientific) and TAX2 (Cat. no. E-EL-0057c; Elabscience) were measured by ELISA kits according to the manufacturer's protocols.

Table 1: Sequences of primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer	Sequence
P-selectin	Forward	5'-CTATACCTGCTCCTGCTACCCA-3'
	Reverse	5'-CTGGAGTCGTAGGCAAAGGC-3'
GAPDH	Forward	5'-CCTCGTCCCGTAGACAAAATG-3'
	Reverse	5'-TGAGGTCAATGAAGGGGTCGT-3'

2.8. Immunofluorescence. The lung tissues were fixed in 4% paraformaldehyde at 4°C overnight. The lung tissue of each group was serially sliced into 4 – μmthick slices and then incubated with 5% goat serum (Beyotime Institute of Biotechnology) at 22°C for 1 h. Lung tissues from each group were sliced into 2 sections, and each sample was incubated with rabbit anti-CD41 primary antibodies (Cat. no., ab63983; both 1 : 100; Abcam, Inc.) at 4°C overnight and subsequently with fluorescein-conjugated mouse anti-rabbit IgG (Cat. no. GB25303; Servicebio, Inc.) for 1 h at room temperature. Following staining with DAPI (Cat. no. G1012; Servicebio, Inc.) for 5 min at room temperature, the samples were imaged using a wide-field fluorescence microscope (Olympus X-cite 120; Olympus Corporation; magnification: ×200).

2.9. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted from mouse lung tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was detected under a UV lamp after formaldehyde-modified agarose gel electrophoresis for 10 min (stained with ethidium bromide, buffered with 1× MOPS, and applied with a constant voltage of 5 V/cm) [23]. cDNA was synthesized according to the Avian Myeloblastosis Virus Reverse Transcriptase Protocol (Promega Corporation) and purified by the PAGE method. GAPDH mRNA was used as the loading control to ensure uniform loading of all RNA samples. The amplification conditions were 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The transcription levels of target genes (Table 1) in all samples were compared with the internal reference gene GAPDH and were analyzed by the 2 - Cq method [24].

2.10. Statistical Analysis. Statistical analysis was performed using SPSS software (version 22.0; SPSS, Inc.). All data are presented as the mean ± standard deviation and were analyzed using Student's t-tests. All experiments were repeated in triplicate, and $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Establishment of an Immunocompromised Mouse Model. Tacrolimus and dexamethasone are two immunosuppressive drugs used by patients after organ transplantation. Tacrolimus and dexamethasone were used to establish immunocompromised mouse models. Mice in the control group, FK506 group and FK506 + DXM group were intraperitoneally injected with normal saline, tacrolimus or tacrolimus plus dexamethasone, respectively, for seven consecutive days. During this period, there was no death in the three groups of mice. The thymus of the control group was plump and moist, the thymus of the FK506 group was atrophied, and the thymus of the FK506 + DXM group was severely atrophied or even disappeared (Figure 1(a)). Compared with the spleen sizes in the control group, the spleen sizes in the FK506 group decreased, and atrophy of the spleens in the FK506 + DXM group was more obvious (Figure 1(b)). Then, we calculated the thymus index and spleen index of the three groups of mice, which suggested that the thymus and spleen sizes in the FK506 group and FK506 + DXM group were markedly atrophied compared with those of the control group ($P < 0.05$). Furthermore, the thymus and spleen sizes in the FK506 + DXM group were smaller than those in the FK506 group ($P < 0.05$; Figure 1(c)–1(d)).

We counted white blood cells (WBCs) and platelets in the three groups of mice using a hematology analyzer. The results indicated that the number of circulating WBCs in the FK506 + DXM group was markedly decreased compared with that in the control group, but there was no significant difference between the control and FK506 groups ($P < 0.05$; Figure 1(e)). Moreover, we found that the inhibitory effect of the combination of tacrolimus and dexamethasone on circulating lymphocytes was better than that of tacrolimus alone ($P < 0.05$; Figure 1(f)). However, tacrolimus and dexamethasone had no significant effect on the numbers of circulating cells in mice ($P > 0.05$; Figure 1(g)). Tacrolimus and dexamethasone had strong immunosuppressive effects on the mice, and the combination of tacrolimus and dexamethasone was superior to that of tacrolimus alone.

3.2. Establishment of a Lung Injury Model in Immunocompromised Mice. The immunocompromised mice were inoculated with Klebsiella pneumoniae at four different concentrations (2×10^8 CFU/ml, 6×10^8 CFU/ml, 2×10^9 CFU/ml or 6×10^9 CFU/ml). Four to six hours after inoculation with Klebsiella pneumoniae mice in the ICH group manifested symptoms such as dispiritedness, polypnea, bleeding around the nose and mouth, and a reduced frequency of eating and drinking water. Mice in the 2×10^8 CFU/ml and 6×10^8 CFU/ml groups survived 24 hours after inoculation with Klebsiella pneumoniae. However, sixty percent of the mice in the 2×10^9 CFU/ml group and eighty percent of the mice in the 6×10^9 CFU/ml group that were inoculated with Klebsiella pneumoniae died before the scheduled test time. Therefore, high inoculation concentrations of Klebsiella pneumoniae caused death in the mice.

In the control group, the lung surfaces of the mice were smooth and slightly white. After the inoculation with Klebsiella pneumoniae, pulmonary congestion and edema were observed on the surface of the lung, and some punctate or flaky hemorrhage was observed under the capsule. Furthermore, the extent of pulmonary hemorrhage and edema increased with the concentration of inoculated bacteria (Figure 2(a)). Then, HE staining was performed on the lung tissues (Figure 2(b)). The structure of the lung tissues in the

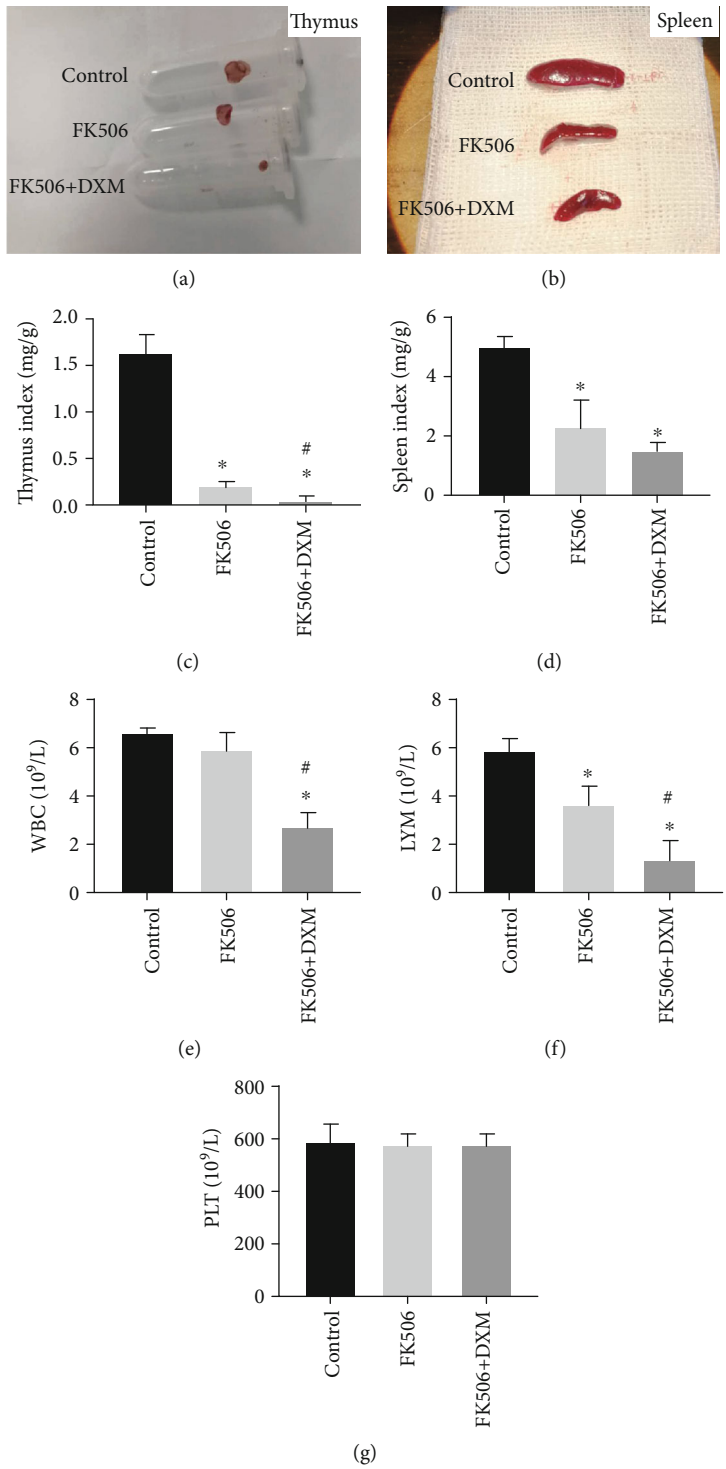


Figure 1: Establishment of immunocompromised mice model. (a) the thymus of mice; (b) the spleen of mice; (c) the thymus index of mice; (d) the spleen index of mice; (e–g) peripheral blood circulation leukocytes, lymphocytes and platelet counts. Values are expressed as mean ± SEM. $*P < 0.05$ vs control; $^{\#}P < 0.05$ vs FK506 group (six mice per group).

control group was clear and complete. After inoculation with Klebsiella pneumoniae, the lungs showed various degrees of inflammatory reactions, including exudation and inflammatory cell infiltration in the bronchi, surrounding bronchi and alveoli, pulmonary interstitial edema, and capillary congestion (Figure 2(b)). As shown in Figure 2(c), histology scores were significantly higher in the KPN + ICH groups than in the control group. Moreover, the histological score of the lung increased with increasing concentrations of Klebsiella pneumoniae inoculation in the mice. However, there was no significant difference between the 2×10^9 CFU/ml and 6×10^9 CFU/ml groups ($P < 0.05$; Figure 2(c)).

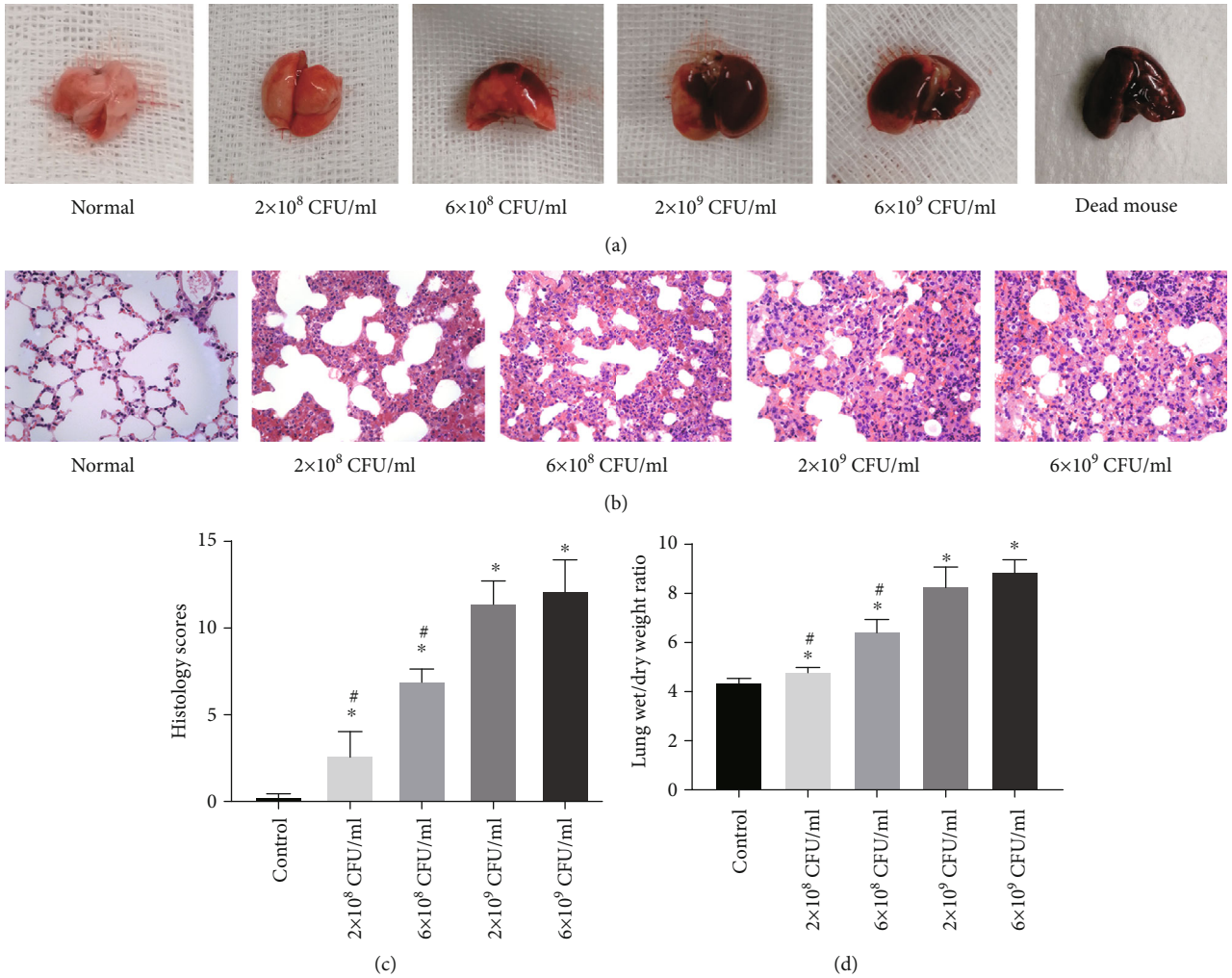


FIGURE 2: Establishment of lung injury model in immunocompromised mice. (a) The lung tissues of mice; (b) representative HE sections of the lung tissues (magnification, ×400); (c) lung histology scores; (d) the Wet/Dry weight ratio of lung tissues. Values are expressed as mean ± SEM. $*P < 0.05$ vs control; $^{\#}P < 0.05$ vs 2×10^9 CFU/ml group (six mice per group).

To evaluate the extent of pulmonary edema in each group, the W/D weight ratio of lung tissues was determined. Consistent with the histological analysis, the lung W/D weight ratios in the 2×10^9 CFU/ml and 6×10^9 CFU/ml groups were significantly higher than those of the 2×10^8 CFU/ml and 6×10^8 CFU/ml groups ($P < 0.05$; Figure 2(d)). However, there was no significant difference between the 2×10^9 CFU/ml and 6×10^9 CFU/ml groups. In general, these results suggest that high concentrations of inoculated Klebsiella pneumoniae induce severe acute lung injury. Therefore, 2×10^9 CFU/ml Klebsiella pneumoniae was used for subsequent experiments.

