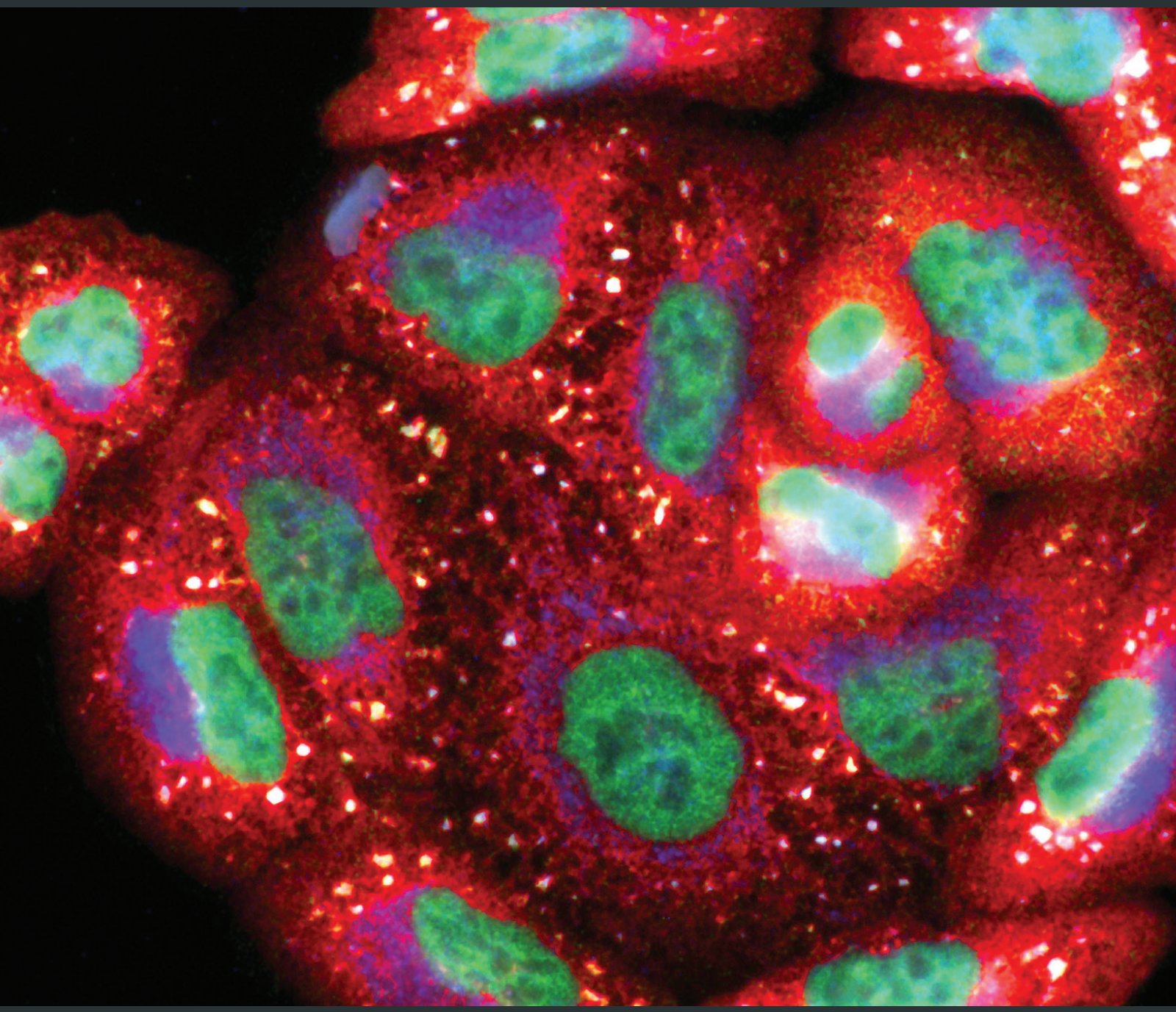
Oxidative Medicine and Cellular Longevity

Oxidative Stress and Membrane Transport Systems

Lead Guest Editor: Angela Marino
Guest Editors: Silvia Dossena, Grazia Tamma, and Sandra Donnini



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Editorial Board

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Editorial
Oxidative Stress and Membrane Transport Systems

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Abnormal intracellular levels of reactive species (RS), including oxygen and nitrogen RS, are recognized as common denominator of numerous pathological conditions including aging and frailty. In the recent years, it has been widely accepted that high levels of intracellular RS can be associated with several chronic disturbs, such as Alzheimer disease (AD), chronic kidney disease, atherosclerosis, and cardiovascular and endothelial dysfunctions.

Emerging concepts point out that RS can influence intracellular transduction pathways by acting as key signal molecules. Therefore, the roles of free radicals have been strongly redefined. Plasma membrane, as the interface between intracellular and extracellular environments, may sense several molecules, including RS, through the action of membrane receptors and channels that also mediate the transport of RS, such as hydrogen peroxide (H_2O_2) and nitric oxide (NO). In turn, membrane transport systems can also be controlled by oxidative signaling. In this respect, posttranslational oxidative modifications translate changes of oxidative intracellular environment under physiological and pathophysiological conditions.

This special issue aimed to depict a link between membrane transport systems and oxidative stress in health and disease. From the manuscripts received, we selected 5 reviews, 1 clinical study, and 1 research article that addressed the main objectives of this issue.

G. Tamma and coauthors reviewed the transport of RS as a novel function of the water channels aquaporins (AQPs). AQPs are widely expressed in the animal and plant kingdom, with 13 different isoforms found so far in mammals. AQPs are highly expressed in tissues displaying high water permeability, such as endothelia, kidney tubules, and secretory glands. However, AQPs are also expressed in skin and fat cells that, normally, are not characterized by relevant fluid transport. In this review, authors reported that AQPs facilitate RS transport in several cell types, including hepatocytes and endothelial cells. Special focus is given to the AQP3-mediated H_2O_2 transport, which plays an important role in modulating sperm mobility as well as cell migration, and AQP1-mediated NO transport, which is involved in vascular senescence. The link between AQP2, oxidative stress, and aging has been also reported. The emerging evidence of clinical implications deriving from RS abnormal permeation through AQPs is also emphasized.

K. Izuhara et al. reviewed the involvement of the anion transporter pendrin/SLC26A4 in inflammatory lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD). In particular, the attention has been focused on the proinflammatory cytokine IL-13, a well-established marker of asthma. IL-13 stimulates the expression as well as the function of pendrin, which in turn facilitates thiocyanate (SCN^-) transport into the airway surface liquid at the apical side of airway epithelium, where it reacts with H_2O_2, thereby producing hypothiocyanite ($OSCN^-$) via the DUOX/peroxidase pathway. $OSCN^-$ is involved in the innate defense of the lumen mucosa. Authors specifically focus on the different intracellular signal transduction pathways, that is, NF-κB signaling and cell necrosis, activated by different $OSCN^-$ concentrations. As such, the review not only highlights

the involvement of pendrin in the pathogenesis of lung disease but, more importantly, also provides a useful basis to identify novel drug targets for oxidative stress-related lung disease.

The review proposed by S. G. Vitale and coauthors highlights the roles of oxidative molecules in the pathogenesis of endometriosis, a chronic disorder affecting about 10–15% of women during the reproductive period. Though the molecular basis of this disease is not completely clarified, several risk factors have been found. In this latter regard, the authors report about inflammatory signals and oxidative molecules concurring to endometriosis. Oxidative molecules alter the permeability of endothelial cells and the expression of adhesion proteins, thereby promoting inflammatory processes. Indeed, oxidative molecules can also modulate the function of different immune cells. Last but not the least, authors reported the involvement of reactive oxygen species (ROS) in modulating epigenetic processes that may increase the risk of ovarian cancer. Overall, the review underscores the crucial role of antioxidant treatment in mitigating endometriosis progression.

G. Sita and coauthors, in their review, discuss the involvement of ROS in the modulation of P-glycoprotein (ABCB1) expression levels. ABCB1 is an ATP-binding cassette transporter, playing a role in the pathogenesis of AD, the most common cause of dementia and mortality in elderly. This neurological disorder is associated with an abnormal accumulation of beta amyloid plaques in the extracellular environment, and the authors clearly define the contribution of oxidative molecules in promoting this adverse phenomenon. Several studies have been discussed in order to identify and clarify the intracellular signal transduction pathways controlled by ROS in AD.

The review article proposed by V. Fisi and coauthors focuses on the interplay between O-GlcNAc and oxidative stress in influencing membrane transport molecules. O-GlcNAc is a highly dynamic and abundant posttranslational modification that may compete with or influence phosphorylation and plays a fundamental role in controlling several physiological processes, such as insulin signaling, glucose transport, and stress adaptation. In addition, alterations of O-GlcNAc levels have been found in several chronic diseases such as AD, diabetes, and inflammation, suggesting that abnormally elevated or reduced O-GlcNAc levels may be a novel marker to predict the risk of degenerative diseases.

The clinical study performed by C. Torino and coauthors reported the effect of vitamin D receptor (VDR) stimulation on the advanced glycosylation end products receptor (AGE/RAGE) system in patients affected by chronic kidney disease (CKD). VDR responsive genes are involved in several cellular functions, such as cell proliferation, differentiation, membrane transport, and oxidative stress. Increased oxidative stress promotes the generation of advanced glycosylation end products (AGE) and related receptors (RAGE), with circulating RAGE contributing to protective responses against cardiovascular and renal diseases. Interestingly, the authors evaluated several biomarkers of oxidative stress in CKD patients, including myeloperoxidase activity, which is a pivotal enzyme involved in endogenous oxidant production. In contrast to previous studies, the evidence that paricalcitol stimulation does not alter the AGE/RAGE system and myeloperoxidase in CKD patients is here for the first time provided.

A research article from C. Prata and colleagues shows the insulin mimetic effect of sweet glycosides extracted from the leaves of the plant *Stevia rebaudiana*. Using several approaches, the authors demonstrate that steviol glycosides hold antioxidant ability stimulating, similarly to insulin, glucose uptake through Glut4 via activation of PI3K/Akt pathway, thus underscoring the emerging role of phytocompounds in facing several chronic and metabolic disturbs related to oxidative stress.

In conclusion, this special issue provides insights into the crosstalk between oxidative molecules and membrane transport in health and disease. Amongst other, phytocompounds are proposed as novel therapeutic approach to face oxidative stress-related pathological conditions.

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Angela Marino
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Review Article

Aquaporin Membrane Channels in Oxidative Stress, Cell Signaling, and Aging: Recent Advances and Research Trends

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Reactive oxygen species (ROS) are produced as a result of aerobic metabolism and as by-products through numerous physiological and biochemical processes. While ROS-dependent modifications are fundamental in transducing intracellular signals controlling pleiotropic functions, imbalanced ROS can cause oxidative damage, eventually leading to many chronic diseases. Moreover, increased ROS and reduced nitric oxide (NO) bioavailability are main key factors in dysfunctions underlying aging, frailty, hypertension, and atherosclerosis. Extensive investigation aims to elucidate the beneficial effects of ROS and NO, providing novel insights into the current medical treatment of oxidative stress-related diseases of high epidemiological impact. This review focuses on emerging topics encompassing the functional involvement of aquaporin channel proteins (AQPs) and membrane transport systems, also allowing permeation of NO and hydrogen peroxide, a major ROS, in oxidative stress physiology and pathophysiology. The most recent advances regarding the modulation exerted by food phytocompounds with antioxidant action on AQPs are also reviewed.

1. Introduction

Reactive oxygen species (ROS) are unstable reactive molecules, physiologically produced by xanthine oxidase, nicotinamide adenine dinucleotide phosphate oxidase, lipoxygenases, and mitochondria [1, 2]. Though oxygen is peremptory for life, imbalances between antioxidant defense mechanisms, overproduction of ROS, or incorporation of free radicals from the environment to living systems lead to oxidative stress. ROS and other reactive species are implicated in a large spectrum of biological conditions, such as mutation, tumorigenesis, degenerative diseases, inflammation, aging, frailty, and development [3]. ROS exert a dual role as both deleterious and beneficial species, the latter being of pivotal importance as signaling molecules. At physiological levels, ROS can improve cellular activities as they are involved in the control of the chemical balance and synaptic plasticity [4], whereas an excess amount of ROS can damage the endothelium, leading to alteration of the intracellular reduction-oxidation homeostasis [5].

Among various mechanisms, the uncoupling of nitric oxide synthase (NOS) in vascular cells has also widely been reported to be involved in ROS generation. In that event, NOS is turned into a peroxynitrite generator, leading to detrimental effects on vascular function, due to lipidic peroxidation [6]. Furthermore, superoxide anions can modify endothelial function by reducing nitric oxide (NO) biosynthesis and bioavailability [7]. This issue is of particular

relevance since changes in NO release could play an important role in endothelial function maintenance, in addition to regulating proliferation of smooth muscle cells, leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone, and hemodynamics. Hence, endothelial dysfunction, a predictor of several cardiovascular diseases (CVDs), is caused by imbalance between vasodilating and vasoconstricting agents, including NO, endothelium-derived hyperpolarizing factor, prostacyclin, or vasoconstrictive factors such as thromboxane (TXA_2) and endothelin-1 (ET-1) [8].

NO is a gas which plays an important role in blood pressure modulation due to its signaling action on renal, cardiovascular, and central nervous system functions [9]. The role of NO in vascular homeostasis also comes from the negative regulation on coagulation and inflammation operated by this signaling molecule.

Throughout the years, ROS and NO have been widely considered to enter cells by freely diffusing through the cell membrane lipid bilayer and not via specific transporters or channels. This notion has been challenged by the discovery of new membrane transport functions, especially those exerted by aquaporins (AQPs), a family of membrane channel proteins widespread in nature [10, 11]. Transport of NO and ROS by AQPs would be required for cell homeostasis to play a critical role in maintaining endothelial function.

This review focuses on an emerging topic, the functional involvement of AQPs in ROS membrane transport, with specific regard to the movement of hydrogen peroxide and NO into and out of cells, in both health and oxidative stress-induced diseases. The emerging information and research trends regarding the modulation exerted by food phytocompounds with antioxidant action on the expression and function of AQPs are also reviewed.

2. Exogenous and Endogenous Source of Oxidants

Reactive species (RS) derive from either endogenous or exogenous sources. Prolonged exercise, ischemia, inflammation, infection, cancer, and aging correlate with production of free radicals. Production of ROS and reactive nitrogen species (RNS) may occur through enzymatic and nonenzymatic reactions [12, 13]. Among enzymatic processes, NADPH oxidase (NOX), xanthine oxidase, and peroxidases play a pivotal role in free radical generation. For example, NOX catalyzes the production of superoxide [14], which represents a master substrate for generation of other RS, such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl). The latter is synthesized in neutrophils by myeloperoxidase, an enzyme oxidizing chloride ions when H_2O_2 is present [15, 16]. Nitric oxide ($NO^\bullet$) is generated in many tissues and results from the oxidation of L-arginine to citrulline through the action of nitric oxide synthase [17], as reported above.

Nonenzymatic reactions can also occur during oxidative phosphorylation in mitochondria, the main RS production site inside the cell [18]. The leakage of electrons at complex I, complex II, or complex III associates with superoxide production. In the mitochondrial matrix and in the cytosol, superoxide can be converted into H_2O_2 by superoxide dismutase and further detoxified by catalases. In addition, ROS stimulates the generation and the release of other RS, thereby causing a vicious circle due to increased permeability of mitochondrial pores by ROS resulting in mitochondrial defects leading to release of further RS [19].