3.3. Treatment with Immunosuppressive Drugs Exacerbates Severe Acute Lung Injury in Mice. Normal and ICH mice were inoculated with 2×10^9 CFU/ml Klebsiella pneumoniae. The extent of lung injury was more severe in the KPN + ICH group than in the KPN group. After inoculation with Klebsiella pneumoniae, there were multiple patchy hemorrhages under the lung capsule in ICH mice, with obvious pulmonary congestion and edema (Figure 3(a)–3(b)). Likewise, lung histology scores and the W/D weight ratio were significantly higher in the KPN + ICH group than in the KPN group (Figure 3(c)–3(d)). Therefore, as shown in Figure 3(e), the survival rate of mice in the ICH group was significantly higher than that of KPN mice within 40 hours. These data indicate that the use of immunosuppressive drugs in the KPN + ICH group exacerbates severe acute lung injury caused by Klebsiella pneumoniae.

The anal temperatures and arterial partial pressure of oxygen (PaO2) decreased and the arterial partial pressure of carbon dioxide (PaCO2) increased significantly in the KPN and KPN + ICH groups compared with those in the control and ICH groups. Compared with KPN group mice, KPN + ICH group mice had lower anal temperatures and PaO2 and higher PaCO2. In addition, severe acute lung injury in mice induced with Klebsiella pneumoniae reduced the numbers of circulating WBCs (Figure 4(d)). Interestingly, Klebsiella pneumoniae-induced severe acute lung injury in ICH mice decreased the number of circulating platelets ($P < 0.05$;

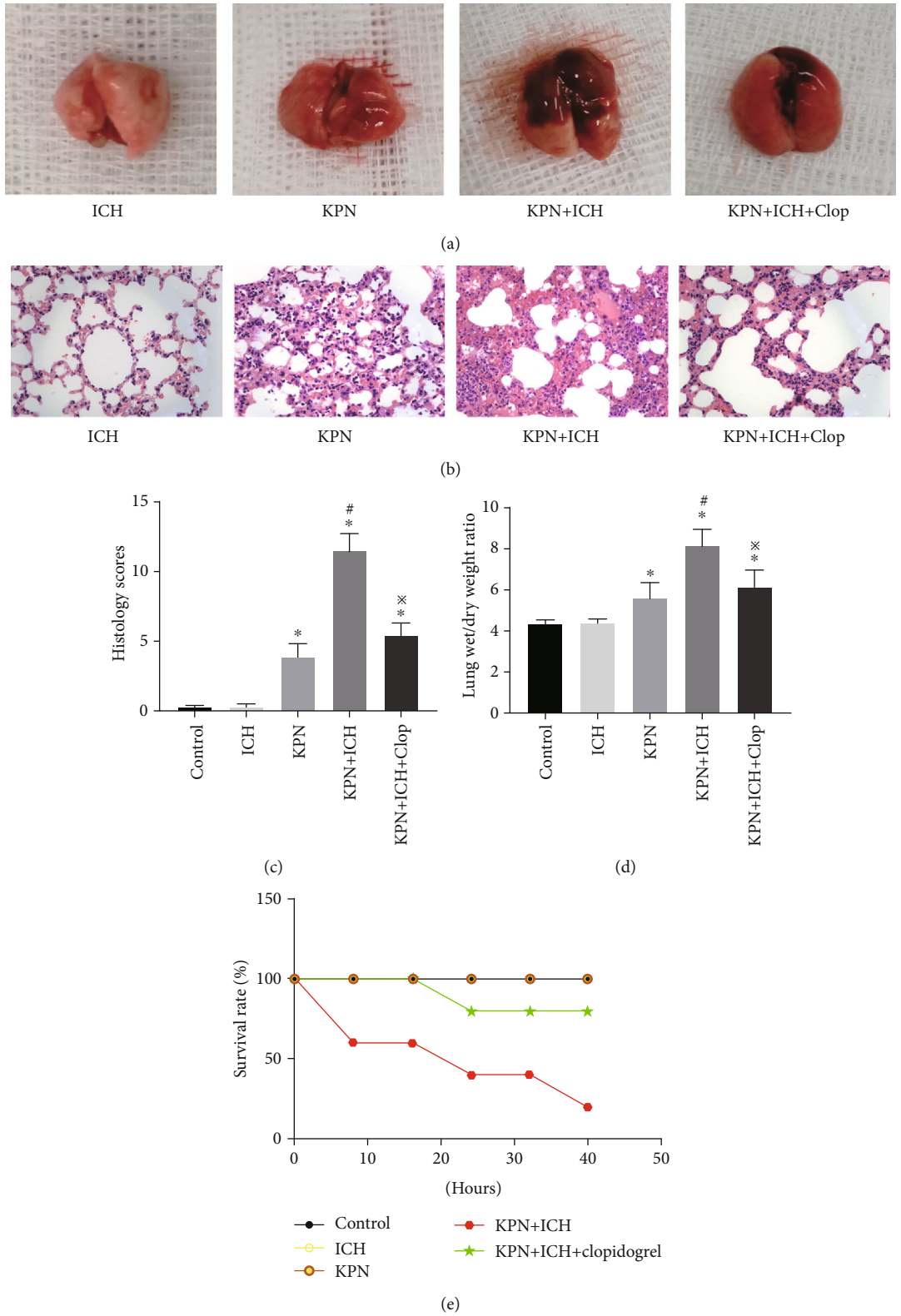


Figure 3: Effect of Clopidogrel pretreatment on lung injury mice. (a) The lung tissues of mice; (b) representative HE sections of the lung tissues (magnification, ×400); (c) lung histology scores; (d) The Wet/Dry weight ratio of lung tissues; (e) the survival curve of mice. Values are expressed as mean ± SEM. $*P < 0.05$ vs control; $^{\#}P < 0.05$ vs KPN group; $^{※}P < 0.05$ vs KPN + ICH group (five mice per group).

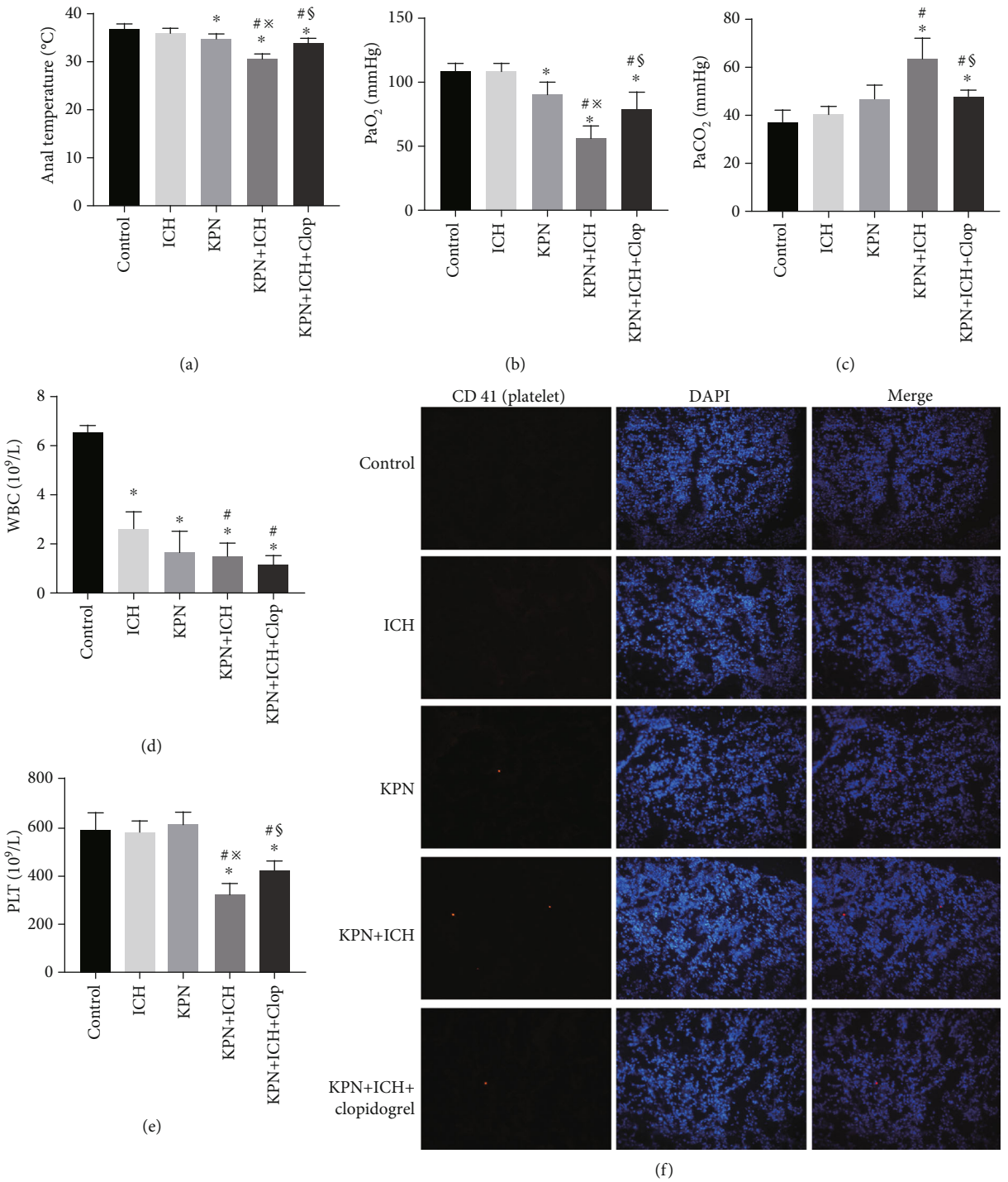


Figure 4: Clopidogrel alleviate acute lung injury and reduce platelet aggregation in lung tissues in mice. (a) Anal temperatures of mice; (b–c) Arterial oxygen partial pressure and carbon dioxide partial pressure of mice; (d–e) peripheral blood circulation leukocyte and platelet counts; (f) immunofluorescence sections of mice lung tissue. Values are expressed as mean ± SEM. $*P < 0.05$ vs control; $^{\#}P < 0.05$ vs ICH group; $^{※}P < 0.05$ vs KPN group; $^{\$}P < 0.05$ vs KPN + ICH group (five mice per group).

Figure 4(e)). However, thrombocytopenia did not occur in the severe acute lung injury model in normal mice in the KPN group (Figure 4(e)).

We stained lung tissues with antibodies against CD41 to detect platelet aggregation in lung tissues. In the control and ICH groups, the lung tissues did not show platelet aggregation. However, platelet aggregation was observed in Klebsiella pneumoniae-inoculated mice. As expected, there were increased platelet aggregates in the lung tissues of the KPN + ICH group mice (Figure 4(f)). These results indicate that the use of immunosuppressive drugs exacerbate severe acute lung injury, decrease the numbers of circulating platelets and increase the formation of platelet aggregates in the lung during pulmonary infection.

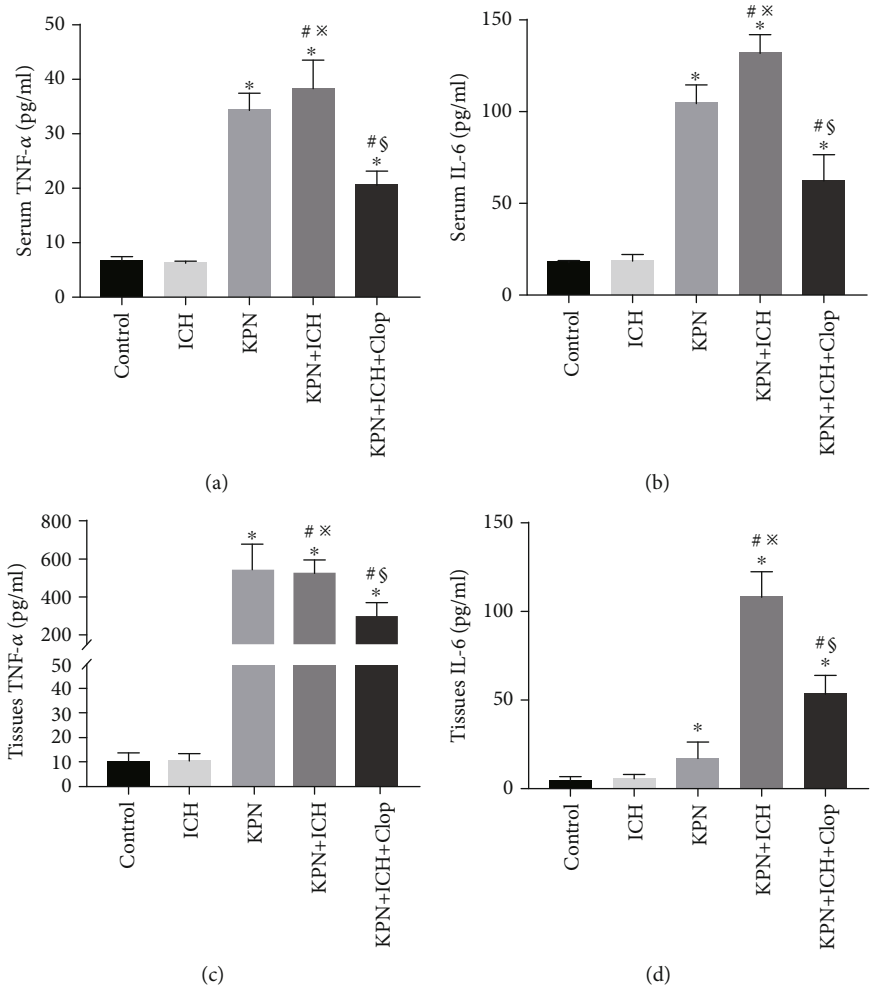
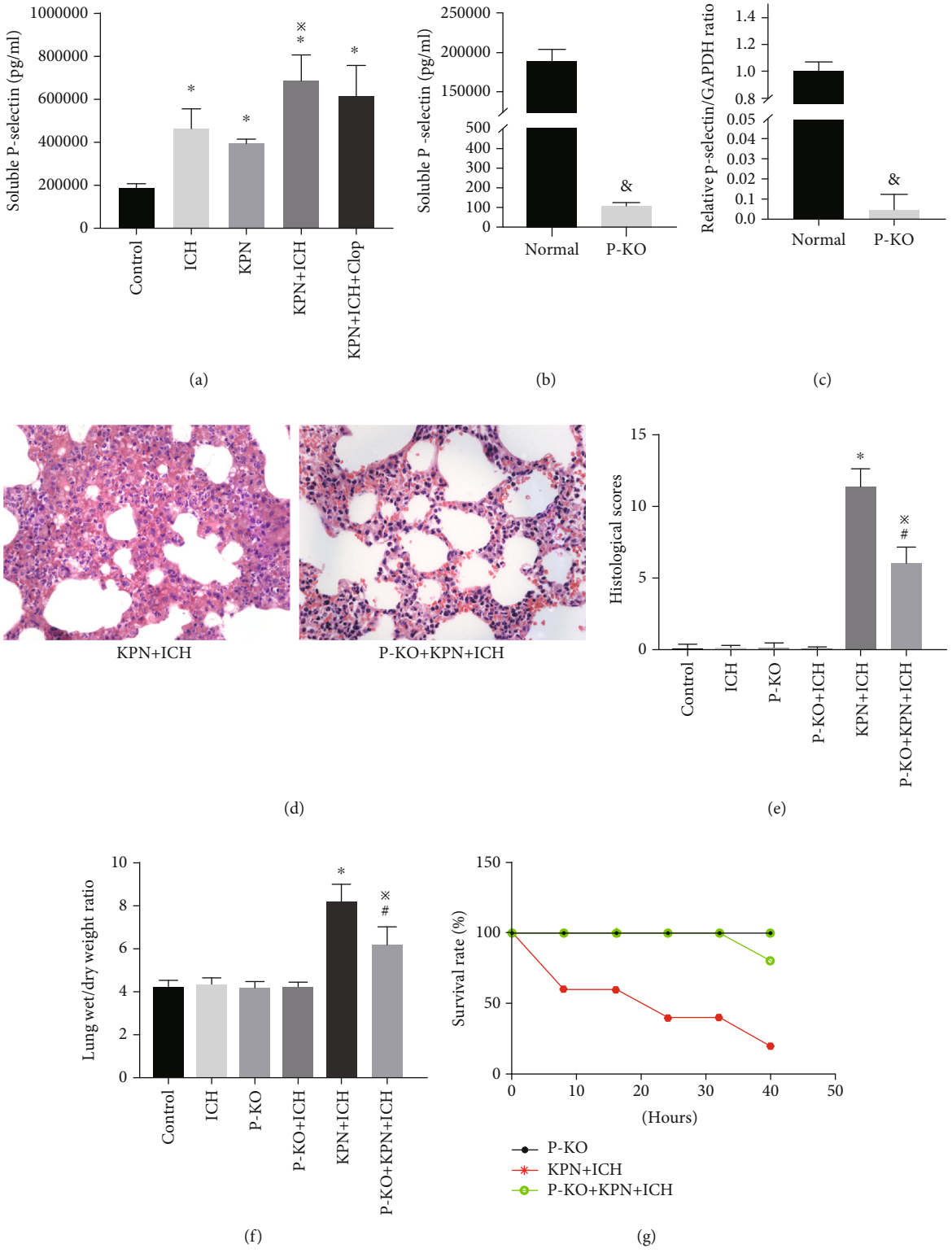