Alternatively, RS also result from reaction with organic compounds subjected to ionizing radiations. Indeed, high doses of ionizing radiation increase the production and release of inflammatory chemokines and RS that, in concert, promote tissue injury [20].

Exogenous RS can originate from water and air pollution, cigarette smoke, pesticides, dioxin, and several drugs. Once in the body, these different compounds are metabolized, generally in the liver, generating free radicals.

3. Aquaporins, Membrane Channel Proteins of Pleiotropic Relevance

Aquaporins (AQPs) are channel proteins widely present in living organisms where they were initially reported to facilitate the transport of water and certain neutral solutes across biological membranes [21, 22]. Mammals possess thirteen distinct AQPs (AQP0–12) that are roughly subdivided into *orthodox aquaporins* (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8) and *aquaglyceroporins* (AQP3, AQP7, AQP9, and AQP10). Orthodox AQPs were initially described to conduct only water, whereas aquaglyceroporins were shown to transport water and some small neutral solutes, particularly glycerol. These peculiarities did not apply to AQP11 or AQP12, due to their distinct evolutionary pathway and primary sequence distinctions, the reason why they have been indicated as *unorthodox aquaporins* [23]. The transport properties and subcellular localization of AQP11 and AQP12 remain unclear and a matter of debate. The functional subdivision of AQPs has become more articulated in the light of transport properties reported in recent years. Some AQPs are also able to conduct H_2O_2 and/or ammonia [24], and, due to these biophysical properties, they are also denoted as *peroxiporins* [25, 26] and *ammoniaporins* (or *aquaammoniaporins*) [25, 27, 28], respectively. The currently identified mammalian AQP homologues allowing passive diffusion of considerable amounts of H_2O_2 are AQP1, AQP3, AQP5, AQP8, and AQP9 [29]. AQPs also facilitate permeation of gases such as CO_2, NO, or O_2 [11, 30, 31], features that have raised a lot of interest due to the potential physiological relevance they may have in permeating gases of biological relevance. This feature would add more knowledge to the physiological importance of gas channels in nature [32].

Expression and modulation of AQPs in all body districts are the subject of intense investigation around the world. Important roles have already been ascribed to this family of membrane channels, in both health and disease [21, 22, 33] (Table 1).

Table 1: Functional relevance of mammalian aquaporins in health and disease.

Physiological functions involving aquaporins
Generation of fluids
(i) Urine [150]
(ii) Cerebrospinal fluid [151]
(iii) Aqueous humor [152]
(iv) Sweat [21]
(v) Saliva [21]
(vi) Tears [21]
(vii) Bile [153]
(viii) Gastrointestinal juices [33]
(ix) Seminal fluid [154]
Immune response and inflammation
(i) Memory T-cell longevity [155]
(ii) Inflammatory response [156]
(iii) Dendritic cell maturation [157]
Metabolic homeostasis and energy balance
(i) Gluconeogenesis [158]
(ii) Triacylglycerol synthesis [158]
(iii) Ammonia detoxification via ureagenesis [159]
Nervous system physiology
(i) Multiple functions [151]
Other functions
(i) Apoptosis [160]
(ii) Oxidative stress [26]
(iii) Cell migration [161]
(vi) Cell volume homeostasis [162]
(v) Angiogenesis [163]
Pathological states involving aquaporins
(i) Cardiovascular diseases [164]
(ii) Renal concentration disorders [165]
(iii) Inflammatory diseases [156]
(iv) Cholestasis [153]
(v) Brain edema [151]
(vi) Cataract [162]
(vii) Immune system disorders (i.e., neuromyelitis optica) [166]
(viii) Malaria [167]
(ix) Obesity, diabetes, liver steatosis [158]
(x) Cancer [168]
(xi) Infertility [169]

4. Involvement of Aquaporins in the Transport System of Reactive Species

4.1. Aquaporin-8 as Peroxiporin Mediating Mitochondrial H_2O_2 Release in Hepatocytes. H_2O_2 is a major ROS constantly generated in mitochondria by the aerobic metabolism. Respiratory chain-linked H_2O_2 is produced by enzymatic dismutation of superoxide radicals [34]. Complex I generates superoxide within the mitochondrial matrix, whereas complex III generates superoxide in the intermembrane space [34, 35]. Hepatic mitochondria are not only important sources for ROS but also important key targets for their potential damage. Under physiological conditions, H_2O_2 is the only ROS that can move out of the mitochondria into the cytoplasm and function as a second messenger in signal transduction pathways [35, 36]. Under oxidative stress, high ROS (H_2O_2) levels can induce loss of mitochondrial membrane potential and mitochondrial dysfunction with the resulting triggering of cell death mechanisms [37, 38].

H_2O_2 had been long thought to be freely diffusible across cellular membranes, a notion that has been challenged by both the existence of H_2O_2 gradients across biological membranes [39, 40] and the finding that membrane permeability is a rate-limiting factor in H_2O_2 elimination by mammalian cells [41]. Limited diffusion of H_2O_2 across mitochondrial membranes has also been suggested [42]. Hence, a protein-facilitated diffusional pathway for H_2O_2 across membranes was proposed [40, 42]. H_2O_2 size and chemical and physicochemical properties are similar to those of water [40], which may explain H_2O_2 passage through channel membrane proteins such as AQPs. Accordingly, initial studies in reconstituted yeast [10] and transfected mammalian cells [43] indicate that AQP8 and some other members of the mammalian AQP family facilitate H_2O_2 passage across plasma membranes. Thus, AQP8 is able to function as peroxiporin.

An initial study demonstrated that AQP3 is required for (NOX)-derived H_2O_2 signaling [43]. More recent studies in diverse nonhepatic cells have reported that plasma membrane AQP8 transports NOX-generated H_2O_2 that participates in intracellular signal transduction pathways [44–47]. In HeLa cells, AQP8 plays a key role in the epidermal growth factor- (EGF-) induced entry of H_2O_2, which in turn initiates intracellular signaling by tyrosine phosphorylation of target proteins [44]. In B lymphocytes, AQP8-mediated H_2O_2 transport has been reported to induce cell activation and differentiation [45], whereas in leukemia cells, it has been found to induce proliferation pathways [46, 47].

In hepatocytes, plasma membrane AQP8 is exclusively expressed on the bile canalicular domain [48]. Therefore, AQP8 cannot be involved in the intracellular transport of H_2O_2 generated by NADPH oxidases at sinusoidal plasma membranes. AQP8 is also expressed in the inner mitochondrial membranes of some cells, including hepatocytes [49, 50]. Experimental evidence in human hepatocyte carcinoma HepG2 cells suggests that mitochondrial AQP8 (mtAQP8) facilitates the diffusional efflux of H_2O_2 [51]. A similar observation was made studying mitochondrial AQP8b, the marine teleost orthologue of human AQP8 [52]. As reviewed below, the involvement of an mtAQP8-mediated H_2O_2 transport in normal human spermatozoa functioning has also been suggested [53].

The knockdown of mtAQP8 expression in HepG2 cells markedly reduces the release of mitochondrially generated H_2O_2, and the resulting mitochondrial ROS accumulation induces mitochondrial depolarization via the mitochondrial permeability transition mechanism and reduced ATP levels [51]. Interestingly, the immunological blockage of AQP8b-mediated mitochondrial H_2O_2 efflux in marine spermatozoa

also causes ROS accumulation, mitochondrial depolarization, and decreased ATP production [52].

The oxidant-induced mitochondrial dysfunction in HepG2 cells causes loss of viability by activating a necrotic death pathway [51, 54]. Interestingly, mtAQP8 silencing causes a minor loss of viability in human hepatoma HuH-7 cells but does not affect viability in neither in normal rat hepatocytes nor in the nonneoplastic human cell lines, renal HK-2, and Chang liver cells [54]. Therefore, carcinoma cells might be particularly susceptible to defective mtAQP8 expression. As the loss of viability in mtAQP8-knockdown HepG2 cells is prevented by the mitochondria-targeted antioxidant MitoTempol [51], a disparity in mitochondrial antioxidant defenses is likely to explain the observed differential susceptibility among mtAQP8-knockdown cells. Nevertheless, it is worth mentioning that, at least for total and reduced mitochondrial glutathione levels, there were no significant differences between HepG2, HuH-7, Chang liver cells, and rat hepatocytes (unpublished data from Raul A. Marinelli's laboratory). Further studies are needed to understand the mechanisms that actually cause differential death in mtAQP8-knockdown cells.

With the use of HeLa cells, the AQP8-mediated plasma membrane H_2O_2 transport has recently been reported to be functionally modulated under stress [55]. AQP8 permeability to H_2O_2 was reversibly inhibited, thus preventing intracellular ROS accumulation during oxidative stress [55]. To the best of our knowledge, as AQP8 expression has not been demonstrated in HeLa cell mitochondria [44, 56], it would be interesting to explore whether hepatocyte mtAQP8 is under this novel regulatory mechanism of cell survival during stress.

Another as-yet-unexplored area of research is the role that mtAQP8-mediated H_2O_2 may play in hepatocyte physiology. We have recently provided evidence suggesting that hepatocyte mtAQP8 expression can be modulated by cholesterol via sterol regulatory element-binding protein (SREBP) transcription factors; that is, mtAQP8 is upregulated in cholesterol-depleted cells and downregulated in cholesterol-loaded cells [57]. As H_2O_2 has been described to stimulate hepatocyte cholesterogenesis via SREBPs [58], our finding might suggest that mtAQP8 plays a role in SREBP-controlled cholesterol biosynthesis. For example, at low cellular cholesterol levels, SREBP-dependent mtAQP8 upregulation could facilitate the mitochondrial H_2O_2 release that would contribute to stimulating cholesterogenesis. Further studies are required to elucidate this issue.

4.2. AQP-Mediated H_2O_2 Transport Is Critical in Sperm Cell Motility and ROS Scavenging. The relevance of AQP-mediated water and H_2O_2 transport in human sperm cells activity has been reported in a recent study investigating the expression, distribution, and role of AQP3, 7, 8, and 11 in subfertile compared with normospermic subjects [53]. The investigated AQPs were found to be implicated in sperm cell volume regulation and ROS scavenging, two functions of critical importance in sperm counts and motility. With the use of AQP blockers, it was suggested that chronic deficiency in AQP-mediated H_2O_2 permeability impairs ROS efflux out of sperm cells and reduces the detoxification efficiency, with consequent loss of sperm functionality. However, although coordinated action of AQPs has been reported to regulate sperm motility in the marine teleost seabream [59], further studies are needed to confirm the suggested pathophysiological relevance of AQPs in human male fertility. The specific AQP homologue that, among AQP3, 7, 8, and 11, may account for sperm cell permeability to H_2O_2 remains elusive. AQP8 features one of the highest conductances to H_2O_2 among peroxiporins. However, the relevance of AQP8 as the major H_2O_2 membrane transport system in human sperm cells remains to be proved. A recent study using HeLa cells showed reduction of AQP-mediated water and H_2O_2 cell permeability following oxidative stress [60]. Interestingly, the diminution was prevented or reversed when the cells were treated with antioxidant phytochemical compounds.

4.3. AQP3 Mediates Hydrogen Peroxide-Dependent Intracellular Signaling, Responses to Environmental Stress, and Cell Migration. AQP3 is also reported to facilitate the uptake of H_2O_2 into mammalian cells [43]. Microimaging studies using peroxy yellow 1 methyl-ester (PY1-ME), a specific fluorescent probe for H_2O_2, showed AQP3-mediated uptake of H_2O_2 in HEK cells [43]. Moreover, it has been demonstrated that T-cell migration towards chemokines is regulated by AQP3-facilitated transport of H_2O_2 that, in turn, stimulates Rho signaling [61]. In primary keratinocytes, H_2O_2 is required to stimulate NF-κB signaling in response to TNF-alpha [62].

Conversely, oxidative signals seem to be important in controlling AQP3 expression. Chrysin and resveratrol, two antioxidant phytocompounds, have been reported to modulate the expression of AQP3 [63, 64]. Accordingly, severe ultraviolet A (UVA) irradiation causes a significant reduction in AQP3 expression secondary to increased oxidative stress [65]. In this regard, a negative correlation between AQP3 expression and age in sun exposed skin has been described, suggesting AQP3 as a biomarker of age-related skin alteration [66].

In the colon, AQP3 is expressed in the epithelial cells where changes in expression were found in response to inflammation, and AQP3-depleted mice experienced impaired recovery after chemical-induced colitis [67]. Interestingly, mice lacking AQP3 showed impaired healing of superficial wounds in the colon. This finding elucidates the signaling mechanism of extracellular H_2O_2 in colonic epithelium and suggests the implication of AQP3-mediated H_2O_2 transport in innate immune responses at mucosal surfaces [68]. AQP3-mediated H_2O_2 transport has also been described to control EGF signaling in epithelial cells [69], playing an important role in T-cell and breast cancer cell migration [70, 71]. However, the exact contribution of the AQP3-mediated H_2O_2 transport to these changes in cellular function remains to be fully elucidated. Involvement of AQP3 in trefoil peptide and EGF-mediated migration, a vital process in inflammatory bowel disease repair in case of excess free radical production, has also been recently shown [72].

4.4. AQP1-Mediated Diffusion of NO in Vasorelaxation

4.4.1. Endothelial NO Release and Oxidative Stress during Aging. With aging, endothelial cells (ECs) undergo considerable remodeling processes [73, 74]. Increased endothelial permeability, alterations in the cytoskeleton, the appearance of β-galactosidase staining, and the expression of several cell cycle inhibitors [75] are also observed. Aging of ECs is associated with an increased release of vasoconstrictors, such as angiotensin II and endothelin, and a reduced release of vasodilators, such as NO and prostacyclin [76].

Among the above factors, NO bioavailability has been suggested to play a central role in maintaining endothelial function [77–79]. NO is the subject of extensive studies as one of the most relevant factors released by the endothelium, playing an outstanding role in maintaining vascular system function [77, 79–81]. NO is produced by endothelial NO synthase (eNOS), which transfers electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to the heme in the amino-terminal oxygenase domain. In this way, the substrate L-arginine is oxidized to L-citrulline and NO. Tetrahydrobiopterin (BH4) is an essential cofactor of eNOS exerting a key role in the progression of NO synthesis (Figure 1). NO formed by the vascular ECs diffuses to the adjacent cells, such as vascular smooth muscle cells (VSMCs), platelets, and leucocytes, where it exerts many of its beneficial actions, such as vasodilation, antithrombotic, anti-inflammatory, and antiproliferative effects [82]. Endothelium-derived NO is known to be particularly important to maintain normal vascular tone, endothelial function, and homeostasis [83], preventing the progression of age-related vascular disorders [80]. Decreased production of endothelium-derived NO during aging is commonly believed to be due to decreased eNOS activity characterizing senescent ECs [78]. In the peroxidative conditions associated with aging, superoxide anion (O_2^-) can also react with NO leading to the formation of peroxynitrites, which, in turn, can promote protein nitration and contribute to EC dysfunction and death [84, 85]. Furthermore, enhanced oxidative stress can lead to eNOS "uncoupling" and cause endothelial dysfunction [86].

BH4 oxidation is one of the possible mechanisms of eNOS "uncoupling." Intracellular BH4 levels depend on the balance between its synthesis and degradation. In particular, oxidative stress may lead to excessive oxidation and depletion of BH4. As a consequence, the flow of electrons within NOS could be "uncoupled" from L-arginine oxidation and O_2^- produced from the oxygenase domain [87]. Hence, eNOS would be converted to a superoxide-producing enzyme with reduced NO production and enhanced preexisting oxidative stress [88, 89].

4.4.2. AQP1 and NO Flow in Vascular Senescence and Atherosclerosis. Free diffusion (simple diffusion) through the phospholipid bilayer composing the plasma membrane had historically been assumed to be the only pathway whereby NO moves into or out of cells. Thus, based on the partition coefficient of NO between lipids and water [90, 91] rather than direct experimental assessment of NO diffusion across the cell membrane, NO was believed to cause vasodilation, antithrombotic, anti-inflammatory, and antiproliferative effects without need of facilitation by channels or transporters. This assumption was not confirmed after measurements of NO fluxes across reconstituted proteoliposomes and transfected cultured cells showing that, in addition to water, the AQP1 channel could conduct NO across plasma membranes and that the plasma membrane represents a significant barrier to NO diffusion [11]. Successively, with the use of thoracic aortas isolated from wild-type ($Aqp^{+/+}$) and $Aqp^{-/-}$ knockout mice, it was shown that AQP1 facilitates NO diffusion out of endothelial cells and NO influx into vascular smooth muscle cells, and that AQP1 conduction of NO is required for full expression of endothelium-dependent vasorelaxation [92]. Regarding vascular aging, changes in AQPs expression have been found in animal models of kidney-clip hypertension [11]. The trafficking of AQPs within cells has also been shown to change during aging, as observed in the parotid gland [93]. The suggested role of AQPs in vascular function regulation and senescence through modulation of NO diffusion across cell membranes opens a new avenue in understanding vascular senescence physiology and pathophysiology. Additional work is, however, needed since a discrepancy has been raised by a study reporting intact NO-dependent vasorelaxation in AQP1-depleted mice [94]. Vascular AQP1 expression was found to undergo positive regulation with the mediation of KLF2, the flow-responsive transcription factor Krüppel-like factor 2 that maintains an anticoagulant, anti-inflammatory endothelium with sufficient NO bioavailability [95]. Both *in vitro* and *in vivo* AQP1 expression was subjected to KLF2-mediated positive regulation by atheroprotective shear stress whereas it proved to be downregulated under inflammatory conditions. While suggesting that endothelial expression of AQP1 characterizes the atheroprotected, noninflamed vessel wall, this finding supports the putative continuous role of KLF2 in stabilizing the vessel wall via cotemporal expression of eNOS and AQP1, helping to prevent or counteract the pathogenesis of atherosclerosis.

5. Role of Vasopressin/AQP2 Axis and Oxidative Stress in Aging

Alterations in plasma osmolality and fluid body volume are observed in the elderly, making old people at high risk of developing disturbances of the water metabolism, which can give rise to several adverse effects. Aging blunts thirst and drinking responses, making older people more vulnerable to body fluid imbalance and dehydration [96], which can compromise cognitive function [97, 98]. Indeed, dehydration is a predisposing factor for confusion in long-term care residents [99]. Furthermore, plasma hypertonicity, a marker of dehydration, increases the risk of ischemic stroke in hospitalized patients [100] and may precipitate cerebral ischemic events in susceptible elderly individuals [101].

The major hormone regulating water metabolism in the body is vasopressin. Vasopressin is a 9-amino acid peptide that is secreted from the posterior pituitary in response to high plasma osmolality and hypovolemia. Vasopressin has important roles in circulatory and water homeostasis

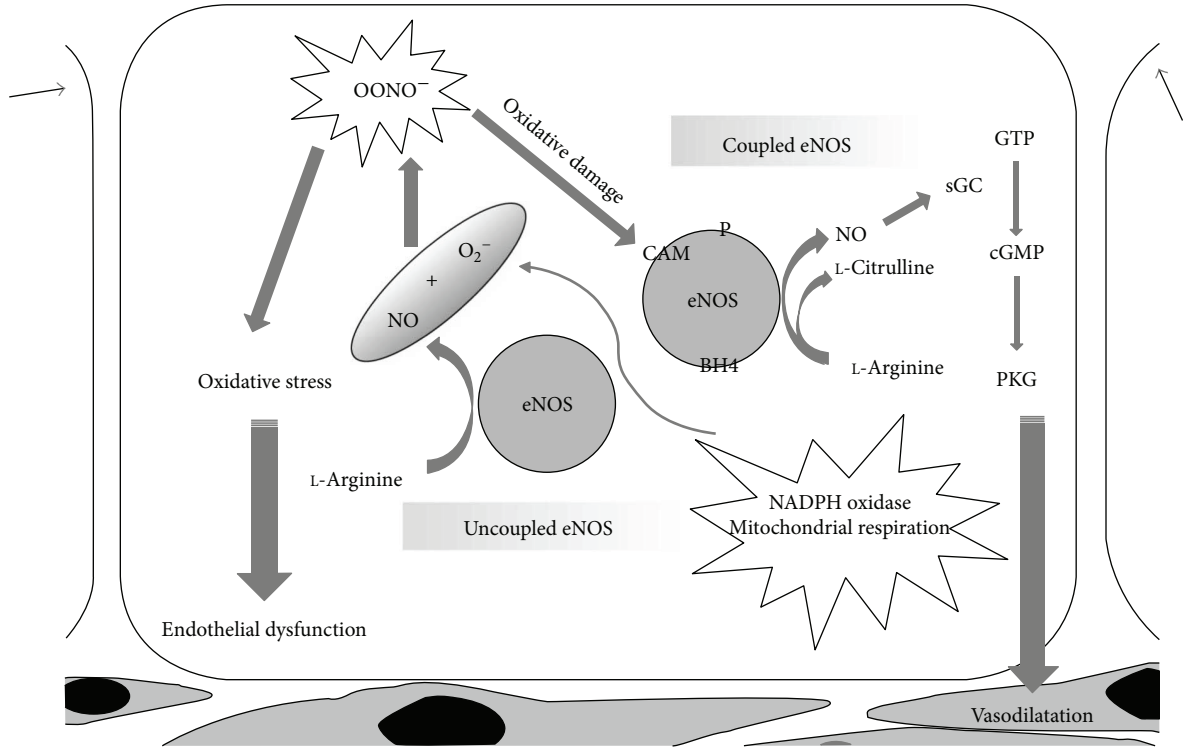

Figure 1: NO release by eNOS in physiological and peroxidative conditions. While "coupled" eNOS is involved in the physiological NO release underlying vasorelaxation, NO release by "uncoupled" eNOS is turned into $OONO^-$ (peroxynitrites) leading to an increase in oxidative stress with consequent endothelial dysfunction. ADMA: asymmetric dimethylarginine; Akt: protein kinase B; BH4: tetrahydrobiopterin; CAM: calmodulin; Cav-1: caveolin 1; eNOS: endothelial NO synthase; cGMP: cyclic guanosine monophosphate; GTP: guanosine triphosphate; GTPCH: guanosine triphosphate cyclohydrolase I; Hsp 90: heat shock protein 90; NADPH: nicotinamide adenine dinucleotide phosphate; NO: nitric oxide; sGC: soluble guanylate cyclase; PKG: protein kinase G.

mediated by vasopressin receptor subtypes V1a (vascular), V1b (pituitary), and V2 (vascular, renal). Therefore, age-related dysfunction of the hypothalamic-neurohypophyseal-vasopressin axis can result in multiple abnormalities in several physiological systems that might promote a variety of morbidity such as cardiovascular and renal diseases [102, 103].

At the renal level, it has been observed that aging is accompanied by a parallel decrease in maximal urine concentrating ability [104]. Individuals aged 60–79 years show an approximately 20% reduction in maximum urine osmolality, a 50% decrease in the ability to conserve solute, and a 100% increase in minimal urine flow rate, when compared to younger age groups. Abnormalities in vasopressin secretion appear to be associated with the decrease in urine concentrating ability with aging: the abundance of many of the key transport proteins responsible for urine concentrating ability is reduced in the kidney medulla of aged rats [105]. The reductions in water, sodium, and urea transport protein abundances, along with their reduced response to water restriction, contribute to the reduced ability of aged rats to concentrate urine and conserve water [104].

The major mechanism by which vasopressin modulates water reabsorption is by regulating the trafficking of the vasopressin-sensitive water channel aquaporin-2 (AQP2) in collecting duct principal cells. Specifically, binding of vasopressin to the V2R increases cAMP levels, resulting in the activation of protein kinase A (PKA). PKA-dependent phosphorylation of the water channel AQP2, at S256, is essential to promote the translocation of AQP2-bearing vesicles from an intracellular pool to the apical plasma membrane [106]. Phosphoproteomic studies have demonstrated that, besides S256, vasopressin stimulation increases S264 and T269 but decreases the phosphorylation of S261 [107].

Several studies performed in animal models have shown that in aged rats, there is a large decrease in the level of AQP2 as well as of its phosphorylated form at S256, which can contribute to the reduced renal concentrating abilities [105, 108]. Furthermore, AQP3 is reduced in aged rats, but no change in the expression of AQP1 and AQP4 has been detected in aged rats. This distinction in the regulation of AQPs abundance may be related to the fact that only AQP2 and AQP3 expressions are under control of vasopressin.

One possible therapy to overcome the decrease in AQP2 abundance in aging might be the administration of vasopressin. A recent study showed that desmopressin (dDAVP), a selective V2R agonist, administered to 10- and 30-month-old Wag/Rij rats, decreases urine output in both rat groups and leads to an increase in AQP2 and AQP3 abundance. These results suggest that a decrease in AQP2 and AQP3 expression levels partially accounts for the diminution in urinary concentrating ability in aging.

In general, due to an impaired ability to conserve water, in the elderly, there is a decrease in total body water content

associated with a reduction of plasma volume. These changes make the elderly much more sensitive to water overload or dehydration resulting in abnormal movement of solutes and, thereby, increasing the possibility of developing hypo- or hypernatremia.

However, an increase in vasopressin levels can also be found in aged people, where it induces water retention and hyponatremia and stimulates calcium release from bone, thus contributing to osteoporosis, as well as affecting the cardiovascular system and blood pressure, thus contributing to the development of hypertension.

For these diseases often associated with the elderly, vasopressin receptor antagonists represent a promising therapeutic tool. Interest in vasopressin has been renewed with the availability of vaptans, new, potent, orally active vasopressin receptor antagonists, initially developed for the treatment of various forms of hyponatremia (often related to vasopressin dysfunction) and proven to be safe in humans [109–111]. Evaluation of the specific aquaretic effect of vaptans in aged patients treated with this antagonist might have a profound impact in understanding the therapeutic effect of vaptan compounds.

Interestingly, vasopressin, the levels of which are increased after water deprivation, stimulates vascular superoxide production through activation of V1aR [112]. Accordingly, it has been shown that water deprivation increases ROS production in the somatosensory cortex, indicating that cerebrovascular dysfunction is related to oxidative stress [113].

5.1. Oxidative Signals and AQP2. Oxidative stress plays a key role in modulating renal functionality, including its diluting and concentrating ability during aging [114]. Oxidative stress increases the risk of developing several age-related diseases because ROS may alter cell signaling, leading to inflammation, apoptosis, and cellular senescence. During aging, significant increases in advanced glycosylation end products (AGE) and other oxidants have been reported in kidneys [115, 116]. Chronic inhibition of nitric oxidase synthase regulates renal water balance by reducing the expression of AQP2 [117, 118]. Importantly, oxidative stress is often associated with disorders linked to redox unbalance.

At a molecular level, ROS can oxidize selective amino acids on target proteins. Oxidative dependent modifications are being shown to be fundamental in transducing several intracellular signals controlling pleiotropic functions such as cell proliferation, apoptosis, autophagy, and membrane transport. These modifications result from reactions between ROS or reactive nitrogen species (RNS) and amino acid residues [26]. Oxidative modifications mainly occur through the switching of the sulfur in target cysteines. However, cysteines are not the only residues involved in oxidative modifications as methionine, lysine, arginine, threonine, and proline residues can also be oxidized to reactive carbonyls [119]. Oxidative sensitive modifications include carbonylation, nitrotyrosinylation, succinylation, S-sulfenation, S-nitrosylation, S-glutathionylation, and disulfide formation.

Reversible glutathionylation results from the reaction between glutathione and cysteine residues (PSSG) upon exposure to RS. S-Glutathionylation is recognized as a crucial modification by which cells translate local changes of reactive species [120].

Using a proteomic approach, Sandoval and coworkers revealed that vasopressin stimulation is associated with increased expression of different oxidative related proteins such as glutathione S-transferase [121]. This observation is likely to indicate that oxidative signaling may somehow play a role in controlling the physiological signal transduction cascade initiated by vasopressin. Studies from several groups have revealed, indeed, that antioxidant compounds such as *N*-acetylcysteine (NAC) rescued the reduction of AQP2 abundance observed in rats subjected to the bilateral ureteral obstruction (BUO) [122]. Conversely, treatment with the oxidant 4-hydroxy-2-hexenal (HHE) decreases the abundance of AQP2 and activates several kinases such as p38-MAPK and ERK [123], which have been proposed to phosphorylate AQP2 at S261 [124–126]. Phosphorylation at S261 is involved in AQP2 ubiquitylation and degradation [125, 127].

However, vasopressin is not the only factor regulating AQP2 expression. In this respect, it has been demonstrated that the oxidant HHE increases the expression of the transcription factors NF-κB and the enzyme NOX4 [123], both involved in modulating the expression level of AQP2. Specifically, NF-κB decreased *AQP2* mRNA and protein abundance [128]. Conversely, NOX4 promotes *AQP2* expression [129]. These findings strongly suggest that AQP2 abundance is the result of a balanced activity between NF-κB and NOX4. However, how oxidative signals modulate the stability of the target proteins remains to be clarified. It appears conceivable that transient oxidative posttranslational modifications may mean there is a molecular signature translating oxidative information signaling and thus controlling the fate of target proteins. In this respect, some of the authors of this review have recently shown that AQP2 undergoes S-glutathionylation. It was also found that the increase in AQP2 glutathionylation is paralleled by higher ROS production. Conversely, low levels of ROS, measured in cells displaying low intracellular calcium concentration, secondary to the expression of the calcium-sensing receptor (CaSR), associates with reduced S-glutathionylation of AQP2 [130]. Whether or not S-glutathionylation of AQP2 is involved in the water imbalance observed during aging remains to be investigated.

6. Modulatory Actions of Food Antioxidant Phytocompounds on Aquaporins

A growing number of food phytochemicals are being found to exert antioxidant and anti-inflammatory actions. Thanks to their ability to interact with pivotal signaling pathways, a number of food and herbal phytochemicals have been found to impart health benefits modulating important cellular functions such as growth, differentiation, death, and volume homeostasis as well as redox, metabolic, and energy balance.

To date, a large number of biologically active phytochemicals have been identified, characterized, and eventually modified as natural sources of novel compounds to prevent, delay, or cure many human diseases. This is an important

achievement since secondary prevention or adjunct therapy through dietary intervention is a cost-effective alternative for avoiding the large burden of health care, especially that associated with chronic illnesses.