Figure 5: The levels of TNF-α and IL-6 in mice. (a–b) The expression of TNF-α and IL-6 in serum; (c–d) the expression of TNF-α and IL-6 in lung tissues. Values are expressed as mean ± SEM. $*P < 0.05$ vs control; $^{\#}P < 0.05$ vs ICH group; $^{※}P < 0.05$ vs KPN group; $^{\$}P < 0.05$ vs KPN + ICH group (five mice per group).

3.4. Blocking Platelet Aggregation with Clopidogrel Alleviates Severe Acute Lung Injury and Reduces Platelet Aggregation in Lung Tissues in Mice. Clopidogrel is a platelet aggregation inhibitor that selectively inhibits the binding of ADP and platelet receptors and inhibits activation of the glycoprotein GPIIb/IIIa complex on platelets [25]. Mice were intraperitoneally injected with 1.25 mg/kg clopidogrel (Salubris; China) for three consecutive days before inoculation with Klebsiella pneumoniae. Clopidogrel pretreatment attenuated Klebsiella pneumoniae-induced histological injury and the extent of lung edema in the KPN + ICH + Clop group compared to that of the KPN + ICH group (Figure 3(a)–3(d)). Furthermore, clopidogrel dramatically improved the survival rate of Klebsiella pneumoniae-inoculated ICH mice (Figure 3(e)). Systemic infection and lung ventilation disorder in KPN + ICH group mice were alleviated by clopidogrel (Figure 4(a)–4(c)). As expected, clopidogrel pretreatment increased the numbers of circulating platelets and reduced the formation of platelet aggregates in the lungs of KPN + ICH group mice (Figure 4(e)–4(f)). These data indicate that clopidogrel plays a positive role in protecting against severe acute lung injury and mitigating induced thrombocytopenia.

3.5. Blocking Platelet Aggregation by Clopidogrel Reduces the Levels of TNF-α and IL-6. TNF-α and IL-6 are two proinflammatory cytokines that are indicators of inflammation [26, 27]. We detected TNF-α and IL-6 expression levels in serum and lung tissue by ELISA. As shown in Figure 5, the TNF-α and IL-6 levels in the KPN and KPN + ICH groups were higher than those in the control and ICH groups ($P < 0.05$). Furthermore, pretreatment with clopidogrel significantly reduced TNF-α and IL-6 expression levels in the KPN and KPN + ICH groups ($P < 0.05$). These data confirmed that platelet blockade induced by clopidogrel pretreatment significantly reduces the extent of inflammation in severe acute lung injury mice.

3.6. Knockout of P-Selectin Alleviates Severe Acute Lung Injury in ICH Mice. P-selectin mediates the rolling of neutrophils and lymphocytes on platelets or activated endothelial cells and is involved in clotting, thrombosis and inflammation [28]. We found that P-selectin expression in mouse serum and lung tissues increased after administration of immunosuppressive drugs or inoculation with Klebsiella pneumoniae ($P < 0.05$; Figure 6(a)). The P-selectin expression



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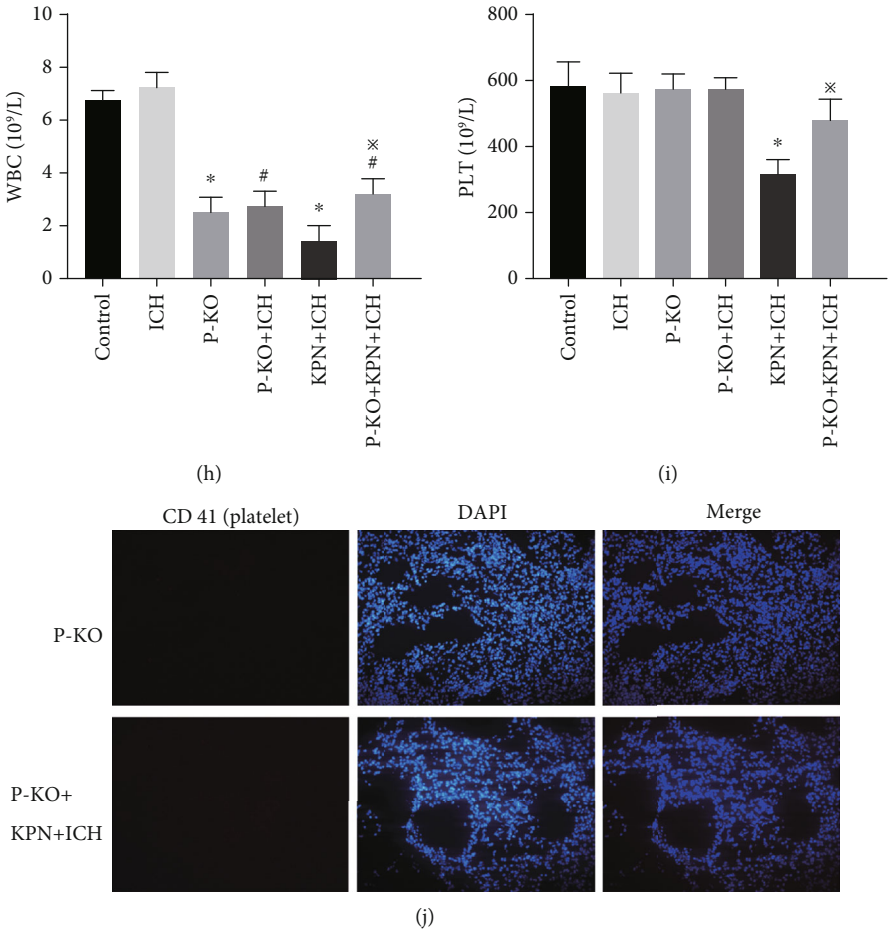

FIGURE 6: Knockout of p-selectin alleviate acute lung injury in ICH mice. (a) The expression of serum P-selectin in mice; (b–c) the expression of serum P-selectin in P-selectin knockout mice detected by Elisa assay and RT-PCR; (d) representative HE sections of the lung tissues (magnification, ×400); (e) lung histology scores; (f) The Wet/Dry weight ratio of lung tissues; (g) the survival curve of mice; (h–i) peripheral blood circulation leukocyte and platelet counts; (j) immunofluorescence sections of mice lung tissue. Values are expressed as mean ± SEM. $*P < 0.05$ vs control; $^{\&}P < 0.05$ vs Normal group; $^{\#}P < 0.05$ vs P-KO group; $^{※}P < 0.05$ vs KPN + ICH group(five mice per group).

in serum and lung tissues of KPN pneumoniae-inoculated ICH mice in the KPN + ICH group was higher than those in the KPN and ICH groups ($P < 0.05$; Figure 6(a)). Then, the effect of P-selectin on severe acute lung injury induced by Klebsiella pneumoniae in immunocompromised mice was evaluated in subsequent experiments. Compared with wild-type mice, p-selectin knockout mice showed low expression in serum and lung tissues ($P \leq 0.001$; Figure 6(b)–6(c)). Likewise, P-selectin knockout mice were used to establish animal models of immunosuppression and severe acute lung injury. The results confirm that P-selectin knockout attenuated Klebsiella pneumoniae-induced severe acute lung injury and improved the survival rate of ICH mice that were inoculated with Klebsiella pneumoniae (Figure 6(d)–6(g)). Furthermore, P-selectin knockout increased the numbers of circulating WBCs and platelets and reduced the formation of platelet aggregates in the lungs of KPN + ICH group mice (Figure 6(h)–6(j)). These data suggest that P-selectin knockout alleviates Klebsiella pneumoniae-induced severe acute lung injury in ICH mice.

4. Discussion

With the wide development of organ transplantation and the wide clinical application of immunosuppressive drugs, the number of immunocompromised hosts is increasing. Refractory respiratory infection caused by immunosuppressive drugs is the main cause of death in ICHs [29]. Immunosuppressive drugs, including tacrolimus and glucocorticoids, are widely used in the immunotherapy of organ transplantation, chemoradiotherapy and immune-related diseases, and recipients often develop immune impairment characterized by leukopenia [30].

At present, many studies on ICHs have established animal models to simulate immunocompromised patients [31–33]. In our experiment, we used tacrolimus and dexamethasone to establish the ICH model in C57BL/6 mice. The ICH mouse model showed spleen atrophy, atrophy or even disappearance of the thymus, and significantly decreased peripheral blood leukocytes ($P < 0.05$).

Klebsiella pneumoniae is widely distributed in nature and is an important pathogen associated with acquired

pneumonia in hospitals. In recent years, the incidence of Klebsiella pneumoniae has been on the rise [34, 35]. Kidney transplant patients are typical immunocompromised hosts. The continuous use of high-dose immunosuppressive drugs leads to a reduction in immune cells, including PMNs. Pulmonary infiltration of PMNs is significantly reduced compared with that of normal host infection. However, the extent of acute lung injury is more serious in ICHs than in normal hosts. Our study suggested that severe acute lung injury caused by pulmonary infection in ICH mice is related to thrombocytopenia, and the extent of thrombocytopenia is related to the extent of lung injury. Moreover, the extent of lung injury in C57 wild-type mice that were treated with clopidogrel was less than that in the control group. Therefore, we believe that platelets play an important role in severe acute lung injury after renal transplantation.

Numerous studies have confirmed that platelets play not only a leading role in coagulation and thrombosis but also a key role in inflammation [36–38]. Platelets in the blood mainly exert anti-infection effects through the following mechanisms: ① After inflammatory stimulation, platelet P-selectin is transferred from the cytoplasm to the surface of the cell membrane, inducing adhesion of PMNs to endometrial cells and promoting the formation of neutrophil extracellular traps [39, 40]. ② Activated platelets produce microparticles and participate in inflammatory reactions [41]. ③ Platelets contain alpha granules, dense granules, lysosomes and other particles. After platelet activation, various mediators are released, including coagulation factors, vasoactive substances and inflammatory mediators. These molecules have a dramatic impact on the permeability of blood vessels [42–44].④ Platelets synthesize molecules such as TNF-α and IL-6 under infection conditions and participate in the inflammatory response. Increasing evidence supports the important role of platelets in severe acute lung injury [45–47].

Graff J. et.al suggested that platelets are easily activated in transplant patients after immunosuppressive therapy [46]. Our study confirmed that the continuous use of high-dose immunosuppressive drugs in mice increases the expression of P-selectin on the platelet surface. Mayadas et.al. showed that the binding of P-selectin and its ligand PSGL-1 mediates the adhesion of platelets to vascular endothelial cells and promotes platelet release and aggregation [47]. Immunosuppressive drugs promote the adhesion of platelets to vascular endothelial cells [48]. Platelets adhere to the vascular endothelium and release some bioactive molecules, resulting in increased permeability of the air-blood barrier.

In our study, p-selectin gene knockout and wild-type mice were selected to establish immunocompromised animal models, and Klebsiella pneumoniae was inoculated into airways to establish an animal model of severe acute lung injury induced by pulmonary infection. Finally, we found that the extent of acute lung injury in p-selectin knockout mice was lower than that in wild-type mice. These results confirmed that p-selectin plays an important role in severe acute lung injury in immunocompromised hosts.

The present study demonstrated that blocking platelet aggregation with clopidogrel reduced severe acute lung injury in mice; however, clopidogrel exerts both platelet-dependent and platelet-independent anti-inflammatory effects [49]. The platelet-independent anti-inflammatory effect of clopidogrel also has a certain effect on severe acute lung injury in mice. Also, platelet aggregation in the lung was not observed in real time. It is necessary to use in vivo fluorescence microscopy imaging technology in subsequent experiments. Platelet lung aggregation in mice with severe acute lung injury can be observed in real time, and we can better understand the effects of platelets and p-selectin on lung permeability and lung tissue injury. Further cellular or animal experiments are required in the future to clarify this issue.

In summary, the use of immunosuppressive drugs and Klebsiella pneumoniae infection upregulate the expression of p-selectin on platelets. The p-selectin on platelets mediates the aggregation of platelets in the lung. Platelets are activated by inflammatory factors, release a large number of active mediators, and significantly increase the permeability of the air-blood barrier. Severe acute lung injury finally occurs with the participation of immune cells. These results may lead to a more in-depth understanding of the mechanism of acute lung injury induced by infection following organ transplantation and provide new ideas for the development of therapeutic treatments against this lethal disease.