Similarly to several other transport systems, AQPs are also modulated by a number of food bioactive phytocompounds [131, 132]. The modulatory effects exerted by beneficial dietary patterns, food phytochemicals, and herbal compounds on AQPs, in both health and disease, is a fast growing topic as their exploitation may help support current medical treatment options to improve the prognosis of several diseases. Flavonoid modulation of AQPs has been reported to ameliorate forms of cerebral and retinal edemas of different origins (AQP4) [132–135], lung injuries (AQP1) [136], and Sjögren syndrome-associated xerostomia (AQP5) [137], to inhibit ovarian tumor growth (AQP5) [89] and protect against UV-induced skin damage (AQP3) [63]. Curcumin influences choroid plexus AQP1 [138], ovarian AQP3 [139], and brain AQP4 and AQP9, reducing intracranial pressure in brain injury, inhibiting ovarian cancer cell migration, and reducing brain edema, respectively [140–142]. Modulation of AQP channel gating by curcumin has been recently reported in a paper describing the use of HeLa cells to investigate the effects of some antioxidant phytocompounds on AQP1, AQP3, AQP8, and AQP11 [60]. Resveratrol, a stilbene compound, was found to inhibit human keratinocytes and ameliorate the ischemia/reperfusion injury acting on AQP3 [64] and AQP4 [143], respectively. The chalcone compound phloretin also acts on the expression of AQP9 to exert its antioxidant and anti-inflammatory actions [144]. Genistein and daidzein, two isoflavonoids, were found to upregulate the expression of uterine AQP1 by increasing the responsiveness to estrogens [145]. The monoterpenoid carvacrol has been reported to reduce the intracerebral hemorrhage-induced brain edema by downregulating brain AQP4 [146]. Triterpenoids have been shown to act on the expression of AQP1 to reduce cancer cell migration, counteracting metastasis, as well as to ameliorate forms of allergic rhinitis and to downregulate kidney AQP2 to protect against renal failures [147]. Capsaicin was found to increase the expression level of submandibular salivary gland AQP5 to ameliorate salivary gland hypofunction [148, 149]. While useful information is already available, further important achievements are expected from the ongoing studies on the modulatory effects exerted by biologically active phytocompounds on the expression and function of AQPs.

7. Conclusions and Future Perspectives

Excess of ROS within the cells and reduction of NO bioavailability can largely promote cellular dysfunction, which is linked to the development of metabolic disorders, cardiovascular and renal diseases, frailty, and aging. Key roles for AQPs as peroxiporins in the signal transduction pathways underlying diverse cellular functions, such as differentiation, proliferation, or mobility, are suggested by the recent evidence of AQP-mediated H_2O_2 transport at the plasma or mitochondrial membrane level. Dysregulation of peroxiporin function can lead to oxidative stress and eventually cell death. Alterations in AQP-mediated ROS and/or NO transport are therefore assuming an increasing translational value in physiology and pathophysiology with promising nutraceutical and pharmacological implications. Indeed, modulation of the peroxiporin and/or NO channel function of AQPs at the vascular, hepatic, testicular, or renal level may prove to be valuable in preventing or treating cardiovascular (vascular stiffness/hypertension, atherosclerosis), metabolic, and reproductive (impaired sperm cell motility) diseases. Last but not least, further work is warranted to investigate the involvement of AQPs in the antioxidant and anti-inflammatory actions exerted by food phytochemical compounds in order to devise new strategies to promote health and improve aging.

Conflicts of Interest

The authors declare they do not have any conflicts of interest.

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References

[1] M. P. Murphy, "How mitochondria produce reactive oxygen species," *The Biochemical Journal*, vol. 417, no. 1, pp. 1–13, 2009.

[2] D. B. Zorov, M. Juhaszova, and S. J. Sollott, "Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release," *Physiological Reviews*, vol. 94, no. 3, pp. 909–950, 2014.

[3] B. Uttara, A. Singh, P. Zamboni, and R. Mahajan, "Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options," *Current Neuropharmacology*, vol. 7, no. 1, pp. 65–74, 2009.

[4] L. Zuo, T. Zhou, B. K. Pannell, A. C. Ziegler, and T. M. Best, "Biological and physiological role of reactive oxygen species—the good, the bad and the ugly," *Acta Physiologica*, vol. 214, no. 3, pp. 329–348, 2015.

[5] M. de la Paz Scribano, M. del Carmen Baez, B. Florencia et al., "Effects of atorvastatin on oxidative stress biomarkers and mitochondrial morphofunctionality in hyperfibrinogenemia-induced atherogenesis," *Advances in Medicine*, vol. 2014, Article ID 947258, 6 pages, 2014.

[6] P. Ferroni, S. Basili, V. Paoletti, and G. Davi, "Endothelial dysfunction and oxidative stress in arterial hypertension," *Nutrition, Metabolism, and Cardiovascular Diseases*, vol. 16, no. 3, pp. 222–233, 2006.

[7] J. Vasquez-Vivar, B. Kalyanaraman, P. Martasek et al., "Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 16, pp. 9220–9225, 1998.

[8] N. Panth, K. R. Paudel, and K. Parajuli, "Reactive oxygen species: a key hallmark of cardiovascular disease," *Advances in Medicine*, vol. 2016, Article ID 9152732, 12 pages, 2016.

[9] V. Bauer and R. Sotnikova, "Nitric oxide—the endothelium-derived relaxing factor and its role in endothelial functions," *General Physiology and Biophysics*, vol. 29, no. 4, pp. 319–340, 2010.

[10] G. P. Bienert, A. L. B. Møller, K. A. Kristiansen et al., "Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes," *The Journal of Biological Chemistry*, vol. 282, no. 2, pp. 1183–1192, 2007.

[11] M. Herrera, N. J. Hong, and J. L. Garvin, "Aquaporin-1 transports NO across cell membranes," *Hypertension*, vol. 48, no. 1, pp. 157–164, 2006.

[12] L. A. Pham-Huy, H. He, and C. Pham-Huy, "Free radicals, antioxidants in disease and health," *International Journal of Biomedical Sciences*, vol. 4, no. 2, pp. 89–96, 2008.

[13] I. Grattagliano, G. Calamita, T. Cocco, D. Q. Wang, and P. Portincasa, "Pathogenic role of oxidative and nitrosative stress in primary biliary cirrhosis," *World Journal of Gastroenterology*, vol. 20, no. 19, pp. 5746–5759, 2014.

[14] K. Bedard, B. Lardy, and K. Krause, "NOX family NADPH oxidases: not just in mammals," *Biochimie*, vol. 89, no. 9, pp. 1107–1112, 2007.

[15] W. Droge, "Free radicals in the physiological control of cell function," *Physiological Reviews*, vol. 82, no. 1, pp. 47–95, 2002.

[16] M. Genestra, "Oxyl radicals, redox-sensitive signalling cascades and antioxidants," *Cellular Signalling*, vol. 19, no. 9, pp. 1807–1819, 2007.

[17] P. Pacher, J. S. Beckman, and L. Liaudet, "Nitric oxide and peroxynitrite in health and disease," *Physiological Reviews*, vol. 87, no. 1, pp. 315–424, 2007.

[18] J. F. Turrens, "Mitochondrial formation of reactive oxygen species," *The Journal of Physiology*, vol. 552, no. 2, pp. 335–344, 2003.

[19] C. Maack and M. Bohm, "Targeting mitochondrial oxidative stress in heart failure throttling the afterburner," *Journal of the American College of Cardiology*, vol. 58, no. 1, pp. 83–86, 2011.

[20] C. Betlazar, R. J. Middleton, R. B. Banati, and G. J. Liu, "The impact of high and low dose ionising radiation on the central nervous system," *Redox Biology*, vol. 9, pp. 144–156, 2016.

[21] P. Agre, "Aquaporin water channels," *Bioscience Reports*, vol. 24, no. 3, pp. 127–163, 2004.

[22] P. Gena, M. Pellegrini-Calace, A. Biasco, M. Svelto, and G. Calamita, "Aquaporin membrane channels: biophysics, classification, functions, and possible biotechnological applications," *Food biophysics*, vol. 6, no. 2, pp. 241–249, 2011.

[23] K. Ishibashi, "New members of mammalian aquaporins: AQP10–AQP12," *Handbook of Experimental Pharmacology*, vol. 190, pp. 251–262, 2009.

[24] T. P. Jahn, A. L. B. Møller, T. Zeuthen et al., "Aquaporin homologues in plants and mammals transport ammonia," *FEBS Letters*, vol. 574, no. 1–3, pp. 31–36, 2004.

[25] A. Almasalmeh, D. Krenc, B. Wu, and E. Beitz, "Structural determinants of the hydrogen peroxide permeability of aquaporins," *The FEBS Journal*, vol. 281, no. 3, pp. 647–656, 2014.

[26] G. Tamma and G. Valenti, "Evaluating the oxidative stress in renal diseases: what is the role for S-glutathionylation?," *Antioxidants & Redox Signaling*, vol. 25, no. 3, pp. 147–164, 2016.

[27] L. M. Holm, T. P. Jahn, A. L. B. Møller et al., "NH_3 and NH_4^+ permeability in aquaporin-expressing *Xenopus* oocytes," *Pflugers Archiv: European Journal of Physiology*, vol. 450, no. 6, pp. 415–428, 2005.

[28] S. M. Saparov, K. Liu, P. Agre, and P. Pohl, "Fast and selective ammonia transport by aquaporin-8," *The Journal of Biological Chemistry*, vol. 282, no. 8, pp. 5296–5301, 2007.

[29] B. Wu and E. Beitz, "Aquaporins with selectivity for unconventional permeants," *Cellular and Molecular Life Sciences*, vol. 64, no. 18, pp. 2413–2421, 2007.

[30] N. L. Nakhoul, B. A. Davis, M. F. Romero, and W. F. Boron, "Effect of expressing the water channel aquaporin-1 on the CO_2 permeability of *Xenopus* oocytes," *American Journal of Physiology-Cell Physiology*, vol. 274, no. 2, pp. C543–C548, 1998.

[31] Y. Wang, J. Cohen, W. F. Boron, K. Schulten, and E. Tajkhorshid, "Exploring gas permeability of cellular membranes and membrane channels with molecular dynamics," *Journal of Structural Biology*, vol. 157, no. 3, pp. 534–544, 2007.

[32] W. F. Boron, "Sharpey-Schafer lecture: gas channels," *Experimental Physiology*, vol. 95, no. 12, pp. 1107–1130, 2010.

[33] G. Calamita, C. Delporte, and R. A. Marinelli, *Hepatobiliary, Salivary Glands and Pancreatic Aquaporins in Health and Disease*, 2015, Place Published.

[34] A. Boveris and E. Cadenas, "Mitochondrial production of hydrogen peroxide regulation by nitric oxide and the role of ubisemiquinone," *IUBMB Life*, vol. 50, no. 4, pp. 245–250, 2000.

[35] H. Sies, "Role of metabolic H_2O_2 generation: redox signaling and oxidative stress," *The Journal of Biological Chemistry*, vol. 289, no. 13, pp. 8735–8741, 2014.

[36] T. Finkel, "Signal transduction by mitochondrial oxidants," *The Journal of Biological Chemistry*, vol. 287, no. 7, pp. 4434–4440, 2012.

[37] R. Solito, F. Corti, C. H. Chen et al., "Mitochondrial aldehyde dehydrogenase-2 activation prevents β-amyloid-induced endothelial cell dysfunction and restores angiogenesis," *Journal of Cell Science*, vol. 126, no. 9, pp. 1952–1961, 2013.

[38] F. Yin, H. Sancheti, and E. Cadenas, "Mitochondrial thiols in the regulation of cell death pathways," *Antioxidants & Redox Signaling*, vol. 17, no. 12, pp. 1714–1727, 2012.

[39] F. Antunes and E. Cadenas, "Estimation of H_2O_2 gradients across biomembranes," *FEBS Letters*, vol. 475, no. 2, pp. 121–126, 2000.

[40] G. P. Bienert and F. Chaumont, "Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1840, no. 5, pp. 1596–1604, 2014.

[41] N. Makino, K. Sasaki, K. Hashida, and Y. Sakakura, "A metabolic model describing the H_2O_2 elimination by mammalian cells including H_2O_2 permeation through cytoplasmic and peroxisomal membranes: comparison with experimental data," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1673, no. 3, pp. 149–159, 2004.

[42] V. G. Grivennikova, A. V. Kareyeva, and A. D. Vinogradov, "What are the sources of hydrogen peroxide production by heart mitochondria?," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 1797, no. 6-7, pp. 939–944, 2010.

[43] E. W. Miller, B. C. Dickinson, and C. J. Chang, "Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream

intracellular signaling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 36, pp. 15681–15686, 2010.

[44] M. Bertolotti, S. Bestetti, J. M. Garcia-Manteiga et al., "Tyrosine kinase signal modulation: a matter of H_2O_2 membrane permeability?," *Antioxidants & Redox Signaling*, vol. 19, no. 13, pp. 1447–1451, 2013.

[45] M. Bertolotti, G. Farinelli, M. Galli, A. Aiuti, and R. Sitia, "AQP8 transports NOX2-generated H_2O_2 across the plasma membrane to promote signaling in B cells," *Journal of Leukocyte Biology*, vol. 100, no. 5, pp. 1071–1079, 2016.

[46] F. Vieceli Dalla Sega, C. Prata, L. Zambonin et al., "Intracellular cysteine oxidation is modulated by aquaporin-8-mediated hydrogen peroxide channeling in leukaemia cells," *BioFactors*, vol. 43, no. 2, pp. 232–242, 2017.

[47] F. Vieceli Dalla Sega, L. Zambonin, D. Fiorentini et al., "Specific aquaporins facilitate Nox-produced hydrogen peroxide transport through plasma membrane in leukaemia cells," *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1843, no. 4, pp. 806–814, 2014.

[48] R. A. Marinelli, G. L. Lehmann, L. R. Soria, and M. J. Marchissio, "Hepatocyte aquaporins in bile formation and cholestasis," *Frontiers in Bioscience*, vol. 16, no. 1, p. 2642, 2011.

[49] D. Ferri, A. Mazzone, G. E. Liquori, G. Cassano, M. Svelto, and G. Calamita, "Ontogeny, distribution, and possible functional implications of an unusual aquaporin, AQP8, in mouse liver," *Hepatology*, vol. 38, no. 4, pp. 947–957, 2003.

[50] G. Calamita, D. Ferri, P. Gena et al., "The inner mitochondrial membrane has aquaporin-8 water channels and is highly permeable to water," *The Journal of Biological Chemistry*, vol. 280, no. 17, pp. 17149–17153, 2005.

[51] M. J. Marchissio, D. E. A. Francés, C. E. Carnovale, and R. A. Marinelli, "Mitochondrial aquaporin-8 knockdown in human hepatoma HepG2 cells causes ROS-induced mitochondrial depolarization and loss of viability," *Toxicology and Applied Pharmacology*, vol. 264, no. 2, pp. 246–254, 2012.

[52] F. Chauvigne, M. Boj, R. N. Finn, and J. Cerda, "Mitochondrial aquaporin-8-mediated hydrogen peroxide transport is essential for teleost spermatozoon motility," *Scientific Reports*, vol. 5, no. 1, p. 7789, 2015.

[53] U. Laforenza, G. Pellavio, A. Marchetti, C. Omes, F. Todaro, and G. Gastaldi, "Aquaporin-mediated water and hydrogen peroxide transport is involved in normal human spermatozoa functioning," *International Journal of Molecular Sciences*, vol. 18, no. 12, 2017.

[54] M. J. Marchissio, D. E. A. Francés, C. E. Carnovale, and R. A. Marinelli, "Evidence for necrosis, but not apoptosis, in human hepatoma cells with knockdown of mitochondrial aquaporin-8," *Apoptosis*, vol. 19, no. 5, pp. 851–859, 2014.

[55] I. Medrano-Fernandez, S. Bestetti, M. Bertolotti et al., "Stress regulates aquaporin-8 permeability to impact cell growth and survival," *Antioxidants & Redox Signaling*, vol. 24, no. 18, pp. 1031–1044, 2016.