Data Availability

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

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Review Article

Role of the eNOS Uncoupling and the Nitric Oxide Metabolic Pathway in the Pathogenesis of Autoimmune Rheumatic Diseases

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Atherosclerosis and its clinical complications constitute the major healthcare problems of the world population. Due to the central role of endothelium throughout the atherosclerotic disease process, endothelial dysfunction is regarded as a common mechanism for various cardiovascular (CV) disorders. It is well established that patients with rheumatic autoimmune diseases are characterized by significantly increased prevalence of cardiovascular morbidity and mortality compared with the general population. The current European guidelines on cardiovascular disease (CVD) prevention in clinical practice recommend to use a 1,5-factor multiplier for CV risk in rheumatoid arthritis as well as in other autoimmune inflammatory diseases. However, mechanisms of accelerated atherosclerosis in these diseases, especially in the absence of traditional risk factors, still remain unclear. Oxidative stress plays the major role in the endothelial dysfunction and recently is strongly attributed to endothelial NO synthase dysfunction (eNOS uncoupling). Converted to a superoxide-producing enzyme, uncoupled eNOS not only leads to reduction of the nitric oxide (NO) generation but also potentiates the preexisting oxidative stress, which contributes significantly to atherogenesis. However, to date, there are no systemic analyses on the role of eNOS uncoupling in the excess CV mortality linked with autoimmune rheumatic diseases. The current review paper addresses this issue.

1. Introduction

Atherosclerosis and its clinical complications constitute the major healthcare problems of the world population [1–3]. Over the last decades, it has become clear that the vascular endothelium plays the central role throughout the atherosclerotic disease process, and all alterations initiating the onset and promoting the progression of the disease depend on the dynamic changes in endothelial cell phenotype. Endothelial dysfunction (ED), the early feature of atherosclerosis, precedes the development of morphologic changes and is the earliest detectable impairment of vascular function [4, 5]. It is a consequence of chronic exposure to cardiovascular (CV) risk factors, and its progression is related to the intensity and duration of these factors [6, 7]. Therefore, ED is regarded as a common mechanism for various CV disorders, and numerous clinical studies have shown that endothelial dysfunction can be an independent predictor of future cardiovascular disease (CVD) progression and acute thrombotic events [8–11].

Patients with autoimmune rheumatic diseases even in the absence of CV risk factors have an almost twofold increase in CV morbidity and mortality than the general population. It is thought that persistent systemic inflammation enhances CV risk through direct or indirect mechanisms leading to accentuation of existing risk pathways [12]. Such evidence has now been implemented in European guidelines (ESC 2016 and 2019, EULAR 2010 with 2015/2016 update) and risk scores [13–16]. Increased production of proinflammatory mediators and cytokines results in enhanced oxidative stress, the hallmark of both autoimmune diseases and atherosclerosis [17–20]. Elevated ROS generation, *via* activation of the transcription factor, nuclear factor *kappa*-light-chain-enhancer of activated B cell (NF-κB) pathway, induces expression of inflammatory and immune genes (cytokines, chemokines, adhesion molecules, acute phase proteins, regulators of

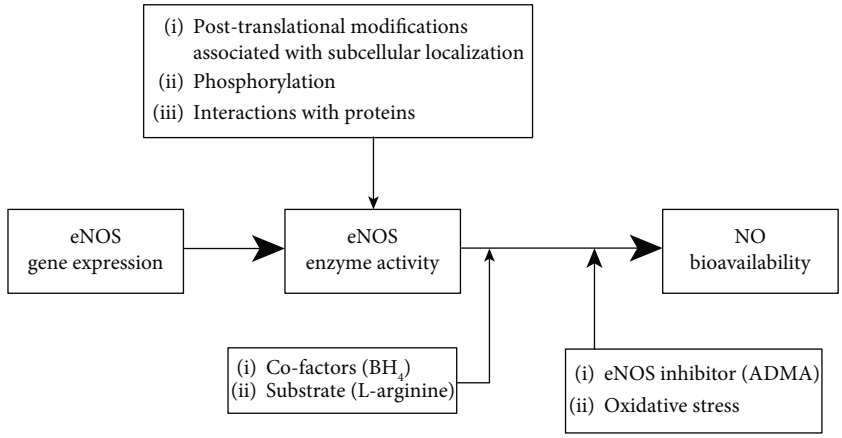

Figure 1: Balance between production and degradation of nitric oxide (NO) by oxidative stress determines endothelial NO bioavailability. Synthesis of NO can be regulated at the endothelial nitric oxide synthase (eNOS) gene expression level and eNOS enzymatic activity level. The eNOS activity depends also on substrate and cofactor availability and the presence of oxidative stress and endogenous inhibitor asymmetric dimethylarginine (ADMA). Adapted from Yang and Ming [177]. Abbreviations: eNOS: endothelial nitric oxide synthase; NO: nitric oxide; BH_4: tetrahydrobiopterin; ADMA: assymetric dimethylarginine.

apoptosis, and cell proliferation). These mediators on one hand promote change in endothelial phenotype, known as endothelial activation; on the other hand, they potentiate inflammation *via* further recruitment of adaptive and innate immune cells and ROS generation, leading to persistence of inflammation and disease progression [21, 22]. It is thought that the destructive loop of oxidative stress and inflammation leads to development of endothelial dysfunction, a fundamental feature of atherosclerosis [23].

Due to the fact that atherosclerosis is a complex disease, no single mechanism can fully explain the endothelial dysfunction. However, decreased nitric oxide (NO) bioavailability with subsequent inability of endothelium to initiate vasodilatation and exhibit multiple antiatherogenic functions appears to play a major role [24]. Decreased NO bioavailability may result from its limited production and/or increased NO degradation by reactive oxygen species (ROS) (Figure 1). Reduced NO generation can be due to decreased endothelial NO synthase (eNOS) expression and/or activity, eNOS uncoupling, impaired NO-mediated signaling events, and oxidative stress. Among these mechanisms, the eNOS uncoupling has recently attracted the gaining attentions. However, there is scarcely no data in the literature on the role of the eNOS uncoupling in atherogenesis in autoimmune rheumatic diseases. The current review paper addresses this gap in literature.

2. Molecular Mechanisms of the eNOS Uncoupling: Pathophysiological Considerations and Potential Therapeutic Implications

2.1. eNOS Uncoupling (Figure 2): General Information. A number of studies have revealed that under pathological conditions, due to the enhanced oxidative stress, the eNOS may become dysfunctional resulting in production of superoxide instead of NO. Moreover, the expression of eNOS is increased by ROS through posttranscriptional and posttranslational modifications, although the NO bioavailability is reduced. This phenomenon contributes significantly to endothelial dysfunction and cardiovascular disease not only by reducing the NO generation but also by triggering the preexisting oxidative stress [25, 26].

Produced by the uncoupled eNOS, superoxide scavenges NO leading to the peroxynitrite formation. Both ROS exert multiple proatherogenic effects, including effects on eNOS function. Peroxynitrite oxidizes tetrahydrobiopterin (BH_4), the eNOS cofactor to the trihydrobiopterin (BH_3) radical, resulting in the eNOS uncoupling, perpetual superoxide production, and subsequent peroxynitrite formation [27]. It also reduces endothelial transport of L-arginine, the exclusive substrate for eNOS, and increases the rate of L-arginine efflux [28]. Peroxynitrite directly oxidizes the reduced glutathione (GSH), its endogenous scavenger, which plays a major role in the cellular defense against reactive oxygen species. Similarly, *via* nitration of superoxide dismutase (SOD), peroxynitrite inactivates the enzyme, leading to diminished antioxidant cellular defense mechanisms and increase in superoxide levels [29, 30]. Elevated superoxide levels are also the result of peroxynitrite action-induced protein phosphatase 2A (PP2A) activation, which leads in turn to the dephosphorylation of eNOS and therefore decrease in enzyme activity and subsequent NO generation [31, 32]. Peroxynitrite and superoxide, the known contributors to endothelial dysfunction, have also multiple indirect effects on the eNOS function. Peroxynitrite inactivates prostacyclin synthase (PGIS), an enzyme that catalyzes the isomerization of prostaglandin H_2 to prostacyclin, widely known for its vasoprotective activity, therefore resulting in formation of vasoconstricting prostaglandins including thromboxane A_2. Recent studies have shown that stimulation of thromboxane receptor (TPr) by thromboxane A_2 and prostaglandin H_2 promotes ROS formation in vascular smooth muscle cells and endothelial cells by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase facilitating eNOS deactivation through increased oxidative stress [33, 34]. Both superoxide and

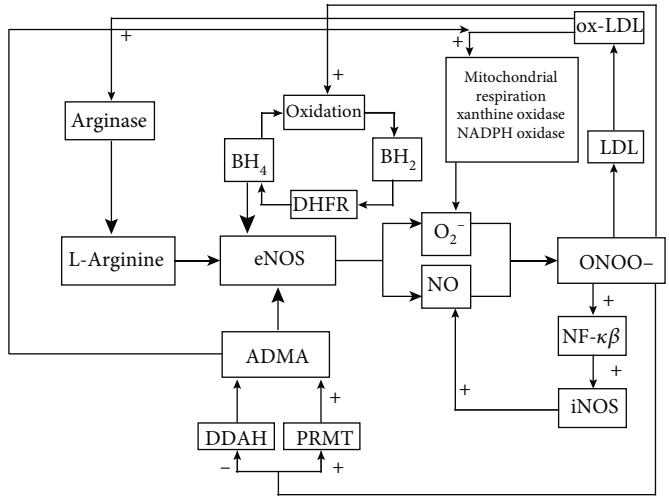

Figure 2: Mechanisms of endothelial nitric oxide synthase (eNOS) uncoupling in endothelial dysfunction. A depletion of eNOS cofactor tetrahydrobiopterin (BH_4), an L-arginine deficiency, and an increase in endogenous eNOS inhibitor, asymmetric dimethylarginine (ADMA), leads to eNOS uncoupling. Produced by the uncoupled enzyme, superoxide scavenges nitric oxide (NO) leading to the peroxynitrite formation. Peroxynitrite (1) oxidizes BH_4, resulting in the eNOS uncoupling and perpetual superoxide production and subsequent peroxynitrite formation; (2) oxidizes low-density lipoproteins (LDL) forming oxidized LDL (ox-LDL) which in turn through the scavenger receptor, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), downregulate the enzyme expression. Furthermore, ox-LDL stimulate nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and xanthine oxidase to produce reactive oxygen species (ROS) in excess and increase arginase activity leading to reduction in L-arginine availability for nitric oxide synthase (NOS) and subsequent eNOS uncoupling and impaired NO generation. Moreover, arginase via increased formation of polyamines and L-proline stimulates vascular smooth muscle cell proliferation and extracellular matrix deposition, thereby contributing to intimal hyperplasia and remodeling processes; (3) nitrosylates the cationic amino acid transporter, therefore inhibiting the L-arginine transport in endothelial cells and increasing the rate of arginine efflux; (4) increases the activity of protein-arginine methyl transferase (PRMTs) and inhibits that of dimethylarginine dimethylaminohydrolase (DDAH), resulting in elevated ADMA levels, which in turn via inhibition of NO synthesis and eNOS uncoupling enhance production of ROS. Oxidative stress in turn upregulates ADMA levels; (5) through activation of transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) induces expression of inducible nitric oxide synthase (iNOS), arginase, and inflammatory and immune genes (cytokines, chemokines, adhesion molecules, acute phase proteins, regulators of apoptosis, and cell proliferation) potentiating inflammation via further recruitment of adaptive and innate immune cells and ROS generation, leading to persistence of inflammation and disease progression. Abbreviations: eNOS: endothelial nitric oxide synthase; NO: nitric oxide; BH_4: tetrahydrobiopterin; ADMA: asymmetric dimethylarginine; O_2^-: superoxide; ONOO-: peroxynitrite; BH_2: dihydrobiopterin; DHFR: dihydrofolate reductase; ox-LDL: oxidized LDL; NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase; LDL: low-density lipoproteins; iNOS: inducible nitric oxide synthase; NF-κB: nuclear factor kappa light-chain-enhancer of activated B cells; DDAH: dimethylarginine dimethylaminohydrolase; PRMT: protein arginine methyl transferase.

peroxynitrite also oxidize low-density lipoproteins (LDL) forming oxidized LDL (ox-LDL), which in turn through the scavenger receptor, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), downregulates the enzyme expression. Furthermore, ox-LDL stimulate NADPH oxidase and xanthine oxidase to produce ROS in excess, promoting a vicious cycle mechanism of oxidative stress and vascular damage [35, 36].

Numerous mechanisms have been proposed to play a role in the eNOS uncoupling in atherosclerosis: depletion of eNOS cofactor BH_4, L-arginine deficiency, and increase in endogenous eNOS inhibitor, asymmetric dimethylarginine (ADMA) [37]. All these mechanisms are discussed below.

2.2. Asymmetric Dimethylarginine (ADMA). ADMA is a naturally occurring amino acid formed from the proteolysis of methylated arginine residues in intracellular proteins that are posttranslationally modified by a class of enzymes known as protein-arginine methyl transferases (PRMTs). Following proteolysis, free methylarginines are released and subsequently converted to citrulline and dimethylamine by dimethylarginine dimethylaminohydrolase (DDAH). ADMA is a key NOS inhibitor—it competes with L-arginine for the binding site in the active center of NOS isoforms, thereby resulting in decreased NO generation [38–40]. Increased ADMA reduces NO bioavailability leading to subsequent inflammation and oxidative stress, the typical features of endothelial dysfunction, contributing substantially to cardiovascular risk [41, 42]. Therefore, increased ADMA levels have been associated with several risk factors and cardiovascular morbidity and mortality. Indeed, numerous studies have confirmed its role as an established independent predictor for cardiovascular events and all-cause cardiovascular mortality [43–45]. In animal models, ADMA levels correlated with vascular function and the degree of atherosclerosis, in humans with cholesterol levels [46, 47]. Elevated ADMA levels are largely due to increased PRMT activity or decreased DDAH activity. The regulation of gene expression and activity of PRMT and DDAH remains predominantly unclear. However, activities of both enzymes are redox sensitive. Oxidative stress has been shown to increase the activity of PRMTs and inhibit that of DDAH, resulting in elevated ADMA levels, which in

turn via inhibition of NO synthesis and eNOS uncoupling enhance production of ROS [48]. Therefore, ADMA promotes superoxide production by eNOS, and the resulting oxidative stress upregulates ADMA levels [49]. Tumor necrosis factor-alpha (TNF-α) and high levels of glucose and homocysteine diminish DDAH activity via induction of oxidative stress. Similarly does oxidized low-density lipoprotein (ox-LDL) [50–52]. Increased ADMA in turn upregulates the LOX-1 expression, the main receptor for ox-LDL in endothelial cells, resulting in enhanced production of oxidized LDL and intracellular generation of reactive oxygen species, creating a vicious cycle mechanism [53, 54].