[56] R. A. Marinelli and M. J. Marchissio, "Mitochondrial aquaporin-8: a functional peroxiporin?," *Antioxidants & Redox Signaling*, vol. 19, no. 8, p. 896, 2013.

[57] M. Danielli, A. M. Capiglioni, J. Marrone, G. Calamita, and R. A. Marinelli, "Cholesterol can modulate mitochondrial aquaporin-8 expression in human hepatic cells," *IUBMB Life*, vol. 69, no. 5, pp. 341–346, 2017.

[58] A. M. Giudetti, F. Damiano, G. V. Gnoni, and L. Siculella, "Low level of hydrogen peroxide induces lipid synthesis in BRL-3A cells through a CAP-independent SREBP-1a activation," *The International Journal of Biochemistry & Cell Biology*, vol. 45, no. 7, pp. 1419–1426, 2013.

[59] M. Boj, F. Chauvigne, and J. Cerda, "Coordinated action of aquaporins regulates sperm motility in a marine teleost," *Biology of Reproduction*, vol. 93, no. 2, p. 40, 2015.

[60] G. Pellavio, M. Rui, L. Caliogna et al., "Regulation of aquaporin functional properties mediated by the antioxidant effects of natural compounds," *International Journal of Molecular Sciences*, vol. 18, no. 12, 2017.

[61] M. Hara-Chikuma, S. Chikuma, Y. Sugiyama et al., "Chemokine-dependent T cell migration requires aquaporin-3-mediated hydrogen peroxide uptake," *The Journal of Experimental Medicine*, vol. 209, no. 10, pp. 1743–1752, 2012.

[62] M. Hara-Chikuma, H. Satooka, S. Watanabe et al., "Aquaporin-3-mediated hydrogen peroxide transport is required for NF-κB signalling in keratinocytes and development of psoriasis," *Nature Communications*, vol. 6, no. 1, p. 7454, 2015.

[63] N. L. Wu, J. Y. Fang, M. Chen, C. J. Wu, C. C. Huang, and C. F. Hung, "Chrysin protects epidermal keratinocytes from UVA- and UVB-induced damage," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 15, pp. 8391–8400, 2011.

[64] Z. Wu, H. Uchi, S. Morino-Koga, W. Shi, and M. Furue, "Resveratrol inhibition of human keratinocyte proliferation via SIRT1/ARNT/ERK dependent downregulation of aquaporin 3," *Journal of Dermatological Science*, vol. 75, no. 1, pp. 16–23, 2014.

[65] H. Xie, F. Liu, L. Liu et al., "Protective role of AQP3 in UVA-induced NHSFs apoptosis via Bcl2 up-regulation," *Archives of Dermatological Research*, vol. 305, no. 5, pp. 397–406, 2013.

[66] I. Seleit, O. A. Bakry, H. S. El Rebey, G. El-Akabawy, and G. Hamza, "Is Aquaporin-3 a determinant factor of intrinsic and extrinsic aging? An immunohistochemical and morphometric study," *Applied Immunohistochemistry & Molecular Morphology*, vol. 25, no. 1, pp. 49–57, 2017.

[67] J. R. Thiagarajah, D. Zhao, and A. S. Verkman, "Impaired enterocyte proliferation in aquaporin-3 deficiency in mouse models of colitis," *Gut*, vol. 56, no. 11, pp. 1529–1535, 2007.

[68] J. R. Thiagarajah, J. Chang, J. A. Goettel, A. S. Verkman, and W. I. Lencer, "Aquaporin-3 mediates hydrogen peroxide-dependent responses to environmental stress in colonic epithelia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 3, pp. 568–573, 2017.

[69] M. Hara-Chikuma, S. Watanabe, and H. Satooka, "Involvement of aquaporin-3 in epidermal growth factor receptor signaling via hydrogen peroxide transport in cancer cells," *Biochemical and Biophysical Research Communications*, vol. 471, no. 4, pp. 603–609, 2016.

[70] P. Ricanek, L. K. Lunde, S. A. Frye et al., "Reduced expression of aquaporins in human intestinal mucosa in early stage inflammatory bowel disease," *Clinical and Experimental Gastroenterology*, vol. 8, pp. 49–67, 2015.

[71] H. Satooka and M. Hara-Chikuma, "Aquaporin-3 controls breast cancer cell migration by regulating hydrogen peroxide transport and its downstream cell signaling," *Molecular and Cellular Biology*, vol. 36, no. 7, pp. 1206–1218, 2016.

[72] T. Marchbank and R. J. Playford, "Trefoil factor family peptides enhance cell migration by increasing cellular osmotic

permeability and aquaporin 3 levels," *The FASEB Journal*, vol. 32, no. 2, pp. 1017–1024, 2018.

[73] K. Asai, R. K. Kudej, Y. T. Shen et al., "Peripheral vascular endothelial dysfunction and apoptosis in old monkeys," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 6, pp. 1493–1499, 2000.

[74] D. S. Celermajer, K. E. Sorensen, D. J. Spiegelhalter, D. Georgakopoulos, J. Robinson, and J. E. Deanfield, "Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women," *Journal of the American College of Cardiology*, vol. 24, no. 2, pp. 471–476, 1994.

[75] Q. Shi, K. Aida, J. L. Vandeberg, and X. L. Wang, "Passage-dependent changes in baboon endothelial cells—relevance to in vitro aging," *DNA and Cell Biology*, vol. 23, no. 8, pp. 502–509, 2004.

[76] M. Wang, J. Zhang, L. Q. Jiang et al., "Proinflammatory profile within the grossly normal aged human aortic wall," *Hypertension*, vol. 50, no. 1, pp. 219–227, 2007.

[77] L. J. Ignarro, G. M. Buga, K. S. Wood, R. E. Byrns, and G. Chaudhuri, "Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 24, pp. 9265–9269, 1987.

[78] F. Paneni, C. Diaz Canestro, P. Libby, T. F. Luscher, and G. G. Camici, "The aging cardiovascular system: understanding it at the cellular and clinical levels," *Journal of the American College of Cardiology*, vol. 69, no. 15, pp. 1952–1967, 2017.

[79] M. A. Potenza, F. Addabbo, and M. Montagnani, "Vascular actions of insulin with implications for endothelial dysfunction," *American Journal of Physiology Endocrinology and Metabolism*, vol. 297, no. 3, pp. E568–E577, 2009.

[80] L. J. Ignarro and C. Napoli, "Novel features of nitric oxide, endothelial nitric oxide synthase, and atherosclerosis," *Current Atherosclerosis Reports*, vol. 6, no. 4, pp. 281–287, 2004.

[81] S. Taddei, A. Virdis, L. Ghiadoni et al., "Age-related reduction of NO availability and oxidative stress in humans," *Hypertension*, vol. 38, no. 2, pp. 274–279, 2001.

[82] A. L. Sverdlov, W. P. A. Chan, N. E. K. Procter, Y. Y. Chirkov, D. T. M. Ngo, and J. D. Horowitz, "Reciprocal regulation of NO signaling and TXNIP expression in humans: impact of aging and ramipril therapy," *International Journal of Cardiology*, vol. 168, no. 5, pp. 4624–4630, 2013.

[83] R. M. J. Palmer, A. G. Ferrige, and S. Moncada, "Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor," *Nature*, vol. 327, no. 6122, pp. 524–526, 1987.

[84] S. Donnini, M. Monti, R. Roncone et al., "Peroxynitrite inactivates human-tissue inhibitor of metalloproteinase-4," *FEBS Letters*, vol. 582, no. 7, pp. 1135–1140, 2008.

[85] M. A. Incalza, R. D'Oria, A. Natalicchio, S. Perrini, L. Laviola, and F. Giorgino, "Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases," *Vascular Pharmacology*, vol. 100, pp. 1–19, 2018.

[86] M. Monti, S. Donnini, A. Giachetti, D. Mochly-Rosen, and M. Ziche, "δPKC inhibition or varepsilonPKC activation repairs endothelial vascular dysfunction by regulating eNOS post-translational modification," *Journal of Molecular and Cellular Cardiology*, vol. 48, no. 4, pp. 746–756, 2010.

[87] U. Forstermann and H. Li, "Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling," *British Journal of Pharmacology*, vol. 164, no. 2, pp. 213–223, 2011.

[88] D.-D. Chen, L.-Y. Chen, J.-B. Xie et al., "Tetrahydrobiopterin regulation of eNOS redox function," *Current Pharmaceutical Design*, vol. 20, no. 22, pp. 3554–3562, 2014.

[89] Y. M. Yang, A. Huang, G. Kaley, and D. Sun, "eNOS uncoupling and endothelial dysfunction in aged vessels," *American Journal of Physiology Heart and Circulatory Physiology*, vol. 297, no. 5, pp. H1829–H1836, 2009.

[90] T. Malinski, Z. Taha, S. Grunfeld, S. Patton, M. Kapturczak, and P. Tomboulian, "Diffusion of nitric oxide in the aorta wall monitored in situ by porphyrinic microsensors," *Biochemical and Biophysical Research Communications*, vol. 193, no. 3, pp. 1076–1082, 1993.

[91] M. Moller, H. Botti, C. Batthyany, H. Rubbo, R. Radi, and A. Denicola, "Direct measurement of nitric oxide and oxygen partitioning into liposomes and low density lipoprotein," *The Journal of Biological Chemistry*, vol. 280, no. 10, pp. 8850–8854, 2005.

[92] M. Herrera and J. L. Garvin, "Novel role of AQP-1 in NO-dependent vasorelaxation," *American Journal of Physiology Renal Physiology*, vol. 292, no. 5, pp. F1443–F1451, 2007.

[93] J. U. Lee, Y. W. Oh, and S. W. Kim, "Altered renal expression of aquaporin-2 water channels in rats with experimental two-kidney, one clip hypertension," *Journal of Korean Medical Science*, vol. 16, no. 4, pp. 462–466, 2001.

[94] V. Montiel, E. Leon Gomez, C. Bouzin et al., "Genetic deletion of aquaporin-1 results in microcardia and low blood pressure in mouse with intact nitric oxide-dependent relaxation, but enhanced prostanoids-dependent relaxation," *Pflugers Archiv: European Journal of Physiology*, vol. 466, no. 2, pp. 237–251, 2014.

[95] R. D. Fontijn, O. L. Volger, T. C. van der Pouw-Kraan et al., "Expression of nitric oxide-transporting aquaporin-1 is controlled by KLF2 and marks non-activated endothelium *in vivo*," *PLoS One*, vol. 10, no. 12, article e0145777, 2015.

[96] W. L. Kenney and P. Chiu, "Influence of age on thirst and fluid intake," *Medicine and Science in Sports and Exercise*, vol. 33, no. 9, pp. 1524–1532, 2001.

[97] J. A. Suhr, J. Hall, S. M. Patterson, and R. T. Niinisto, "The relation of hydration status to cognitive performance in healthy older adults," *International Journal of Psychophysiology*, vol. 53, no. 2, pp. 121–125, 2004.

[98] J. A. Suhr, S. M. Patterson, A. W. Austin, and K. L. Heffner, "The relation of hydration status to declarative memory and working memory in older adults," *The Journal of Nutrition, Health & Aging*, vol. 14, no. 10, pp. 840–843, 2010.

[99] P. Voyer, S. Richard, L. Doucet, and P. H. Carmichael, "Predisposing factors associated with delirium among demented long-term care residents," *Clinical Nursing Research*, vol. 18, no. 2, pp. 153–171, 2009.

[100] L. Nadav, A. Y. Gur, A. D. Korczyn, and N. M. Bornstein, "Stroke in hospitalized patients: are there special risk factors?," *Cerebrovascular Diseases*, vol. 13, no. 2, pp. 127–131, 2002.

[101] G. J. Rodriguez, S. M. Cordina, G. Vazquez et al., "The hydration influence on the risk of stroke (THIRST) study," *Neurocritical Care*, vol. 10, no. 2, pp. 187–194, 2009.

[102] L. E. Cowen, S. P. Hodak, and J. G. Verbalis, "Age-associated abnormalities of water homeostasis," *Endocrinology and Metabolism Clinics of North America*, vol. 42, no. 2, pp. 349–370, 2013.

[103] G. Tamma, N. Goswami, J. Reichmuth, N. G. De Santo, and G. Valenti, "Aquaporins, vasopressin, and aging: current perspectives," *Endocrinology*, vol. 156, no. 3, pp. 777–788, 2015.

[104] J. M. Sands, "Urine concentrating and diluting ability during aging," *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, vol. 67, no. 12, pp. 1352–1357, 2012.

[105] S. Combet, S. Gouraud, R. Gobin et al., "Aquaporin-2 down-regulation in kidney medulla of aging rats is posttranscriptional and is abolished by water deprivation," *American Journal of Physiology Renal Physiology*, vol. 294, no. 6, pp. F1408–F1414, 2008.

[106] T. Katsura, C. E. Gustafson, D. A. Ausiello, and D. Brown, "Protein kinase A phosphorylation is involved in regulated exocytosis of aquaporin-2 in transfected LLC-PK1 cells," *The American Journal of Physiology*, vol. 272, no. 6, Part 2, pp. F817–F822, 1997.

[107] J. D. Hoffert, T. Pisitkun, G. Wang, R. F. Shen, and M. A. Knepper, "Quantitative phosphoproteomics of vasopressin-sensitive renal cells: regulation of aquaporin-2 phosphorylation at two sites," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 18, pp. 7159–7164, 2006.

[108] S. Combet, L. Teillet, G. Geelen et al., "Food restriction prevents age-related polyuria by vasopressin-dependent recruitment of aquaporin-2," *American Journal of Physiology Renal Physiology*, vol. 281, no. 6, pp. F1123–F1131, 2001.

[109] T. Berl, F. Quittnat-Pelletier, J. G. Verbalis et al., "Oral tolvaptan is safe and effective in chronic hyponatremia," *Journal of the American Society of Nephrology*, vol. 21, no. 4, pp. 705–712, 2010.

[110] O. Devuyst and V. E. Torres, "Osmoregulation, vasopressin, and cAMP signaling in autosomal dominant polycystic kidney disease," *Current Opinion in Nephrology and Hypertension*, vol. 22, no. 4, pp. 459–470, 2013.

[111] R. W. Schrier, P. Gross, M. Gheorghiade et al., "Tolvaptan, a selective oral vasopressin V2-receptor antagonist, for hyponatremia," *The New England Journal of Medicine*, vol. 355, no. 20, pp. 2099–2112, 2006.

[112] L. Li, J. J. Galligan, G. D. Fink, and A. F. Chen, "Vasopressin induces vascular superoxide via endothelin-1 in mineralocorticoid hypertension," *Hypertension*, vol. 41, no. 3, pp. 663–668, 2003.

[113] G. Faraco, T. S. Wijasa, L. Park, J. Moore, J. Anrather, and C. Iadecola, "Water deprivation induces neurovascular and cognitive dysfunction through vasopressin-induced oxidative stress," *Journal of Cerebral Blood Flow and Metabolism*, vol. 34, no. 5, pp. 852–860, 2014.

[114] V. M. Mendoza-Nunez, M. Ruiz-Ramos, M. A. Sanchez-Rodriguez, R. Retana-Ugalde, and J. L. Munoz-Sanchez, "Aging-related oxidative stress in healthy humans," *The Tohoku Journal of Experimental Medicine*, vol. 213, no. 3, pp. 261–268, 2007.

[115] R. Bucala, "Diabetes, aging, and their tissue complications," *The Journal of Clinical Investigation*, vol. 124, no. 5, pp. 1887-1888, 2014.

[116] J. F. Reckelhoff, V. Kanji, L. C. Racusen et al., "Vitamin E ameliorates enhanced renal lipid peroxidation and accumulation of F2-isoprostanes in aging kidneys," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 274, no. 3, pp. R767–R774, 1998.

[117] M. F. Albertoni Borghese, M. P. Majowicz, M. C. Ortiz, M. F. Delgado, N. B. Sterin Speziale, and N. A. Vidal, "Renal sodium-glucose cotransporter activity and aquaporin-2 expression in rat kidney during chronic nitric oxide synthase inhibition," *Nephron Physiology*, vol. 107, no. 3, pp. p77–p86, 2007.

[118] N. Arreche, A. Fellet, M. Lopez, J. Lopez-Costa, C. Arranz, and A. M. Balaszczuk, "Hypovolemic state: involvement of nitric oxide in the aged related alterations of aquaporins-2 abundance in rat kidney," *Vascular Pharmacology*, vol. 49, no. 1, pp. 19–25, 2008.

[119] R. C. Bollineni, R. Hoffmann, and M. Fedorova, "Proteome-wide profiling of carbonylated proteins and carbonylation sites in HeLa cells under mild oxidative stress conditions," *Free Radical Biology and Medicine*, vol. 68, pp. 186–195, 2014.

[120] A. Pastore and F. Piemonte, "S-Glutathionylation signaling in cell biology: progress and prospects," *European Journal of Pharmaceutical Sciences*, vol. 46, no. 5, pp. 279–292, 2012.

[121] P. C. Sandoval, D. H. Slentz, T. Pisitkun, F. Saeed, J. D. Hoffert, and M. A. Knepper, "Proteome-wide measurement of protein half-lives and translation rates in vasopressin-sensitive collecting duct cells," *Journal of the American Society of Nephrology*, vol. 24, no. 11, pp. 1793–1805, 2013.

[122] M. H. M. Shimizu, A. Danilovic, L. Andrade et al., "*N*-Acetylcysteine protects against renal injury following bilateral ureteral obstruction," *Nephrology Dialysis Transplantation*, vol. 23, no. 10, pp. 3067–3073, 2008.

[123] E. H. Bae, S. Y. Joo, S. K. Ma, J. Lee, and S. W. Kim, "Resveratrol attenuates 4-hydroxy-2-hexenal-induced oxidative stress in mouse cortical collecting duct cells," *The Korean Journal of Physiology & Pharmacology*, vol. 20, no. 3, pp. 229–236, 2016.

[124] P. W. Cheung, L. Ueberdiek, J. Day, R. Bouley, and D. Brown, "Protein phosphatase 2C is responsible for VP-induced dephosphorylation of AQP2 serine 261," *American Journal of Physiology-Renal Physiology*, vol. 313, no. 2, pp. F404–F413, 2017.

[125] P. I. Nedvetsky, V. Tabor, G. Tamma et al., "Reciprocal regulation of aquaporin-2 abundance and degradation by protein kinase A and p38-MAP kinase," *Journal of the American Society of Nephrology*, vol. 21, no. 10, pp. 1645–1656, 2010.

[126] M. M. Rinschen, M. J. Yu, G. Wang et al., "Quantitative phosphoproteomic analysis reveals vasopressin V2-receptor-dependent signaling pathways in renal collecting duct cells," *Proceedings of the National Academy of Sciences*, vol. 107, no. 8, pp. 3882–3887, 2010.

[127] G. Tamma, J. H. Robben, C. Trimpert, M. Boone, and P. M. T. Deen, "Regulation of AQP2 localization by S256 and S261 phosphorylation and ubiquitination," *American Journal of Physiology-Cell Physiology*, vol. 300, no. 3, pp. C636–C646, 2011.

[128] U. Hasler, V. Leroy, U. S. Jeon et al., "NF-κB modulates aquaporin-2 transcription in renal collecting duct principal cells," *Journal of Biological Chemistry*, vol. 283, no. 42, pp. 28095–28105, 2008.

[129] E. Feraille, E. Dizin, I. Roth et al., "NADPH oxidase 4 deficiency reduces aquaporin-2 mRNA expression in cultured renal collecting duct principal cells via increased PDE3 and PDE4 activity," *PLoS One*, vol. 9, no. 1, article e87239, 2014.

[130] G. Tamma, M. Ranieri, A. Di Mise, M. Centrone, M. Svelto, and G. Valenti, "Glutathionylation of the aquaporin-2 water channel: a novel post-translational modification modulated by the oxidative stress," *Journal of Biological Chemistry*, vol. 289, no. 40, pp. 27807–27813, 2014.

[131] P. Portincasa, I. Cataldo, A. Maggio et al., "Modulation of aquaporins by dietary patterns and plant bioactive compounds," *Current Medicinal Chemistry*, vol. 24, 2017.

[132] D. Fiorentini, L. Zambonin, F. V. Dalla Sega, and S. Hrelia, "Polyphenols as modulators of aquaporin family in health and disease," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 196914, 8 pages, 2015.

[133] M. Gao, S. Y. Zhu, C. B. Tan, B. Xu, W. C. Zhang, and G. H. Du, "Pinocembrin protects the neurovascular unit by reducing inflammation and extracellular proteolysis in MCAO rats," *Journal of Asian Natural Products Research*, vol. 12, no. 5, pp. 407–418, 2010.

[134] B. Kumar, S. K. Gupta, T. C. Nag et al., "Retinal neuroprotective effects of quercetin in streptozotocin-induced diabetic rats," *Experimental Eye Research*, vol. 125, pp. 193–202, 2014.

[135] B. Kumar, S. K. Gupta, B. P. Srinivasan et al., "Hesperetin rescues retinal oxidative stress, neuroinflammation and apoptosis in diabetic rats," *Microvascular Research*, vol. 87, pp. 65–74, 2013.

[136] B. Zhang, Z. Liang, S. He et al., "The mechanism underlying alpinetin-mediated alleviation of pancreatitis-associated lung injury through upregulating aquaporin-1," *Drug Design, Development and Therapy*, vol. 10, p. 841, 2016.

[137] K. Saito, S. Mori, F. Date, and G. Hong, "Epigallocatechin gallate stimulates the neuroreactive salivary secretomotor system in autoimmune sialadenitis of MRL-Faslpr mice via activation of cAMP-dependent protein kinase A and inactivation of nuclear factorκB," *Autoimmunity*, vol. 48, no. 6, pp. 379–388, 2015.

[138] K. Oshio, H. Watanabe, Y. Song, A. S. Verkman, and G. T. Manley, "Reduced cerebrospinal fluid production and intracranial pressure in mice lacking choroid plexus water channel aquaporin-1," *The FASEB Journal*, vol. 19, no. 1, pp. 76–78, 2005.

[139] C. Ji, C. Cao, S. Lu et al., "Curcumin attenuates EGF-induced AQP3 up-regulation and cell migration in human ovarian cancer cells," *Cancer Chemotherapy and Pharmacology*, vol. 62, no. 5, pp. 857–865, 2008.

[140] B. F. Wang, Z. W. Cui, Z. H. Zhong et al., "Curcumin attenuates brain edema in mice with intracerebral hemorrhage through inhibition of AQP4 and AQP9 expression," *Acta Pharmacologica Sinica*, vol. 36, no. 8, pp. 939–948, 2015.

[141] L. S. Yu, Y. Y. Fan, G. Ye et al., "Curcumin alleviates brain edema by lowering AQP4 expression levels in a rat model of hypoxia-hypercapnia-induced brain damage," *Experimental and Therapeutic Medicine*, vol. 11, no. 3, pp. 709–716, 2016.

[142] J. Zu, Y. Wang, G. Xu, J. Zhuang, H. Gong, and J. Yan, "Curcumin improves the recovery of motor function and reduces spinal cord edema in a rat acute spinal cord injury model by inhibiting the JAK/STAT signaling pathway," *Acta Histochemica*, vol. 116, no. 8, pp. 1331–1336, 2014.

[143] I. Lozić, R. V. Hartz, C. A. Bartlett et al., "Enabling dual cellular destinations of polymeric nanoparticles for treatment following partial injury to the central nervous system," *Biomaterials*, vol. 74, pp. 200–216, 2016.

[144] M. Aliomrani, M. R. Sepand, H. R. Mirzaei, A. R. Kazemi, S. Nekonam, and O. Sabzevari, "Effects of phloretin on oxidative and inflammatory reaction in rat model of cecal ligation and puncture induced sepsis," *DARU Journal of Pharmaceutical Sciences*, vol. 24, no. 1, p. 15, 2016.

[145] F. J. Moller, P. Diel, O. Zierau, T. Hertrampf, J. Maass, and G. Vollmer, "Long-term dietary isoflavone exposure enhances estrogen sensitivity of rat uterine responsiveness mediated through estrogen receptor α," *Toxicology Letters*, vol. 196, no. 3, pp. 142–153, 2010.

[146] Z. Zhong, B. Wang, M. Dai et al., "Carvacrol alleviates cerebral edema by modulating AQP4 expression after intracerebral hemorrhage in mice," *Neuroscience Letters*, vol. 555, pp. 24–29, 2013.

[147] E. J. Sohn, D. G. Kang, and H. S. Lee, "Protective effects of glycyrrhizin on gentamicin-induced acute renal failure in rats," *Pharmacology & Toxicology*, vol. 93, no. 3, pp. 116–122, 2003.

[148] Q. W. Ding, Y. Zhang, Y. Wang et al., "Functional vanilloid receptor-1 in human submandibular glands," *Journal of Dental Research*, vol. 89, no. 7, pp. 711–716, 2010.

[149] Y. Zhang, X. Cong, L. Shi et al., "Activation of transient receptor potential vanilloid subtype 1 increases secretion of the hypofunctional, transplanted submandibular gland," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 299, no. 1, pp. G54–G62, 2010.

[150] I. D. Weiner, "New insights into the molecular regulation of urine concentration," *American Journal of Physiology-Renal Physiology*, vol. 311, no. 1, pp. F184–F185, 2016.

[151] M. C. Papadopoulos and A. S. Verkman, "Aquaporin water channels in the nervous system," *Nature Reviews. Neuroscience*, vol. 14, no. 4, pp. 265–277, 2013.

[152] T. L. Tran, S. Hamann, and S. Heegaard, "Aquaporins in the eye," *Advances in Experimental Medicine and Biology*, vol. 969, pp. 193–198, 2017.

[153] P. Portincasa and G. Calamita, "Water channel proteins in bile formation and flow in health and disease: when immiscible becomes miscible," *Molecular Aspects of Medicine*, vol. 33, no. 5-6, pp. 651–664, 2012.

[154] L. Pei, G. Yang, J. Jiang et al., "Expression of aquaporins in prostate and seminal vesicles of diabetic rats," *The Journal of Sexual Medicine*, vol. 10, no. 12, pp. 2975–2985, 2013.

[155] G. Cui, M. M. Staron, S. M. Gray et al., "IL-7-induced glycerol transport and TAG synthesis promotes memory CD8+ T cell longevity," *Cell*, vol. 161, no. 4, pp. 750–761, 2015.

[156] V. Rabolli, L. Wallemme, S. Lo Re et al., "Critical role of aquaporins in interleukin 1β (IL-1β)-induced inflammation," *The Journal of Biological Chemistry*, vol. 289, no. 20, pp. 13937–13947, 2014.

[157] M. G. Song, S. Y. Hwang, J. I. Park, S. Yoon, H. R. Bae, and J. Y. Kwak, "Role of aquaporin 3 in development, subtypes and activation of dendritic cells," *Molecular Immunology*, vol. 49, no. 1-2, pp. 28–37, 2011.

[158] A. Rodriguez, R. A. Marinelli, A. Tesse, G. Fruhbeck, and G. Calamita, "Sexual dimorphism of adipose and hepatic aquaglyceroporins in health and metabolic disorders," *Frontiers in Endocrinology*, vol. 6, 2015.

[159] L. R. Soria, J. Marrone, G. Calamita, and R. A. Marinelli, "Ammonia detoxification via ureagenesis in rat hepatocytes involves mitochondrial aquaporin-8 channels," *Hepatology*, vol. 57, no. 5, pp. 2061–2071, 2013.

[160] M. Yasui, S. M. Zelenin, G. Celsi, and A. Aperia, "Adenylate cyclase-coupled vasopressin receptor activates AQP2 promoter via a dual effect on CRE and AP1 elements," *The American Journal of Physiology*, vol. 272, no. 4, pp. F443–F450, 1997.

[161] A. S. Verkman, "More than just water channels: unexpected cellular roles of aquaporins," *Journal of Cell Science*, vol. 118, no. 15, pp. 3225–3232, 2005.

[162] P. Agre, "Aquaporin water channels (Nobel Lecture)," *Angewandte Chemie International Edition*, vol. 43, no. 33, pp. 4278–4290, 2004.

[163] S. Saadoun, M. C. Papadopoulos, M. Hara-Chikuma, and A. S. Verkman, "Impairment of angiogenesis and cell migration by targeted aquaporin-1 gene disruption," *Nature*, vol. 434, no. 7034, pp. 786–792, 2005.

[164] L. Tie, D. Wang, Y. Shi, and X. Li, "Aquaporins in cardiovascular system," *Advances in Experimental Medicine and Biology*, vol. 969, pp. 105–113, 2017.

[165] H. B. Moeller, C. H. Fuglsang, and R. A. Fenton, "Renal aquaporins and water balance disorders," *Best Practice & Research Clinical Endocrinology & Metabolism*, vol. 30, no. 2, pp. 277–288, 2016.

[166] E. Benavente and S. Paira, "Neuromyelitis Optica-AQP4: an update," *Current Rheumatology Reports*, vol. 13, no. 6, pp. 496–505, 2011.

[167] Y. Liu, D. Promeneur, A. Rojek et al., "Aquaporin 9 is the major pathway for glycerol uptake by mouse erythrocytes, with implications for malarial virulence," *Proceedings of the National Academy of Sciences*, vol. 104, no. 30, pp. 12560–12564, 2007.

[168] M. C. Papadopoulos and S. Saadoun, "Key roles of aquaporins in tumor biology," *Biochimica et Biophysica Acta (BBA) - Biomembranes*, vol. 1848, no. 10, pp. 2576–2583, 2015.

[169] R. Bernardino, R. Marinelli, A. Maggio et al., "Hepatocyte and sertoli cell aquaporins, recent advances and research trends," *International Journal of Molecular Sciences*, vol. 17, no. 12, 2016.

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Review Article

The Role of Oxidative Stress and Membrane Transport Systems during Endometriosis: A Fresh Look at a Busy Corner

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Endometriosis is a condition characterized by the presence of endometrial tissue outside the uterine cavity, leading to a chronic inflammatory reaction. It is one of the most widespread gynecological diseases with a 10–15% prevalence in the general female population, rising up to 30–45% in patients with infertility. Although it was first described in 1860, its etiology and pathogenesis are still unclear. It is now accepted that inflammation plays a central role in the development and progression of endometriosis. In particular, it is marked by an inflammatory process associated with the overproduction of an array of inflammatory mediators such as prostaglandins, metalloproteinases, cytokines, and chemokines. In addition, the growth and adhesion of endometrial cells in the peritoneal cavity due to reactive oxygen species (ROS) and free radicals lead to disease onset, its ensuing symptoms—among which pain and infertility. The aim of our review is to evaluate the role of oxidative stress and ROS in the pathogenesis of endometriosis and the efficacy of antioxidant therapy in the treatment and mitigation of its symptoms.