Negative regulation of NO synthesis can also be mediated through overproduction of methylated arginine analogues such as ADMA. Recently, many clinical studies have demonstrated that plasma levels of ADMA were elevated in RA patients regardless of the presence of cardiovascular disease [55–67]. Furthermore, ADMA has been reported to be related to indices of endothelial dysfunction or subclinical atherosclerosis in some [56, 65, 68], but not all, conducted studies [58, 60, 61, 67, 68]. In patients with high disease activity and no overt atherosclerotic disease or classic risk factors, high plasma ADMA levels significantly correlated with IMT [67], coronary flow reserve (CFR) [56], and pulse wave velocity (PWV) [58], whereas in those with evident atherosclerosis and CV risk factors, negative correlation between ADMA and FMD [65] and carotid IMT (cIMT) [62, 68] was found. In the latter subgroup of patients also, a positive relation was observed between the ADMA : SDMA ratio (suggested as the index of dimethylarginine dimethylaminohydrolase activity) and microvascular endothelial function [68] and arterial stiffness [62]. However, these relationships were not observed in RA patients with low and moderate disease activities—although structural and functional changes in vessels and heart were detected by means of multiple noninvasive, validated methods including cIMT, FMD, CFR, PWV, laser Doppler, and subendocardial viability ratio (SEVR), no associations between dimethylarginines and assessments of vascular morphology and function were found [56, 61, 65]. As an explanation of these findings, it has been suggested that inflammatory mechanisms responsible for synovial lesions might also occur in the vascular wall and promote the development of advanced atherosclerosis. Oxidative stress induced by proinflammatory cytokines has been shown to increase the activity of PRMTs and inhibit that of DDAH, resulting in elevated ADMA levels. The latter can be also due to increased endothelial cell turnover with potential liberation of ADMA during cell catabolism. Increased ADMA in turn contributes directly to oxidative stress by causing endothelial NOS uncoupling and switching it to a superoxide synthase. ADMA also significantly increases TNF-α levels in human endothelial cells and thus participates in the pathogenesis of vascular injury in RA [56, 62, 66, 67, 69]. Hence, these data indicate close interactions between endothelial injury and systemic inflammation.

2.3. Tetrahydrobiopterin (BH_4). BH_4 is a critical cofactor for all the NOS isoforms and a regulator of their function [37]. It has been shown that NO generation and eNOS correlate closely with the intracellular concentration of BH_4 [70]. It is synthesized de novo from guanosine triphosphate (GTP) in a multistep pathway that involves GTP cyclohydrolase I (GTPCH I), 6-pyruvoyltetrahydropterin synthase, and sepiapterin reductase, respectively. GTPCH I is a rate-limiting enzyme for BH_4 biosynthesis and therefore plays a major role in controlling the NOS function [71, 72]. Many lines of evidence indicate that oxidative degeneration of BH_4 by ROS leads to the eNOS uncoupling, reduction in NO bioavailability, and increased reactive oxygen species production [73, 74]. Indeed, oxidation of BH_4 forms dihydrobiopterin (BH_2) and biopterin. BH_2 binds with fairly high affinity to eNOS without supporting its catalytic activity [75]. The uncoupled enzyme generates superoxide rather than NO leading to further limitation of BH_4 availability. However, BH_2 can be recycled to BH_4 by dihydrofolate reductase (DHFR), which regulates the rate of BH_4 regeneration [76]. Therefore, BH_4 bioavailability is determined by enzymatic *de novo* synthesis, recycling, and oxidative degradation. There is little information on regulatory mechanisms of GTPCH and DHFR gene expression or activity. BH4 and high concentrations of BH_2 inhibit GTPCH-1 and subsequently de novo synthesis of BH_4, while insulin and mediators such as interferon gamma (IFN-γ), TNF-α, and interleukin-1 beta (IL-1β) can upregulate its activity and expression [77–80]. Expression of DHFR can be downregulated by angiotensin II [81]. It is thought that among these two enzymes, DHFR is critical to eNOS function, especially in cells that do not contain the apparatus required for efficient synthesis of BH_4 or under conditions of low total biopterin levels, as recycling it can reduce eNOS-dependent oxidation of BH_4 that would further decrease BH_4 levels and enhance eNOS uncoupling [82]. In endothelial dysfunction and many models of cardiovascular disease, the BH_4 levels have been found decreased. Therefore, recent studies have shown that pharmacological supplementation of BH_4 improves vascular function in patients with diabetes, essential hypertension, and hypercholesterolemia and in chronic smokers [83–95].

Recently, it has been demonstrated that deficiency of BH_4 may contribute in part to formation of the uncoupled eNOS. Indeed, a decrease in serum levels of BH_4 in AIA rats compared to the control group was reported, and administration of BH_4 restored endothelial function. Therefore, the authors suggested that eNOS contributes to amplification of oxidative stress in vasculature, and this contribution is mediated by the loss of BH_4 availability [96, 97]. Beneficial effects of oral BH_4 supplementation were then investigated in humans. Therapy with BH_4 in patients with active RA improved endothelial function as assessed by vasodilatory response to reactive hyperemia. A decrease in BH_4 levels in RA patients was attributed by the authors to increased expression and activation of inducible nitric oxide synthase (iNOS) in endothelial cells during chronic inflammation, which leads to eNOS uncoupling via limiting BH_4 availability for eNOS. Also, ROS generated by myeloperoxidase released from activated neutrophils contribute to decreased BH_4 levels via their oxidation to inactive BH_2 [98, 99]. In an animal model of arthritis, serum BH_4 levels besides supplementation can be increased upon administration of fluvastatin [97]. It has been

reported in the general population that statins upregulate eNOS expression by stabilizing its mRNA and induce phosphorylation and activation of eNOS via the protein kinase Akt pathway. The authors showed that fluvastatin decreased expression of p22phox mRNA, a membrane-associated component of NADPH oxidase, resulting in inhibition of enzyme activity and decreased ROS generation. Therefore, they indicated that increase in BH_4 availability due to decreased ROS production achieved with fluvastatin therapy prevents eNOS uncoupling [97, 100]. A similar mechanism of action presents etanercept, a TNF inhibitor [101].

Interestingly, methotrexate (MTX) inhibits NF-κB activation through blockade of BH_4 synthesis. It inhibits tetrahydrofolate reductase which recycles BH_2 to BH_4, leading to eNOS uncoupling and ROS production. In turn, increased ROS generation activates the Jun-N-terminal kinase (JNK) and JNK-dependent induction of tumor protein p53 (p53) and cyclin-dependent kinase inhibitor 1 (p21) resulting in decreased NF-κB activation. Therefore, MTX may contribute to reduced BH_4 bioavailability in the endothelium. However, the study investigating the impact of BH_4 supplementation on endothelial function found no difference between patients on MTX and those not receiving MTX. Furthermore, patients treated with MTX had a greater increase in flow-mediated dilatation (FMD) following BH_4 administration probably due to reduced levels of inflammation. Although the study was not powered to look at this difference, it has been reported recently that in activated T cells, inhibition of BH_4 synthesis decreases production of the proinflammatory IFN-γ and increases production of the anti-inflammatory IL-4. MTX induces a similar shift and therefore downregulates expression of proinflammatory cytokines IL-1, IL-2, IL-6, and IFN-γ and upregulates expression of anti-inflammatory cytokines such as IL-4 and IL-10 in RA patients [98, 102, 103].

2.4. L-Arginine Deficiency. A semiessential amino acid L-arginine is the exclusive substrate for nitric oxide synthase, and its availability is one of the rate-limiting factors in cellular NO production [37]. Since reduction in L-arginine availability has emerged as an important mechanism underlying decreased NO bioavailability and endothelial dysfunction, many clinical and experimental studies during the past decade have shown beneficial effects of L-arginine supplementation in both animal studies and humans [104–112]. However, most recent studies are inconsistent with these findings showing no sustained effect or no effect of L-arginine administration on endothelial function [113–119]. This could be due to the complex biochemical metabolism of L-arginine [120]. L-Arginine is derived from dietary intake, protein breakdown, or endogenous de novo synthesis from L-citrulline catalyzed by the enzymes argininosuccinate synthase (ASS) and arginine-succinate lyase (ASL) [121]. Afterwards, it is converted into ornithine and urea by arginase, agmatine by L-arginine decarboxylase (ADC), and NO by NOS. Therefore, arginine metabolism and availability depend on the level of its dietary intake and endogenous synthesis on the one hand and the extent of catabolism on the other hand [122]. Whereas diminished bioavailability of NO is a common mechanism of various vascular disorders and endothelial dysfunction, the deficiency of L-arginine available for eNOS has been recently related to enhanced arginase activity [123]. The latter notion has been supported by findings in atherosclerosis and other cardiovascular disorders where arginase expression or activity has been found increased, suggesting that it plays a predominant role in these conditions [124–127]. However, information on exact regulatory mechanisms of arginase gene expression or activity is still missing. Its expression can be upregulated by proinflammatory factors: TNF-α and interferon-γ, ROS, oxidized LDL via the LOX-1 receptor, hyperglycaemia, thrombin, hypoxia, and angiotensin II. Arginase, both isoforms I and II, is expressed in endothelial and smooth muscle cells of the vascular wall and competes with NOS for the substrate L-arginine [128]. Increased activity of arginase leads to reduction in L-arginine availability for NOS, thereby decreasing the production of NO and resulting in eNOS uncoupling. Uncoupled enzyme produces superoxide instead of NO which further increases arginase activity and impair NO generation via oxidation of tetrahydrobiopterin [129–132]. Arginase also inhibits the L-arginine transport in endothelial cells further exacerbating L-arginine deficiency and downregulating NO production [133]. Moreover, arginase, by increased formation of polyamines and L-proline, stimulates vascular smooth muscle cell proliferation and extracellular matrix deposition, thereby contributing to intimal hyperplasia and remodeling processes. Similarly, promoting abnormal remodeling and neointimal hyperplasia reduced NO bioavailability [134, 135].

eNOS uncoupling resulting in reduced NO bioavailability and increased oxidative stress causes and aggravates dysregulation of endothelial function. New therapeutic strategies for atherosclerosis are aimed at preventing or reversing the endothelial dysfunction, before clinical manifestations and disease progression will occur. For better understanding of pathophysiology of endothelial dysfunction, novel pharmacological approaches focused on eNOS recoupling are being investigated. Several drugs currently in clinical use, inhibitors of the renin-angiotensin-aldosterone system, statins, and nebivolol, show many pleiotropic actions. Recently, it has been demonstrated that they may prevent or reverse the eNOS uncoupling and improve endothelial function and NO bioavailability in animal models. Similarly do resveratrol, sepiapterin, folic acid, AVE3085, and AVE9488 (enhancers of endothelial nitric oxide synthase acting on the eNOS gene transcription). All these compounds target eNOS through multiple direct and indirect mechanisms; however, the detailed mechanisms of their action are beyond the scope of this review and are comprehensively reviewed elsewhere [25, 37, 136]. Despite beneficial effects in animal models, applying these experimental results to clinical treatment still requires further studies and more extensive investigation.

Emerging evidence has suggested the deficiency of L-arginine available for eNOS as an etiology for endothelial dysfunction and has related it to enhanced arginase activity [137]. In an animal model of arthritis (adjuvant-induced arthritis), it has been shown that arginase II isoform expression and activity were significantly increased and correlated

with disease activity [138, 139]. Nevertheless, high plasma arginase levels failed to correlate with plasma levels of IL-6. Although it is clearly recognized that systemic inflammation with increased proinflammatory cytokine production induces arginase expression, the exact regulatory mechanisms of enzyme activity or gene expression in the endothelial cells still remain elusive. In contrast, eNOS activity was found decreased with no change in its expression, and the authors attributed this discrepancy between eNOS activity and expression to decreased availability of the substrate for the enzyme. Moreover, they indicated that limiting L-arginine accessibility for NOS arginase upregulation contributes to enzyme uncoupling and endothelial dysfunction. Indeed, impaired endothelial function assessed by the vasodilating response to acetylcholine (Ach) was found in AIA rats, and arginase inhibition with a selective inhibitor N_w-hydroxy-nor-L-arginine (nor-NOHA) restored vascular function. The effect of a curative treatment with nor-NOHA on vascular function of AIA rats was further investigated, and authors indicated that it is mediated by an increase in NOS activity and endothelium-derived hyperpolarizing factor (EDHF) production; a decrease in cyclooxygenase 2 (COX-2), thromboxane (TX), and prostaglandin I2 (PGI_2) synthase and NADPH oxidase activities; and a decrease in superoxide production and secreted vascular endothelial growth factor (VEGF) levels. Both enhanced NOS activity and reduced superoxide production can be due to the decrease in vascular eNOS uncoupling, thanks to the beneficial effect of arginase inhibition and restored L-arginine bioavailability. Interestingly, arginase inhibition had no impact on disease severity assessed by clinical, histological, and radiological parameters, whereas it fully reversed endothelial dysfunction in AIA rats. Furthermore, plasma IL-6 levels did not correlate with endothelial dysfunction. Taking these findings into account, the authors conclude that endothelial dysfunction is not the consequence of the disease, at least in the chronic phase of the AIA model. Since the reduction of endothelial dysfunction seems to be possibly independent of RA disease activity, they indicated that the benefits provided by nor-NOHA are related to the direct modulation of endothelium-derived vasorelaxant pathways rather than an anti-inflammatory effect [138, 139]. It is noteworthy that this beneficial effect of arginase activity inhibition can also be obtained with statins, diclofenac, and etanercept [101, 140, 141].

3. Clinical Implications: eNOS Uncoupling and Autoimmune Rheumatic Disease

It is well established that patients with rheumatic autoimmune diseases are characterized by significantly increased prevalence of cardiovascular morbidity and mortality than the general population. The current European guidelines on cardiovascular disease (CVD) prevention in the clinical practice recommend to use a 1,5-factor multiplier for CV risk in rheumatoid arthritis as well as in other autoimmune inflammatory diseases. However, mechanisms of accelerated atherosclerosis in these diseases, especially in the absence of traditional risk factors, still remain unclear.

3.1. Systemic Lupus Erythematosus (SLE). It is now clearly recognized that SLE patients are at high risk of developing CVD, and this excessive risk is especially pronounced in premenopausal women. Traditional risk factors do not fully account for this association, and the disease itself is considered an independent CV risk factor [142]. Therefore, precocious atherosclerosis is likely attributable to the consequences of inflammation, which is in line with observations that disease duration, higher damage index score, and less aggressive immunosuppression are associated with increased CVD burden in SLE patients. Till now, there are no biomarkers that predict CV events in SLE patients, and the known ones used to assess CV risk in the general population have limited or no value in SLE [143–145]. However, patients with SLE show enhanced endothelial dysfunction, which is regarded as a common mechanism for various CV disorders and considered the first step in atherosclerosis. Underlying mechanisms and its pathogenesis in SLE are still poorly understood [146, 147]. It is thought that the common denominator for multiple mechanisms contributing to the development of endothelial dysfunction is diminished activity of endothelial nitric oxide synthase and loss of nitric oxide production. Recent analyses have shown that SLE-specific circulatory factors, TNF-α, interleukin-17, interferons, ligand of cluster of differentiation 40 (CD40L), and C-reactive protein (CRP), lead to endothelial dysfunction via promotion of abnormal eNOS function and enhanced oxidative stress [148]. Among these mediators, type I interferons gained considerable attention. Continuous inflammatory production of interferon-alpha (IFN-α) and subsequent increased expression of IFN-α-regulated genes, referred as IFN signature, due to activation of plasmacytoid dendritic cells by immune complexes, consisting of autoantibodies in combination with deoxyribonucleic acid- (DNA-) or ribonucleic acid- (RNA-) containing autoantigens, have been reported in SLE patients. Studies on animals and humans have provided evidence that IFN accelerate atherosclerosis on multiple stages [149–151]. To date, no studies were conducted to determine direct effects of IFN on eNOS function and NO generation. However, IFN type I has been reported to have impact on enzyme cofactors, its specific transcription factors, and oxidative stress pathways [151]. Animal and human studies indicate that IFN-α leads to depletion of BH_4 via oxidation, serving as a potential mediator of eNOS uncoupling and oxidative stress [152, 153]. There are also scarce studies investigating the role of interferon on L-arginine availability. However, a study conducted in patients with high-risk melanoma showed that therapy with pegylated IFN-α results in a marked decrease in the synthesis of NO and arginine availability [154, 155]. Information on impact on arginase activity is also missing. However, recently, a significant increase in serum arginase 1 activity was detected in the SLE patients. Its levels were positively correlated with the disease severity and IL-17. Furthermore, arginase 1 was found to enhance T helper 17 (Th17) cell differentiation both in vitro and in vivo, augmenting inflammation [156]. Although direct effect of these observations on eNOS function warrants further research, it is thought that inflammatory and immune process characteristics for SLE contribute to the development

of premature atherosclerosis [157]. As reported, disease marker anti-Smith (anti-Sm) and anti-ribonucleoprotein (anti-RNP) antibodies stimulate IFN type I production by plasmacytoid dendritic cells [158]. Surprisingly, an inverse correlation between the presence of atherosclerosis in SLE (evaluated as arterial stiffness and presence of carotid plaque) and anti-nuclear antibodies was observed. Actually, patients with plaque had less frequent anti-Sm and/or anti-RNP antibodies than those without plaque [159]. Therefore, further studies are needed to clarify why patients with anti-nuclear antibodies have less pronounced subclinical atherosclerosis, even having more systemic and severe course of disease, interspersed with episodes of acute disease flares.

Besides IFN, anti-DNA autoantibodies are the hallmark of SLE. It has been implicated that anti-double-stranded DNA (anti-dsDNA) antibodies may have a role in the development of cardiovascular disease in SLE by enhancing ADMA production and by potentiating the inflammatory reaction. Indeed, it has been demonstrated *in vitro* that in the presence of anti-dsDNA, methylation of arginine residues in proteins by PRMT I is increased; therefore, anti-dsDNA antibodies may be a trigger for enhanced ADMA production in SLE [160]. Results of in vitro studies were confirmed by findings observed in vivo, where there are high plasma levels of complement (C3 and C4), measures of disease activity and organ damage, CV events, and prednisone use [161, 162]. Although ADMA is significantly associated with risk factors for CVD in the general population, no such correlation was found in SLE patients [161, 162].

Subclinical atherosclerosis in SLE has been reported and described by different methods. In SLE patients without CVD, the ADMA was independently associated with the coronary calcium score and arterial stiffness [159, 162]. Nevertheless, no association with the presence or extent of carotid atherosclerosis (assessed by carotid ultrasonography—intima-media thickness (IMT) and plaque) was found [159, 162]. These inconsistent findings are attributed by authors to differential effect of ADMA on distinct vascular beds. There are two major mechanisms proposed underlying vascular disease in SLE: IFN-induced reduction of endothelial cell proliferation and survival with subsequent impaired repair and remodeling and ADMA-induced inhibition of eNOS [159]. Nevertheless, further studies are needed to investigate the role of the NO pathway and its components in atherogenesis in SLE.

3.2. Rheumatoid Arthritis (RA). Similar to SLE, endothelial dysfunction has been reported in RA patients, even in the very early stages of disease [163]. Since it has been speculated that RA-related inflammation might contribute to endothelial dysfunction, anti-TNF therapy has been shown to improve vascular function, which strongly indicates involvement of systemic inflammation in the development of premature atherosclerosis [164]. Recent studies on animals showed that endothelial function in adjuvant-induced arthritis (AIA) rats is significantly depressed without any histologic damage, supporting the idea that endothelial dysfunction occur before overt vascular damage [96]. The mechanism of endothelial dysfunction in RA remains still incompletely understood, but decreased NO bioavailability along with increased ROS production has been suggested. Besides NADPH oxidase, uncoupling eNOS has been identified as an important source of ROS and its expression was significantly increased at both messenger RNA (mRNA) and protein levels in AIA rats. Furthermore, incubation of homogenates of AIA rat aortas with L-arginine led to overproduction of superoxide.

Although RA disease-related inflammation may contribute to elevated ADMA levels and increased CVD risk in RA, the association between ADMA and disease activity has been an issue of debate, as previous studies are heterogenous in results. It has been demonstrated that baseline disease status (mainly elevated erythrocyte sedimentation rate (ESR) and also CRP) and cumulative inflammatory burden (6 years of follow-up) had a positive correlation with current ADMA levels as—according to authors—patients with longer periods of uncontrolled disease are more prone to develop endothelial dysfunction due to the higher cumulative inflammatory burden on the vasculature [165]. A few studies have reported a positive correlation between ADMA and inflammatory markers (CRP and ESR), disease activity (DAS 28) and duration, and clinical parameters of disease status (tender and swollen joints, morning rigid) independently of the presence of classical risk factors and CVD [61, 64–66, 165–168], not confirmed by other studies [60, 61, 63, 66, 169]. Similar results were obtained concerning RA disease-specific markers—rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) [60, 61, 63–66, 168]. Interestingly, no association was found with traditional risk factors [55, 68], apart from homeostatic model assessment (HOMA) referred to as the indicator of insulin resistance, being the only independent predictor of elevated ADMA levels in RA patients [169]. Additionally, ADMA has been shown to correlate with other biomarkers of vascular dysfunction such as endothelial progenitor cell (EPC) count [67].

The studies investigating a possible impact of the disease-modifying antirheumatic drugs (DMARDs) on ADMA levels also provided conflicting results. In a small prospective study conducted on treatment-naïve patients with early RA, a significant decrease in ADMA serum levels after 12 months of immunosuppressive treatment with synthetic and biologic DMARDs along with glucocorticoids was reported [60]. However, no increase in carotid IMT was observed after 12 months of DMARD therapy. These findings were not confirmed by another study performed in a similar subgroup of patients treated for 18 months with either methotrexate or adalimumab [170]. Although an improvement in CFR was found, both carotid IMT and plasma ADMA levels did not show significant changes after therapy. The lack of effect of methotrexate and TNF inhibitors (etanercept, adalimumab, and infliximab) on plasma concentrations of ADMA was also demonstrated in long-standing RA patients [58, 61]. Data from studies determining the impact of short-term anti-TNF administration are also inconsistent. One report described similar to baseline ADMA values after 2 weeks and 3 months of anti-TNF treatment with etanercept, infliximab, or adalimumab [167], whereas others demonstrated a significant reduction of dimethylarginine in the group of

patients receiving etanercept or adalimumab [166, 171]. In the latter study by Spinelli et al., besides a decrease in ADMA plasma concentrations, anti-TNF therapy restored circulating endothelial progenitor cell levels, although a not significant increase of FMD was observed. Finally, it has been shown that TNF inhibitors improved the L-arginine/ADMA ratio due to the increase in L-arginine, and the L-arginine/ADMA ratio was longitudinally related to PWV after initiation of anti-TNF-α therapy [58]. Acute and chronic oral treatments with glucocorticoids have also different effects on arginine metabolites; while acute prednisolone therapy has no impact, chronic prednisolone treatment reduces ADMA and SDMA plasma concentrations [57].

Given the evident role of TNF in atherosclerosis and RA pathogenesis and its inhibitory effect on DDAH leading to ADMA accumulation, a beneficial effect of TNF inhibition has been postulated; however, results of conducted studies did not demonstrate a consistent decrease in ADMA levels with subsequent improvement in vascular morphology and function suggesting that the ADMA level does not seem to be a straightforward indicator of endothelial dysfunction and subclinical atherosclerosis in rheumatic diseases. Due to the complexity of the processes observed in RA and atherosclerosis, contributions of traditional and disease-related risk factors cannot be excluded as well as other mechanisms of DMARD action compared with increased NOS activity/expression. The heterogeneity of the study population and methods used to assess subclinical atherosclerosis may also account for the lack of concordance of the results and limit the usefulness of ADMA as a marker for atherosclerotic risk stratification [61, 165, 171].

In accordance with results from animal studies, an increase in plasma arginase activity with a significant decrease in arginine bioavailability was reported in patients with RA [55]. Similar observations were made regarding the catabolic product of arginase (L-ornithine) and catabolic product of NOS (L-citrulline). Interestingly, it has been shown that elevated arginase activity was associated with prior history of CVD in a subgroup of patients with RA, but it did not show any correlation with traditional risk factors. In contrast to animal studies, significant increase in arginase activity seemed to be independent of disease activity; however, patients in the analyzed cohort had relatively low RA disease activity reflected by Disease Activity Score (DAS28). On the other hand, systemic inflammatory conditions can increase arginase expression in endothelial and immune cells, and therefore, authors indicate that elevated arginase levels can be due to higher turnover of these cells. Although the relationship between systemic inflammation in RA and arginase activity warrants further research, authors avail this disconnection between arginase activity and RA disease activity for the clinical practice and proposed arginase activity as a potential biomarker of increased CVD risk independent of the patient's disease state [55].

3.3. Primary Sjogren Syndrome (pSS). There is mounting evidence that primary Sjogren syndrome, similar to SLE and RA, has increased morbidity of CVD [172]. However, limited evidence is available for primary SS regarding premature atherosclerosis and endothelial dysfunction. Previous studies examining subclinical CVD measured by different techniques were heterogenous in results. Nevertheless, their results indicate a subclinical vascular damage that would explain higher CV risk [173]. There are scarcely no studies determining the eNOS function and NO generation in pSS. However, some recent data shows increased oxidative stress in pSS and association of disease with IFN-I signature, which could exert indirect effects as described above [174–176]. Therefore, pSS emerges due to the similarity to SLE and RA and also due to the fact that most patients are out on medication, as an interesting model to study atherosclerosis in autoimmune diseases.

4. Conclusions

The role of oxidative stress has been well established in the development and progression of atherosclerosis, and eNOS uncoupling appears to be an important mechanism contributing to increased ROS generation. Moreover, eNOS uncoupling is also mediated by excessive ROS formation ("ROS-induced ROS formation").

Chronic systemic inflammation is considered an independent CV risk factor, and it contributes significantly to oxidative stress. Due to the close interaction between inflammation and oxidative stress, autoimmune rheumatic diseases are associated with increased CV morbidity and mortality even when traditional risk factors are absent. However, the exact role of eNOS uncoupling in premature atherogenesis in rheumatic diseases is still not fully elucidated. Similarly, there is scarcity of data on the interactions between the NO metabolic pathway and disease-related factors. The best-studied mechanisms thus far are the depletion of eNOS cofactor BH_4, L-arginine deficiency, and increase in endogenous eNOS inhibitor, ADMA. Therefore, the thorough understanding of molecular mechanisms underlying impaired NO bioavailability and eNOS dysfunction may help to identify the best and most effective approach to prevent and manage CV complications in rheumatic diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest to declare.

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Review Article

Intraplatelet L-Arginine-Nitric Oxide Metabolic Pathway: From Discovery to Clinical Implications in Prevention and Treatment of Cardiovascular Disorders

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Despite the development of new drugs and other therapeutic strategies, cardiovascular disease (CVD) remains still the major cause of morbidity and mortality in the world population. A lot of research, performed mostly in the last three decades, revealed an important correlation between "classical" demographic and biochemical risk factors for CVD, (i.e., hypercholesterolemia, hyperhomocysteinemia, smoking, renal failure, aging, diabetes, and hypertension) with endothelial dysfunction associated directly with the nitric oxide deficiency. The discovery of nitric oxide and its recognition as an endothelial-derived relaxing factor was a breakthrough in understanding the pathophysiology and development of cardiovascular system disorders. The nitric oxide synthesis pathway and its regulation and association with cardiovascular risk factors were a common subject for research during the last decades. As nitric oxide synthase, especially its endothelial isoform, which plays a crucial role in the regulation of NO bioavailability, inhibiting its function results in the increase in the cardiovascular risk pattern. Among agents altering the production of nitric oxide, asymmetric dimethylarginine—the competitive inhibitor of NOS—appears to be the most important. In this review paper, we summarize the role of L-arginine-nitric oxide pathway in cardiovascular disorders with the focus on intraplatelet metabolism.