1. Introduction

Endometriosis is a chronic gynecological disorder defined by the presence of endometrial tissues outside the uterine cavity; these lesions encompass glands and stroma that respond to local, exogenous, and endogenous hormones [1]. It affects about 10–15% of all women within the reproductive age group and has a significant impact on their quality of life and psychological well-being [2–7]. The prevalence of women with pelvic pain ranges from 30 to 45% of the infertile population [1, 8]. Nevertheless, since diagnosis necessitates surgical confirmation, the factual prevalence of the disease is most likely underestimated. There are three different phenotypes of endometriosis, graded from the least to most

severe: (1) superficial peritoneal endometriosis (SUP), (2) ovarian endometrioma (OMA), and (3) deeply infiltrating endometriosis (DIE)—the latter being the most aggressive form, characterized by the involvement of the muscularis propria, irrespective of the anatomical location [9–12].

The etiology of endometriosis is complex and indeed still poorly understood. Various theories have been postulated, such as menstrual blood regurgitation, persistent Müllerian duct abnormality, and coelomic metaplasia [13, 14]. The most commonly accepted theory regarding the origin of endometriosis was first postulated by Sampson in 1927. In his work, Sampson described the elements generally present in this condition: retrograde menstruation, viable cells within retrograde menstruation, and the implantation of viable endometrial tissue within the peritoneum [15]. Retrograde menstruation is the backflow of menstrual blood into the peritoneal cavity through the fallopian tubes. Interestingly, retrograde menstruation is not a unique phenomenon to endometriosis and occurs in most women [16]. Normally, the immune system will eliminate these cells, preventing their implantation in the peritoneal cavity. In failing to do so, the patient develops endometriosis. It is now accepted that inflammation plays a central role in the development and progression of endometriosis [17–19]. In particular, the underlying condition is an inflammatory process leading to the overproduction of a wide range of inflammatory mediators, that is, prostaglandins, metalloproteinases, cytokines, and chemokines [20, 21]. Moreover, reactive oxygen species (ROS) and free radicals favor the growth and adhesion of endometrial cells in the peritoneal cavity and consequently disease onset, its related symptoms, pain, and infertility [22–24]. The aim of this review is to evaluate the role of oxidative stress in endometriosis.

2. Materials and Methods

The aim of this review is to evaluate the role of oxidative stress in women presenting with endometriosis. Electronic database searches were conducted (MEDLINE, Scopus, Embase, and ScienceDirect) to retrieve relevant studies conducted over the last 20 years. The search terms used were as follows: "endometriosis," "oxidative stress endometriosis," "endometriosis," "infertility," "infertility in endometriosis," "oocyte quality," and "In Vitro Fertilization (IVF)." We only included manuscripts in English. In addition, the references of all articles retrieved were examined to identify studies that had not been identified by electronic searches.

The electronic search and eligibility of the studies were independently evaluated by five coauthors (V.L.L.R., G.V., F.S., G.S., and D.R.). Discrepancies were settled by four different coauthors (I.P., A.V., M.N., and R.A.). Two authors (S.G.V. and G.Z.) independently evaluated both inclusion criteria and study selection. Lastly, additional divergences were evaluated by a third external reviewer (A.S.L.). Data abstraction was completed by two independent investigators (S.C. and S.L.), each of whom independently extracted the data from each single study. The data retrieved from eligible studies were extracted without modifying the original data.

3. Results

3.1. Oxidative Stress (OS). Oxidative stress (OS) develops as a consequence of an imbalance between the generation of free radicals and the scavenging capacity of antioxidants. Free radicals are defined as any species with one or more unpaired electrons in the outer orbit [25]. There are two types of free radicals: reactive oxygen species (ROS) and reactive nitrogen species (RNS). The main free radicals are the superoxide radical, hydrogen peroxide, hydroxyl, and singlet oxygen radicals. ROS are intermediate products of normal oxygen metabolism. Oxygen is required to support life; however, its metabolites can alter cell functions and/or endanger cell survival [26, 27]. Almost all major classes of biomolecules—including lipids, proteins, and nucleic acids—are potential targets for ROS. Hydroxyl radicals are the best-known reactive free radical species; they are able to react with a wide range of cell constituents, among which amino acid residues, purine, and pyrimidine DNA bases, and attack membrane lipids to trigger a free radical chain reaction known as lipid peroxidation. Therefore, ROS must be continuously inactivated to preserve the least amount needed to retain normal cell function. Enzymatic and nonenzymatic antioxidant systems scavenge and deactivate excessive free radicals, in an attempt to prevent cell damage. The dietary intake of nonenzymatic antioxidants, that is, manganese, copper, selenium and zinc, beta-carotenes, vitamin C, vitamin E, taurine, hypotaurine, and group B vitamins, is all able to influence the body's complex antioxidant system [28]. On the other hand, the body also produces several antioxidant enzymes such as catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase, as well as molecules like glutathione and nicotinamide adenine dinucleotide (NADH). The glutathione produced by the cell has a crucial role in maintaining the normal balance between oxidation and antioxidation. NADH is known to play an antioxidant role in biological systems, through its high reactivity with some free radicals, its high intracellular concentrations, and its remarkable power to reduce all biologically active compounds [29]. Any disruption in the balance between ROS production and antioxidant defense generates higher ROS levels, which might lead to OS and the consequent harmful effects. OS is implicated as a major player in the pathophysiology of endometriosis [30].

3.2. Endometriosis and Oxidative Stress. Murphy et al. were one of the first groups to highlight the active role played by OS in the pathogenesis and development of endometriosis [30].

OS results from an imbalance between ROS and antioxidants. ROS molecules are characterized by an unpaired electron and stabilize themselves by extracting electrons from different molecules in the body, such as lipids, nucleic acids, and proteins. Antioxidants are a defense mechanism created by the body to neutralize ROS. Serving as signaling molecules, ROS modify reproductive processes such as tubal function, oocyte maturation, and folliculogenesis [27].

Van Langendonckt et al. described a positive correlation between prevention of endometriosis onset in rabbits and

Table 1: Markers of OS in endometriosis patients.

Reference	Type of biospecimen	OS marker
Rong et al. 2002 [40]	Peritoneal Fluid	↑ lipoproteins, particularly low-density lipoprotein (LDL)
Polak et al. 2013 [48]	Peritoneal fluid	↑ 8-iso-$PGF_{2\alpha}$ that promotes vasoconstrictor, but has also mitogenesis and cell adhesion
Santulli et al. 2015 [49]	Peritoneal fluid	↑ thiols, advanced oxidation protein products, protein carbonyl, and nitrates/nitrites in deep endometriosis
Jackson et al. 2005 [22]	Peritoneal fluid	↓ antioxidants ↑ lipid peroxides

increase antioxidant levels. They also found higher levels of ROS release by macrophages, higher peritoneal levels of oxidized low-density lipoproteins and their by-products, an altered expression of endometrial prooxidant and antioxidant enzymes, and the consumption of peritoneal fluid vitamin E [31].

Retrograde menstruation seems to be associated with highly prooxidant factors—that is, heme and iron—within the peritoneal cavity, in addition to apoptotic endometrial cells known to induce OS. The free or catalytic iron induces ROS production through a Fenton reaction, thereby inducing OS [32]. Iron release results from the metabolism of hemoglobin and heme by macrophages. Recent studies have provided evidence of an iron overload in various components of the peritoneal cavity of affected patients, such as peritoneal fluid, macrophages, and endometriotic lesions, strongly suggesting a disruption in iron homeostasis within the peritoneal cavity [33, 34].

Alizadeh et al., in a case-control study conducted in 2015, observed that serum iron level in patients with endometriosis was significantly higher than that in the control group [35]. Severe hemolysis occurring during retrograde menstruation, a defective or overwhelmed peritoneal disposal system in the presence of increased menstrual reflux, induced an iron overload in the peritoneal environment, which in turn caused the attachment and growth of endometrial cells or fragments. These iron stores might have numerous cytotoxic effects; indeed, they could disrupt the balance between free radical production and antioxidant defense, leading to a causal role of OS in the pathogenesis of endometriosis [36]. Iron toxicity might catalyze the production of an array of free radical damaging species, inducing the deregulation of cellular processes, cell dysfunction, and apoptosis or necrosis through lipid peroxidation, protein, and DNA damage [37–39].

There is evidence that the peritoneal fluid (PF) of women with endometriosis is characterized by high lipoprotein levels, particularly low-density lipoproteins (LDL) that generate oxidized lipid components in a macrophage-rich inflammatory milieu [40]. F2-isoprostanes are a complex family of compounds generated by the nonenzymatic peroxidation of arachidonic acid [41] on cell membranes [42] and LDL particles [43]. Various studies have documented increased 8-iso-prostaglandin and a specific biomarker of lipid peroxidation in vivo [44–46]. The measurement of F2-isoprostanes is the most reliable approach to assess OS status in vivo, and the products of the isoprostane pathway have been found to exert strong biological actions, possibly acting as pathophysiological mediators of the disease [44]. The 8-iso-$PGF_{2\alpha}$ not only acts as a vasoconstrictor, but has also been shown to promote mitogenesis and cell adhesion of monocytes and polymorphonuclear cells to endothelial cells and to induce endothelial cell necrosis [47]. Polak et al. observed higher PF 8-OHdG and 8-isoprostane concentrations in patients in the advanced stages of endometriosis, laparoscopically and histopathologically confirmed, compared with patients with simple serous and dermoid ovarian cysts [48].

In a large study by Santulli et al., protein OS markers (thiols, advanced oxidation protein products, protein carbonyl, and nitrates/nitrites) were evaluated in the peritoneal fluid on the basis of surgical classification [49]. The markers were significantly higher only in women with deep endometriosis compared with controls (P1/4.001 and P1/4.05, resp.), whereas other forms of endometriosis (peritoneal and ovarian) showed nonstatistically significant increases. These authors failed to find any difference in protein OS markers between women with peritoneal or ovarian endometriosis and control subjects. Moreover, the fact that the control group included women who had undergone surgery for benign gynecological conditions possibly associated with altered peritoneal protein OS markers is also one of the limitations of the study, which may have led to a bias, as acknowledged by the authors themselves [49] (Table 1).

Recent studies have investigated the role of the immune system and oxidative stress in the development of endometriosis [50]. In particular, some women with endometriosis seem to have an inefficient cleansing mechanism, possibly attributable to a failure of the cellular and humoral immune response whose role is to inhibit the implantation of ectopic endometrial tissue [51].

It has been hypothesized that natural killer (NK) cells may subserve this function. NK cells are the effector cells that usually recognize and destroy tumor cells, virus-infected host cells, and transplanted foreign cell lines. Oosterlynck et al. were the first to provide evidence of decreased NK activity and cytotoxicity against autologous endometrial cells in affected women, which correlated with disease stage [52]. These authors went on to show that peritoneal fluid from women with endometriosis, compared to that from fertile controls, presented significantly more marked NK cell suppressive activity [53]. Other authors also found similar findings in the serum [54] and pelvic fluid [55] of endometriosis patients.

Khan et al. were the first to report that peritoneal lesions involving early, active endometriosis and the adjacent peritoneum harbor large amounts of macrophages possibly involved in the onset of endometriosis [56]. Whereas the role of macrophages should be to clear the peritoneal cavity from endometrial cells, in this condition, they seem to enhance their proliferation by secreting growth factors and cytokines. Against this evidence, whether these immunological alterations induce endometriosis or are its consequence has yet to be unraveled [57–59].

3.3. Endometriosis Oxidative Stress and Female Infertility. Despite the wealth of literature on the possible association between endometriosis and infertility, a direct causal relationship has yet to be confirmed [60–63]. Endometriosis is generally considered a cause of infertility, through the mechanical hindrance of sperm–egg encounter caused by adhesions, endometrioma, and pelvic anatomy disruption [50]. However, in less severe cases, where there is no pelvic anatomical distortion, the underlying cause of the decreased fertility is poorly understood. Numerous mechanisms have been proposed to account for fertility impairment. Indeed, endometriosis can cause ovulatory dysfunction, poor oocyte quality, luteal phase defects with implantation failure, and abnormal embryogenesis, all of which may lead to poor fertilization [60, 61].

Marcoux et al., in 1997, and Parazzini, in 1999, in randomized, controlled trials evaluated whether laparoscopic surgery (LPS) enhanced fecundity in infertile women with minimal or mild endometriosis. The authors concluded that even if LPS improved natural fecundity, it was much below the rate expected in fertile women and suggested that other factors might interfere with fertility in early-stage endometriosis [62, 63].

In particular, poor oocyte quality can represent a possible mechanism involved.

Simon et al., in a retrospective study, observed a significantly lower implantation rate (IR) in recipients without endometriosis who received egg from women with endometriosis [64].

Recently, spindle morphology has emerged as a marker of oocyte quality and has led to remarkable technological progress in the ability to visualize this structure.

There are several studies describing the extreme sensitivity of the meiotic spindle to various factors, such as OS. A large body of evidence has shown significant DNA damage and increased anomalies in the microtubules and chromosomes of oocytes incubated with PF from endometriosis patients [65, 66]; as this damage could be prevented supplementing the culture medium with the antioxidant L-carnitine, it has been suggested that impaired oocyte quality in endometriosis may be mediated by oxidative stress [66].

In a prospective study carried out in 2009, Barcelos et al. investigated meiotic spindle and chromosome distribution of in vitro mature (IVM) oocytes from patients with and without endometriosis who underwent IVF. They observed a higher proportion of telophase I oocytes in the endometriosis group and suggested a potential delay or impairment of meiosis I during IVM in the presence of endometriosis, as a consequence of OS [67].

However, the evidence in this field does not allow sound conclusions to be drawn, especially in relation to the different stages of endometriosis and previous treatments received, on oocyte quality.

Oocyte competence depends not only on the quality of the follicular microenvironment, but also on the presence of adequate bidirectional cumulus cell-to-oocyte signaling [68–73].

Cumulus cells (CCs) form a group of closely associated cells surrounding the oocyte in the antral follicle, whereas mural granulosa cells form a lining on the follicular wall.

Granulosa cells (GCs) play an essential role in follicular differentiation, providing optimal conditions for oocyte development, ovulation, fertilization, and subsequent implantation [74]. Moreover, the bidirectional communication between the oocyte and these cells occurs all through follicular development [75–80] and is essential for the acquisition of developmental competence in mammalian oocytes [81–83].

Aromatase is present in GCs and plays a fundamental role in follicle maturation and in determining oocyte quality. In vitro studies using luteinized granulosa cell culture from women with and without endometriosis who underwent ovarian stimulation for IVF showed decreased aromatase activity in the GCs of affected women, which might have induced defects in GCs steroidogenesis and abnormal oocyte functioning [84].

Barcelos et al., in 2015, compared the expression of the aromatase gene (CYP19A1 gene) in CCs of infertile women with and without endometriosis who underwent ovarian stimulation for IVF. The authors demonstrated a lower CYP19A1 expression in CCs of infertile patients with endometriosis compared with infertile women without endometriosis. They also observed a lower number of fertilized oocytes in these women. Based on these results, they hypothesized that the reduced CYP19A1 gene expression in cumulus cells might partly account for the impaired oocyte quality associated with endometriosis [85].

Sanchez et al., in a recent review, evaluated all the aspects that might affect oocyte quality in endometriosis patients. They observed that, compared to other causes of infertility, endometriosis is consistently associated with a reduced number of mature oocytes retrieved, whereas a reduction in fertilization rates is more likely associated with minimal/mild rather than moderate/severe endometriosis [86].