1. Introduction

After establishing the real nature of EDRF by Furchgott et al. [1, 2], which appeared to be nitric oxide (NO), numerous other groups were working on the nitric oxide synthesis pathway and its potential role in human (patho)physiology. This led to the discovery of the nitric oxide synthase [3] which produces nitric oxide from L-arginine with flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH4), and heme with a zinc atom as cofactors. From that time, numerous functions of NO were established which can generally be divided into three groups:

(1) Group associated with neuronal transmission, where the NO plays an inhibitory role as a mediator in peripheral nonadrenergic noncholinergic (NANC) neurotransmission (causing relaxation mainly in the gastrointestinal tract, penile corpus cavernosum, and bladder) [4]

(2) Group playing an inflammatory role, where NO is produced by the inducible isoform of nitric oxide synthase (iNOS)

(3) Group related to the cardiovascular system

2. Nitric Oxide in Cardiovascular Disorders

Despite the development of new drugs and other therapeutic strategies, cardiovascular disease (CVD) remains still the major cause of morbidity and mortality in the world population [5]. A lot of research, performed mostly in the last three decades, revealed an important correlation between

"classical" demographic and biochemical risk factors for CVD (i.e., hypercholesterolemia [6], hyperhomocysteinemia [7], smoking [8], renal failure [9], aging [10], diabetes [11], and hypertension [12]) with endothelial dysfunction associated directly with the nitric oxide deficiency. In the vascular endothelium, NO is produced by the endothelial isoform of nitric oxide synthase (eNOS = NOS3) which is constitutively active, allowing the maintenance of appropriate vascular tone by constant vasodilating action [13]. The other functions of NO are inhibition of platelet aggregation, inhibition of smooth muscle proliferation, and leucocyte interaction with the vascular wall [14]. All of these properties place nitric oxide as a key modulator of vascular homeostasis. Nowadays, endothelial dysfunction, defined as a reduction in the endothelial NO bioavailability, can be measured noninvasively by the change in blood flow (e.g., EndoPAT 2000 and brachial flow-mediated dilation) or appropriate agonists (e.g., reaction to acetylcholine administered by iontophoresis measured by laser Doppler flowmetry) [15]. There are several mechanisms which can limit the bioavailability of NO. One of them is a decrease in the eNOS expression in endothelial cells which occurs in advanced atherosclerosis [16] and in smokers [17]. Decreased NO production can also be an effect of L-arginine deficiency or nitric oxide synthase cofactors. A lot of studies have been performed on the oxidative stress as a factor limiting the NO bioavailability [18]. An imbalance between the creation of reactive oxygen species (ROS) and their scavenging by antioxidants promotes the reaction between NO and $O2^-$ which results in the peroxynitrite formation. Peroxynitrite is a potent oxidative compound which promotes posttranslational modifications of proteins (including the eNOS protein) [19], alterations in the main metabolic pathways [20], or eNOS uncoupling which results in the production of superoxide anion instead of NO [21, 22]. Increased formation of peroxynitrite and other reactive oxygen species has been demonstrated in established cardiovascular system disorders [23] and is associated with a vast majority of CVD risk factors such as hypertension [24], diabetes [25], tobacco use [26], and hypercholesterolemia [27]. Another mechanism responsible for nitric oxide deficiency, which is deeply investigated, is connected with competitive inhibition of nitric oxide synthase by asymmetric dimethylarginine (ADMA)—a naturally occurring amino acid circulating in plasma and present in various tissues and cells.

3. ADMA as the Most Potent Inhibitor of the L-Arginine-Nitric Oxide Pathway

The first mention about asymmetric dimethylarginine presence comes from the study by Kakimoto and Akazawa who have isolated its crystalline form, among other substances, by ion-exchange chromatography of the aliphatic basic amino acid fraction of human urine [28]. By the fact that its concentration in urine is not affected by arginine administered orally, the authors assumed that this compound may be a derivate from endogenous protein proteolysis. In 1992, Leone et al. proposed its potential pathophysiological role by providing *in vitro* and *in vivo* evidence that ADMA inhibits NO synthesis [29]. In addition, they described the accumulation of dimethylarginines by the lack of urine production in patients with end-stage chronic renal failure as a potential mechanism of hypertension and immune dysfunction in this group of patients.

Methylated derivates of arginine are produced as a result of proteolysis of endogenous methylated proteins, i.e., histones. This methylation is catalysed by two isoforms of the arginine methyltransferases (PMRTs)—PMRT-1 and PMRT-2 proteins—with S-adenosylmethionine as a donor of methyl residues. As an effect of PMRT-1—the main isoform present in the vascular wall (endothelial and smooth muscle cells)—asymmetrically dimethylated and monomethylated arginine residues are formed. PMRT-2 is also capable of mono- and dimethylation of arginine residues, but in this case, residues are dimethylated symmetrically [30, 32]. After protein degradation, methylarginine compounds appear at the beginning in the cytosol but also in plasma [31]. Monomethylated arginine (L-NMMA) and asymmetric dimethylarginine (ADMA) are inhibitors of all nitric oxide synthase isoforms whereas symmetric dimethylarginine (SDMA) is not (Figure 1). The inhibitory effect of ADMA and L-NMMA on NOS is similar [33], but considering that plasma concentration of ADMA is up to tenfold higher than that of L-NMMA, ADMA was an object of research for the last decades. The inhibition of NOS may not be the only effect of asymmetric dimethylarginine in human. There are reports that at high concentrations, both ADMA and SDMA may compete in the transport through the Y-amino acid transporter with arginine [34] and also may inhibit the Na+/K+ ATPase [35]. However, concentrations required for these actions seem to be too high to be clinically relevant. Murray-Rust et al. proposed another potential target for ADMA, which is the arginine-glycine amidinotransferase. The structure of this enzyme is similar to that of dimethylarginine dimethylaminohydrolases (DDAHs) that metabolize ADMA [36]. However, Vallance and Leiper suggest that ADMA is only a poor inhibitor of this transferase [37].

All of the methylated arginine derivates are eliminated by kidneys. In contrast to SDMA, which is excreted completely by kidneys, ADMA and L-NMMA are also degraded by DDAH [38, 40]. As a result, citrulline and the monomethylamines are formed. The catalytic site of DDAH involves cysteine residue. Its nitrosylation by reactive nitrogen species renders the enzyme inactive which can be the potential homeostatic mechanism especially in reactions involving the inducible NOS (iNOS) (increased production of NO leads to the accumulation of ADMA by inhibiting the DDAH) [39] (Figure 2). In addition, this cysteine residue is susceptible to the action of the number of oxidative stress-related cardiovascular risk factors such as hypercholesterolemia, hypertension, renal failure [41], hyperhomocysteinemia, hyperglycaemia [42], and tobacco smoking [43], which also results in the accumulation of ADMA. This can be a pathway, allowing different factors to affect endothelial function [44]. There are two known isoforms of DDAH. DDAH-1 accompanies the neuronal NOS and is present in the liver, kidneys, and lungs (its action contributes to circulating

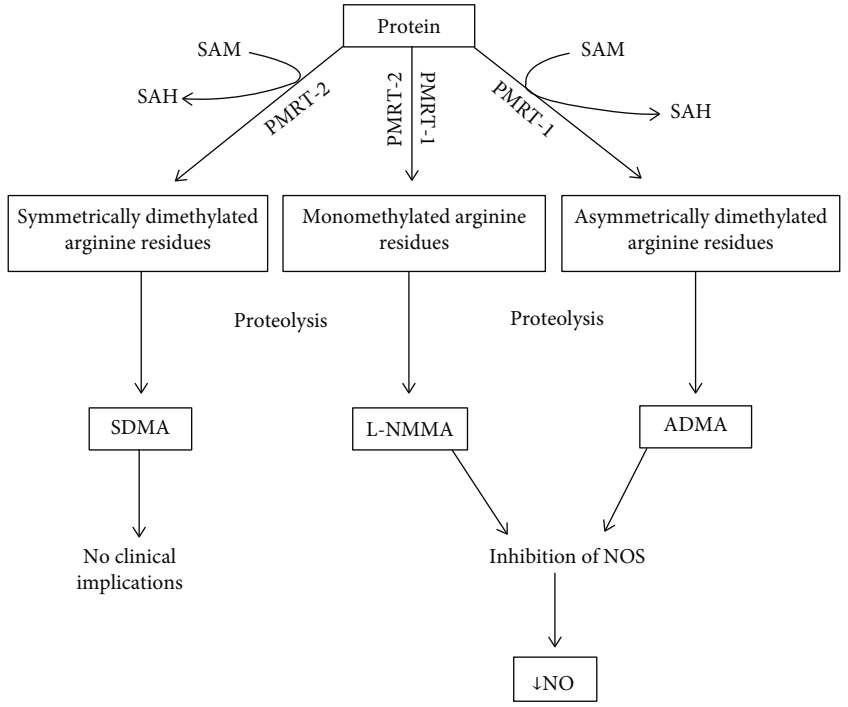

Figure 1: Synthesis of ADMA from methylated proteins. SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; PMRT-1: protein methyltransferase-1; PMRT-2: protein methyltransferase-2; SDMA: symmetric dimethylarginine; L-NMMA: monomethylated arginine; ADMA: asymmetric dimethylarginine; NOS: nitric oxide synthase; NO: nitric oxide. Based on [30–32].

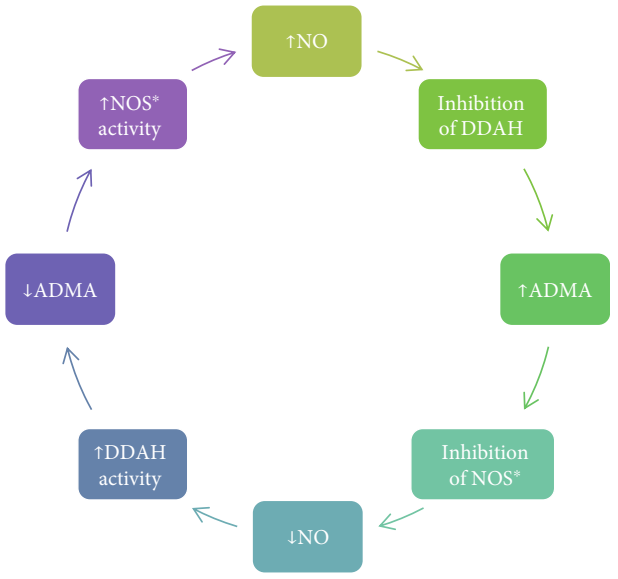

Figure 2: Potential homeostatic mechanism of autoregulation of nitric oxide production. NO: nitric oxide; NOS: nitric oxide synthase; ADMA: asymmetric dimethylarginine; DDAH: dimethylarginine dimethylaminohydrolase; ∗: reactions involving inducible nitric oxide synthase. Based on [38, 39].

ADMA concentration), while DDAH-2 is present in tissues expressing endothelial and inducible NOS and dominates in vessels, especially endothelium and smooth muscle cells (its role is connected with the located regulation of the amount of ADMA) [38, 45].

Concentrations of ADMA in plasma, which are connected with biologic action, are approximately 10-fold higher than concentrations observed under physiological condition. It suggests that even if plasma concentration of ADMA indicates its amount in the whole body, it does not mean that the same concentrations are present in all tissues [31, 46]. In the study by Cardounel et al., where the inhibition of NO generation by bovine endothelial cells was measured, the effect of raising ADMA concentrations was greater than expected on the basis of kinetic studies [47]. It suggests that there should be a mechanism of methylarginine uptake by cells, which, according to Bogle et al., could be the y^+ transport system [34]. The regulation of transport may be the explanation for "L-arginine paradox." This term is used to refer situations where exogenous administration of L-arginine led to enhancement of endothelial vasodilatory function, which is present despite the fact that its plasma concentration is up to 30-fold higher than necessary to completely saturate the NOS [48]. In physiological conditions, plasma level of ADMA is insufficient to compete with L-arginine in transport through the endothelial cell membrane [49]. However, in subjects with developed CVD or with a high CVD risk profile, elevated ADMA concentrations are able to affect the eNOS activity. The restoration of eNOS activity and improvement of vasodilatory function of the endothelium by the addition of exogenous L-arginine in pathological conditions, without effects in healthy subjects, point to the L-arginine/ADMA ratio, instead of L-arginine and ADMA concentrations alone, as the main factor regulating the NO bioavailability [50, 51].

4. The Role of ADMA in Cardiovascular Disease Development

After the discovery of ADMA and the establishment of its function in the L-arginine → nitric oxide → cGMP pathway, the research focused on the connection of elevated ADMA concentrations with CVD and classic cardiovascular risk factors. One of the first studies evaluating the role of ADMA was performed by Bode-Böger et al. They showed that elevated concentrations of asymmetric dimethylarginine are found in hypercholesterolemic rabbits and it is the first biochemical abnormality observed at the early stage of atherosclerosis [52]. The following studies led to the discovery that elevated ADMA plasma concentrations are present in humans with hypercholesterolemia and with vascular disease. This finding was associated with endothelial dysfunction and impairment in the NO production measured by lower excretion of nitrates in the urine and worse NO-dependent forearm vasodilation [53]. It led to the conclusion that elevated ADMA concentration is an early marker of endothelial dysfunction known as a prognostic marker of severe cardiovascular events. At the end of the previous century, Cardillo et al. discovered that hypertension is associated with a defect in NO synthesis [54]. As a result, impaired endothelium-dependent vasodilation occurred, but the reaction for isoproterenol and sodium nitroprusside, which both enhance the NO concentration, was preserved. It means that endothelial dysfunction in hypertension is an effect of the selective decrease in NO bioavailability. Other studies proved that in early stages, hypertension is connected with the elevated plasma level of ADMA. Sonmez et al. compared the population of healthy adults with a demographically matched group of people with a recent diagnosis of hypertension, yet without any medical intervention [55]. Their work indicates that even in the initial stage of the disease, hypertensive patients have a higher level of plasma ADMA concentrations when other factors such as age, BMI, smoking history, CRP level, total cholesterol, LDL cholesterol, and triglyceride levels were similar between the two groups. Other studies on the link between ADMA and hypertension are in line with the previous results [56]. In addition, Curgunlu et al. demonstrated that ADMA concentration is elevated not only in hypertensive subjects but also in individuals with white coat hypertension, which indicates endothelial dysfunction presence in this state. Numerical values of ADMA concentrations place people with white coat hypertension in the continuum between normotensive (lower) and hypertensive (higher ADMA concentrations) subjects [57].

Aging is one of the main risk factors for cardiovascular disease and is connected with the progression of endothelial dysfunction. This resulted in the hypothesis that aging may be associated with increased ADMA concentrations. Gates et al. compared ADMA levels in a group of young adults (18-26 years old) with those in older ones (52-71 years old) without accompanying diseases except for impaired endothelial function measured by forearm flow-mediated dilation. The lack of difference in ADMA concentrations between these groups points to another reason for the dysfunction of endothelium during aging other than competitive inhibition of NOS [58].

One of the main risk factors for coronary artery disease and other cardiovascular disorders is tobacco smoking. Sobczak et al. investigated the influence of active and passive smoking for ADMA concentration in healthy volunteers without other CVD risk factors. The ADMA concentrations were higher in active and passive smokers when compared to the nonsmoking control group. However, these differences were not statistically significant [59]. Other research on the effect of tobacco smoking on the NO bioavailability presented similar results, but most of them were performed on a population with already developed cardiovascular disease [60, 61]. Despite the fact that some authors observed higher ADMA plasma concentrations in healthy people smoking >20 cigarettes daily [62], it seems that endothelial dysfunction and higher CVD risk related to tobacco use are not connected with alterations in NO bioavailability.

In contrast to smoking, hyperhomocysteinemia is among the risk factors that probably cause endothelial dysfunction by elevating plasma levels of asymmetric dimethylarginine. There are some hypotheses regarding the exact pathway in which concentrations of these compounds are connected. The first of them is that hyperhomocysteinemia causes increased methylation of proteins when ADMA is a product of proteolysis [63]. This hypothesis was initially supported by detecting higher ADMA concentrations after the hyperhomocysteinemic meal in humans [64]. However, there are other possible mechanisms such as stimulating SAM-dependent activity of PRMTs, decreased renal excretion, or inhibiting the DDAHs—enzymes responsible for ADMA degradation. Supporting the last hypothesis, Stühlinger et al. found that higher ADMA concentrations after exposure to homocysteine or methionine are connected with decreased activity of the DDAH isoforms [42]. The same authors observed direct inhibition of recombinant human DDAH activity by homocysteine in cell-free systems. What is more, other studies showed that mice with hyperhomocysteinemia had decreased levels of mRNA for the two major DDAH enzymes [65]. Considering the above-given reports, further research is needed to establish the exact mechanism of ADMA concentration elevation by homocysteine.

Metabolic disorders such as obesity, insulin resistance, and diabetes mellitus (DM) are known to be the risk factors for cardiovascular disease development. In all of them, endothelial dysfunction appears to play a pivotal role. It has been proposed that elevated levels of plasma ADMA concentration are responsible for the impairment in the NO bioavailability in metabolic disorders (Figure 3). Almost every diabetic person (with the exception of young subjects with type 1 diabetes) is considered a patient with a high CVD risk profile. In this population, even with no atherosclerosis and other organ damage, elevated ADMA levels are obtained [66]. The ADMA concentrations rise not only in general but also in dynamic situations. Fard et al. investigated changes in ADMA level 5 hours after ingestion of a high-fat meal. Their study demonstrated acute elevation of its concentration followed by a decreased vasodilatory response of the brachial artery measured by flow-mediated dilation

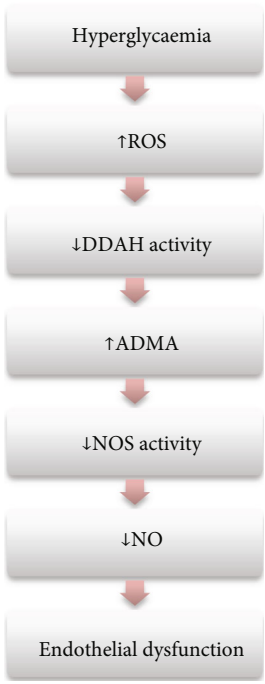

Figure 3: The effect of hyperglycaemia on the L-arginine-nitric oxide pathway. ROS: reactive oxygen species; DDAH: dimethylarginine dimethylaminohydrolase; ADMA: asymmetric dimethylarginine; NOS: nitric oxide synthase; NO: nitric oxide. Authors' modification based on [66–69].

which can be another factor promoting the development of atherosclerosis [67]. Higher ADMA level is a predictor of poor prognosis in patients with DM and already developed coronary artery disease [68] and is also a predictor of acute cardiovascular events in DM patients without vascular changes [69]. Endothelial dysfunction connected with elevated ADMA concentration is present also in prediabetic states such as obesity and insulin resistance [70–72] and is considered the first step in the development of atherosclerosis. The intensity of its dysfunction is higher in insulin-resistant subjects than in obese but insulin-sensitive ones [73]. Some research has shown that weight loss (provided by bariatric surgery followed by diet or only by diet), as well as reduction of insulin resistance (by pharmacological and nonpharmacological treatment), resulted in lowering of plasma ADMA levels and in the improvement of endothelial function [71, 73]. Searching for an explanation of elevated ADMA concentrations in metabolic disorders, Lin et al. conducted a study in which the possible pathways of ADMA accumulation in diabetic rats were investigated [74]. The discovery was that this accumulation is connected with reduced endothelial DDAH activity. However, the amount of DDAH found in the aortic endothelium of both groups was comparable. This suggests that these effects are reversible which is consistent with the studies mentioned above. As DDAH is sensitive to oxidative stress [36], hyperglycaemia-induced release of free radicals may be responsible for the elevation of ADMA concentration and endothelial dysfunction in metabolic disorders.

5. NOS Pathway in Platelets (Figure 4)

Shortly after the discovery of the L-arginine-nitric oxide pathway, the presence of nitric oxide synthase activity in human platelets was reported by Radomski et al. [75]. The tests performed on the specially prepared platelet cytosol showed that an increase in cGMP concentration was shown not only with direct NO donors (sodium nitroprusside) but also with L-arginine, known as a substrate of nitric oxide synthase. The effect of L-arginine is dependent on the presence of NADPH which indicates the enzymatic character of this reaction. The formation of NO in the platelet cytosol was inhibited by the addition of competitive NOS inhibitors such as L-NMMA which provides evidence of the presence of the nitric oxide synthesis pathway in human platelets. The L-arginine addition to the platelet cytosol did not increase the basal level of cGMP when platelets were not activated by collagen. It shows that the exogenous substrate, such as L-arginine, can be used by platelet NOS only after activation which probably potentiates the transport of the substrate through the platelet membrane [76]. However, the presence of nitric oxide synthase and all NO pathway components was questioned by some authors. Subjects to doubt were, among others, contamination of the probes with another blood cells [77], lack of specificity of used assays [78], measurement of cGMP activity or the amount of L-citrulline as indicators of NOS activity without considering other metabolic pathways [79, 80], or measurement of inorganic NO metabolites [81]. Finally, Cozzi et al. directly visualized the nitric oxide production by collagen-induced platelets using an NO-specific fluorescent agent—4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) [82]. This agent reacts with NO and provides a fluorescent signal. The specificity of this compound was tested by incubation in an NO donor and H_2O_2—fluorescence occurred only in solution with an NO donor. What is more, in tests conducted on platelets from eNOS-knockout mice, fluorescence was not observed—it further confirms the specificity of DAF-FM. The results of this study confirmed the presence of eNOS in platelets by the increase in DAF fluorescence in platelets adhering to type I collagen under flow. The intensification of the signal was dependent on the presence of the NOS substrate (L-arginine), as well as of the competitive NOS inhibitor (L-NMMA).

Despite the low concentrations of nitric oxide produced in platelets (compared to the endothelial cells) [82], it appears to have an important role in the aggregation of thrombocytes. According to Tymvios et al., platelet aggregation is regulated by endogenous NO. Inhibiting of all the endogenous NO action resulted in fatal thromboembolism in mice, but the deletion of the eNOS gene did not affect platelet reactivity [84]. This suggests that other sources of NO production, identified to be platelet-derived, are responsible for the regulation of aggregation and recruitment of thrombocytes. The increase in the amount of platelet-derived nitric oxide (PDNO) works as a negative feedback mechanism limiting the number of recruited thrombocytes during thrombus formation [75]. Impaired platelet NO production results in intensified surface P-selectin expression,

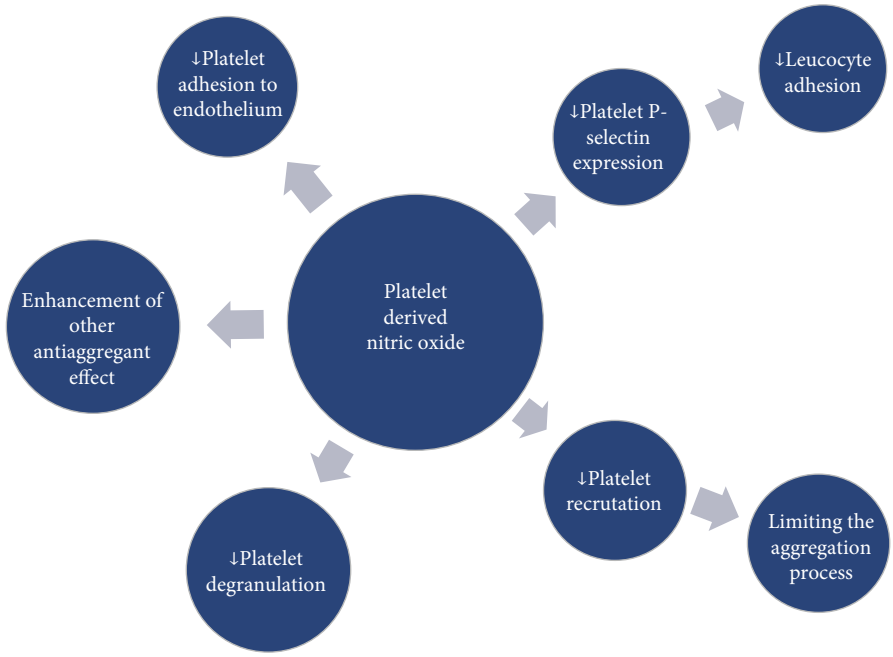

Figure 4: The known functions of platelet-derived nitric oxide. Authors' modification on the basis of [83].

which promotes coagulation by enhancing the expression of the tissue factor [85]. Considering the fact that platelet-derived NO release upon activation is delayed, its role is more complex. It not only limits the process of aggregation but also allows the recruitment of the required amount of cells to form the hemostatic clot [86]. Alterations of this subtle mechanism can play a crucial role in the development of cardiovascular diseases. Several studies provided data that impaired platelet-derived nitric oxide availability is connected with the intensity of coronary disease risk factors. Ikeda et al. showed the inverse correlation of PDNO with age, mean arterial pressure, hypercholesterolemia, and smoking [87]. What is more, the decrease in PDNO production was also correlated with a number of risk factors present in each individual. The impaired platelet-derived nitric oxide release is present also in already developed cardiovascular disorders such as coronary artery disease [88]. LDL cholesterol reduces L-arginine transport into platelets which is followed by reduction of the NO production [89]. It has been shown that statins have the potential to restore the PDNO release, which results in an improvement in the regulation of platelet aggregation [90, 91]. Platelet NOS activity is also impaired in subjects suffering from diabetes, both types 1 and 2 [92].

Patients with type 2 diabetes are characterized by impaired production of NO and cGMP with no changes in the amount of nitric oxide synthase. Intraplatelet level of cGMP presents an inverse correlation with glycated haemoglobin and blood glucose levels [93]. It suggests that impairment in platelet-derived NO production in this population may be associated with glycaemia-dependent suppression of platelet NOS activation [83].

Intraplatelet NO signalling is also affected in essential hypertension. Some studies show decreased platelet-derived NO release [94] and downregulation of receptors ($\dot{y}^{+}$ transport system) responsible for membrane L-arginine transport [95]. The inhibition of L-arginine transport, according to Brunini et al., is connected with elevated levels of ADMA and L-NMMA [96]. What is more, the use of specific agonists for NOS3 with different pathways of action did not result in an increase in its activity in hypertensive subjects [97]. It indicates the enzyme defect as the main reason for the impairment of platelet NO release. Although plasma ADMA concentrations are elevated in a hypertensive subject compared to a healthy subject, Tymvios with colleagues demonstrated that elevated plasma ADMA level does not alter platelet NO production [84]. It suggests the existence of another mechanism controlling PDNO release. On the contrary, De Meirelles et al. found that plasma ADMA and L-NMMA are capable of decreasing the intraplatelet cGMP concentration which corresponds with lower NOS activity [98]. Previously cited, Cozzi et al. [82] showed an impact of the competitive NOS inhibitor—L-NMMA—on the release of nitric oxide by platelets. There is a possibility that disruptions of the NO synthesis process in the abovementioned situations are the effect of the accumulation of NOS inhibitors in thrombocytes. Further research is necessary to test this hypothesis and evaluate its clinical importance.

Recent studies have shown another interesting aspect regarding PDNO release and its potential role in the hemostasis and thrombus generation. The discovery of two subpopulations of platelets, with and without the presence of intraplatelet eNOS, allowed the determination of a new hypothesis on a thrombus generation mechanism. In response to vascular injury, $eNOS^{neg}$ platelets (about 20% of all thrombocytes) adhere to the damaged area. This process is facilitated by the lack of endogenous NO production by this subpopulation. $eNOS^{neg}$ platelets, by the secretion of metalloproteinase-2, recruit $eNOS^{pos}$ ones (80% of the

thrombocyte population), which, by their higher COX-1 content and higher thromboxane production, form the majority of the emerging aggregate. However, their ability to produce NO results in the limitation of the thrombus size [99] [100]. In vitro studies showed that increase in the $eNOS^{neg}/eNOS^{pos}$ ratio, as well as inhibition of eNOS, promotes platelet aggregation. Changes in this ratio may be responsible for the impairment of blood coagulation homeostasis and may predispose individuals to developing CVD. It has been shown that platelets from patients after acute coronary syndrome produce less NO when compared to those from healthy ones [88]. Further research is needed to fully understand and determine the role of alterations in platelet subpopulations or their potential function as a target for new therapeutic strategies.

Little is known about the effect of antiplatelet drugs on NO release by thrombocytes. Inhibition of the GPIIb/IIIa receptor (responsible for fibrinogen binding during platelet aggregation) resulted in the enhancement of NO production and reduction of the formation of superoxide anion [101]. Acetylsalicylic acid (ASA) has different effects on NOS activity dependent on dose-dependent mechanisms of action and duration of the treatment. On the one hand, ASA reduces NOS activity by limiting the NOS-activating response to stimulation of platelet beta-adrenergic receptors—this effect is shared with other nonsteroidal anti-inflammatory drugs so it appears to be mediated through COX inhibition. On the other hand, acute in vivo and in vitro action of aspirin results in the acetylation of the platelet NOS and thereby in COX-independent activation of this enzyme. Of clinical relevance, chronic administration of small doses of ASA (75 mg per day) did not enhance platelet NOS activity in a COX-independent mechanism, but the response to beta-adrenergic stimulation remains reduced [102, 103]. What is more, Rothwell et al. showed that an optimal dose of ASA depends on bodyweight and that for subjects above 70 kg, a daily dose of 75 mg is insufficient to reduce cardiovascular events properly [104]. It suggests that the methodology of already conducted studies should be carefully revised.

6. Conclusions

The knowledge regarding the exact pathogenesis of impaired production of platelet-derived nitric oxide may have important clinical implications. Cardiovascular disorders are frequently related to enhanced thrombus formation. What is more, several conditions, despite the proper antiplatelet treatment, are associated with an elevated incidence of cardiovascular events. Identification of the patients with higher risk, for example, by assessment of platelet-derived nitric oxide production impairment or changes in the $eNOS^{neg}/eNOS^{pos}$ ratio, may enable the application of the more appropriate, individualized treatment or early implementation of proper prevention. More research on the exact relation between cardiovascular disorders and the amount of nitric oxide synthesized by platelets is necessary to fully determine their clinical importance. Finally, the knowledge about the biochemistry and exact pathways of PDNO actions may serve as a basis for creating new or using already known drugs in new indications.

Disclosure

The sponsors of the study had no role in the study design, data collection, data analysis, and manuscript writing.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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