Several studies have shown that women with endometriosis have significantly lower concentrations of antioxidant components such as vitamin A, vitamin C, and selenium, in their follicular fluid (FF) [87–91], as well as lower concentrations of superoxide dismutase (SOD) and vitamin E in both serum and FF [88, 89]. FF as a location for reflecting the metabolic process surrounding a mature oocyte prior to ovulation plays a critical role in the reproductive performance of oocytes [92].

Prieto et al. evaluated vitamins C and E, malondialdehyde, and superoxide dismutase concentrations in plasma

and FF. They found lower vitamin C (potent natural antioxidant) values in FF from patients with endometriosis compared with unaffected infertile patients. Furthermore, they found a statistically significant negative correlation between plasma vitamin C levels and the number of oocytes retrieved, number of mature oocytes, and number of fertilized oocytes. The authors concluded that the lower concentrations of plasma vitamin C may reflect an excess consumption aimed at neutralizing ROS. They also found a lower superoxide dismutase concentration in the plasma of endometriosis patients compared with controls, which suggests a decreased antioxidant capacity in patients with endometriosis [89].

This increase in OS status in FF has been recently found also in follicles surrounding an endometrioma, as assessed using proteomics by mass spectrometry, and has been proposed to account for spindle disruption [93]. Similarly, in FF of women with both mild and severe endometriosis, Da Broi et al. found increased follicular 8-hydroxy-2′-deoxyguanosine, an indicator of oxidative DNA damage [94, 95].

Recent studies have reported a role of epigenetic modifications in ROS-induced oxidative stress processes leading to development or progression of aging, pancreatitis, fatty liver disease, stroke, diabetes, cancer, and also endometriosis [96–99].

The altered chromatin conformation represents the basis of epigenetic regulation, since the pattern of gene expression undergoes modifications without altering the genomic sequence. Chromatin conformation can be affected by DNA methylation and posttranslational modifications of histones. OS may partly mediate changes in epigenetic marks such as DNA methylation and histone modifications. Endometriotic cells express variable levels of the DNA methyltransferase enzymes (DNMTs), which introduce and maintain DNA methylation on the C5 position of cytosine in CpG dinucleotides [100]. Abnormal DNA methylation in endometriosis affects the expression of several genes, including homeobox A10 (HOXA10), estrogen receptor beta (ESR2), steroidogenic factor 1 (NR5A1), and aromatase (CYP19A1); these, in turn, alter steroid signaling and responsiveness and are critically involved in development and decidualization.

Furthermore, iron oxidation blocks the catalytic activity of the jumonji gene (JMJ, also known as JARID2). The JMJ protein uses Fe^{2+} and αKG as cofactors in an oxidative demethylation reaction via hydroxymethyl lysine. Reactive oxygen species oxidize Fe^{2+} to Fe^{3+}, thereby attenuating the activity of the JMJ histone demethylases [101]. Hydrogen peroxide also inhibits histone demethylase activity where increased Fe^{2+} rescued this inhibition. Another epigenetic enzyme utilizing Fe^{2+} is members of the ten–eleven translocation (TET) family of hydroxylases. The ten–eleven translocation protein is an active CpG demethylase converting 5-methylcytosine to 5-hydroxymethylcytosine [98]. Members of the TET protein family play a role in the DNA methylation process and in gene activation. Ten–eleven translocation genes (TET1, TET2, and TET3) are downregulated in endometriosis [102, 103]. Ten–eleven translocation–mediated DNA demethylation may act as a protection against oxidative stress [104].

3.4. Endometriosis Associated Ovarian Cancer (EAOC). Many epidemiological studies underline a possible association between endometriosis and invasive epithelial ovarian cancer, on the basis of the high prevalence and incidence of epithelial ovarian cancer in women with endometriosis [105–107].

Endometriosis associated ovarian cancers (EAOCs) represent a subclass of ovarian neoplasms with specific clinical characteristics that include histology, FIGO stage, CA125 levels, patient age, menopausal status at diagnosis, and survival outcomes [108].

Studies suggest that the most EAOCs are endometrioid and clear cell subtypes, with endometriosis found in 30–55% of clear cell cancers and 30–40% of endometrioid ovarian cancers [109–113].

A meta-analysis on 28 studies showed that the standardized incidence ratio (SIR, defined as observed cases/expected cases after adjusted for age) for epithelial ovarian cancer in women with surgical or histologically diagnosed endometriosis was 1.43–8.95, with a 1.34 odds ratio (OR). The prevalence of epithelial ovarian cancer in women with endometriosis was 2.0–17.0%, and the prevalence of endometriosis in women with epithelial ovarian cancer was 3.4–52.6% [114].

In a recent review, Kobayashi described the possible conditions that could result in ovarian cancer consequent to endometriosis [115]. The autoxidation of hemoglobin in the extracellular milieu releases heme and iron, inducing cellular oxidative damage by promoting reactive oxygen species formation; this, in turn, results in DNA damage and mutations (ovarian cancer initiation from endometriosis). On the other hand, persistent antioxidant production could favor a protumoral microenvironment, resulting in cancer progression [115].

Several gene mutations have been identified concurrently in endometriosis lesions and in EAOCs.

Many studies have assessed LOH at 10q23.3 (such as loss of heterozygosity (LOH) and the mutations that lead to the functional inactivation of the phosphatase and tensin homolog (PTEN) tumor suppressor gene located on chromosome 10q23.3) and microsatellite instability (MSI) (leading to the functional inactivation of the PTEN gene) in EAOC.

In 2000, Sato et al. were the first to understand that the inactivation and loss of heterozygosity (LOH) of the tumor suppressor gene PTEN (locus 10q23.3) due to mutations were associated with both endometrioid and clear cell carcinomas in endometrial and ovarian cancers [116].

Subsequent research identified inactivation of PTEN as an early event in the malignant transformation of endometriosis to EAOC, possibly accounting for the development of as many as 14–20% of epithelial ovarian cancers [117, 118].

Two independent studies carried out in 2010 showed that clear cell and endometrioid ovarian cancers were due to somatic mutations in AT-rich interaction domain-1A (ARID1A) [119, 120]. In most cases, ARID1A mutations are either frameshift or nonsense mutations, suggesting their role as tumor suppressor gene, and BAF250a—the protein encoded—is part of a multiprotein SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling complex

involved in the regulation of cellular processes including differentiation, proliferation, DNA repair, and tumor suppression [121, 122].

ARID1A mutations were found in 46% (55/119) of ovarian clear cell carcinomas, in 30% (10/33) of endometrioid carcinomas, but in none of serous carcinomas. ARID1A mutations and loss of BAF250a expression were both identified in the tumor and in the contiguous atypical endometriosis but could not be detected in distant endometriotic lesions [119].

ARID1A and PIK3CA mutations were particularly important in the clear cell carcinoma subtype of EAOC. Anglesio et al., using whole-genome and targeted deep sequencing, found concurrent ARID1A and PIK3CA mutations in ovarian clear cell carcinoma and in tumor-adjacent and distant endometriotic lesions, independently of cytological atypia [123].

In a study that included 23 clear cell carcinomas with synchronous putative precursor lesions (i.e., endometriosis adjacent to carcinoma, with or without cytological atypia), as many as 43% (10/23) of ovarian clear cell carcinomas and 90% (9/10) of the coexisting endometriotic epithelium adjacent to the clear cell carcinoma presented PIK3CA gene mutations [124].

Loss of ARID1A and PIK3CA was a common finding in 130 cases of ovarian clear cell carcinoma (56.2% and 45.0%, resp.). Loss of ARID1A was quite frequent (76.9%, 20/26) in clear cell carcinoma with concurrent endometriosis. PIK3CA expression was not related to clinical features or survival of clear cell carcinoma. However, loss of ARID1A, along with low-level HNF-1b expression, was common in patients with cancer recurrence and was correlated with late-stage and worse survival outcome [125].

Another study that included 35 pure-type (73.9% with endometriosis) and 11 mixed-type clear cell carcinomas (45.5% with endometriosis) showed that both ARID1A and p53 were mutually altered in pure-type clear cell carcinoma, at immunohistochemical analysis. Altered expression of p53 in these clear cell carcinomas was associated with significantly worse prognosis than in the case of ARID1A ($P < 0.001$) [126].

Catenin (cadherin-associated protein) beta-1 (CTNNB1) mutations are quite peculiar to ovarian endometrioid carcinoma, at variance with other types of ovarian carcinoma. Mutations in exon 3 of the β-catenin gene have been identified in 60% (21/35) of endometrioid carcinoma; these mutations have also been detected in the coexisting nonatypical (52.4%) and atypical (73.3%) endometriosis, in most cases with identical single-nucleotide substitutions. Conversely, no evidence of these mutations was found in clear cell carcinomas and the coexisting endometriosis [127].

Using an animal model, Wu et al. demonstrated that inactivation of PTEN and β-catenin pathways in the murine surface epithelium resulted in adenocarcinoma formation with similar morphology as human ovarian endometrioid carcinoma [128].

In an interesting report published in 2015, Winarto et al. studied the effects of oxidative stress—purportedly associated with malignant transformation—on the *ARID1A* gene, by assessing its expression in endometriotic, EAOC, and non-EAOC tissue samples, as well as in endometriotic primary cell cultures [129]. They measured oxidative stress through the activity of the antioxidant enzyme manganese dismutase (MnSOD), malondialdehyde (MDA), and *ARID1A* gene expression in tissue samples from patients with endometriosis, EAOC or nonendometriosis-associated ovarian cancer (non-EAOC). In addition, they added H_2O_2 to induce OS in cultured cells from patients affected by primary endometriosis and assessed possible alteration of *ARID1A* gene expression based on different H_2O_2 concentrations. The results showed that in endometriotic tissue, the expression of *ARID1A* mRNA was lower than that in normal endometrial (control) and non-EAOC tissues, whereas its protein expression was lower in controls but higher than EAOC and non-EAOC tissues. These findings provide evidence of an early decrease in *ARID1A* expression, especially in its mRNA in endometriotic tissue. Furthermore, oxidative stress seems to play a role in the decreased expression of the *ARID1A* protein and mRNA levels in endometriotic cells. Apparently, oxidative stress suppresses *ARID1A* expression in endometriotic cells; conversely, the low *ARID1A* gene activity occurring in endometriosis could increase the susceptibility of these lesions to malignant transformation [129] (Table 2).

3.5. Use of Antioxidants in the Treatment of Endometriosis. The identification of OS as a major player in endometriosis pathophysiology has been noted in various studies addressing the influence of OS reduction as treatment goal. To determine the effects of vitamins E and C, as many as 46 women with endometriosis-related pain were given a two-month treatment with vitamin E (1200 IU) and vitamin C (1000 mg) [130]. Vitamin E is a fat-soluble antioxidant that prevents the formation of vitamin E radicals. Vitamin C was also added to this regimen for its action in recycling vitamin E radial to vitamin E. At the end of this randomized controlled trial, 43% of the patients reported a reduction in chronic pelvic pain, suggesting that vitamins E and C administration might lead to a noticeable pain reduction, even in the short term ($P = 0.0055$). Conversely, control patients did not experience any decrease in pain [130]. Santanam et al. attributed the effects of vitamin supplementation to its antioxidative and anti-inflammatory properties, although they did not describe any clear physiological mechanism underlying this effect [130]. Some insight come from Durak et al. who experimentally induced endometriotic cysts in a rat model [131] subsequently treated with differing doses of vitamin C (0.5 mg, 1.25 mg, and 2.5 mg) to determine whether vitamin C supplementation altered lesion volume and weights. At the end of treatment, cystic lesions in the group treated with 2.5 mg vitamin C were significantly reduced in weight and volume [131], suggesting that antioxidants, such as vitamin C, are able to reduce endometriosis symptoms by reducing lesion size.

Mier-Cabrera et al., in a randomized, double-blind trial, treated endometriosis patients with vitamins C and E or a placebo for 6 months and further evaluated the levels of malondialdehyde (MDA) and lipid hydroperoxides (LOOHs) as

Table 2: Major genetic alterations/mutations in different stages of endometriosis associated ovarian cancer (EAOC).

Factor	Genetic alteration	Current data
Tumor suppressor genes	PTEN	Phosphatase and tensin homolog is mutated in many cancers, particularly in endometrial and endometrioid ovarian cancer; its inactivation occurs early during tumorigenesis [102]. PTEN somatic mutations are frequently found in endometriotic cysts [101].
	ARID1A	ARID1A mutations are significantly more common in two ovarian cancer subtypes associated with endometriosis (clear cell and endometrioid). Endometriosis synchronous with ovarian cancer presented more frequent mutations in clones derived from endometriosis samples directly adjacent of the tumor than in those from distant endometriotic lesions [104].
DNA repair	hMLH1	hMLH1 corrects errors in DNA replication; hypermethylation of its promoter occurs early in endometriosis malignant transformation [102].
Loss of heterozygosity (LOH)		A trend to increased LOH frequencies has been reported in solitary endometriosis lesions, endometriosis-associated carcinoma, and endometrioid ovarian cancer. Common LOH events can be identified in endometriosis synchronous with ovarian cancer [101].
	ARID1A and PIK3CA	ARID1A and PIK3CA mutations were found in ovarian clear cell carcinoma and in tumor-adjacent and distant endometriotic lesions, regardless of cytological atypia [112–114].
	ARID1A and p53	Both ARID1A and p53 were mutually altered in pure-type clear cell carcinoma at immunohistochemical analysis. Altered expression of p53 in these clear cell carcinomas was associated with significant worse prognosis than that of ARID1A ($P < 0.001$) [115].
	β-Catenin	*β-Catenin* mutations and overexpression are very common in ovarian endometrioid carcinoma; approximately 50% of endometrioid carcinoma has β-catenin alterations [116]. Endometrioid carcinoma containing *β-catenin* mutations is low grade and associated with better prognosis. As many as 90% of endometrioid borderline tumors harbor *β-catenin* mutations.

peripheral OS markers. Significantly decreased levels of MDA and LOOHs were observed after 4 and 6 months, respectively, confirming that vitamins C and E supplementation is associated with a reduction in OS markers in women diagnosed with endometriosis. However, despite OS marker reduction, pregnancy rate did not improve during or after the intervention [132].

Resveratrol (trans-3,5,40-trihydoxystilbene) is a natural polyphenolic flavonoid synthesized by plants following exposure to ultraviolet radiation. Resveratrol is largely present in seeds and the skin of grapes, in mulberries, and in red wine. Amaya et al. evaluated a possible dose-dependent impact on the endometrium. In addition to its antioxidant properties, resveratrol acts as a phytoestrogen, and its estrogen action appears to be related to the concentrations. At low concentrations, it acts agonistically, whereas at high concentrations, it plays an antagonistic role. As endometriosis is an estrogen-dependent disease, high levels of resveratrol were able to reduce xenograft proliferation of human endometrium in mice [133].

Melatonin is another naturally produced hormone with possible powerful effects on endometriotic lesions. Melatonin has several properties; in addition to its action as free radical scavenger, it stimulates antioxidant production and increases the efficacy of the electron chain function [134, 135]. In humans, it is produced in the pineal gland and has been shown to decrease oxidative damage. To understand the role of melatonin, Yilmaz et al. implanted endometriotic lesions in twenty rats and treated ten with melatonin and ten with a saline solution (control). The outcome measures of this study were changes in lesion volume and weight. In the experimental group, lesion volume ($P < 0.01$) and weight significantly decreased ($P < 0.05$), showing that melatonin is able to induce lesion regression [135].

Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol found in green tea. It has strong antioxidative, antimitotic, and antiangiogenic properties [136]. Leaves of the tea plant *Camellia sinensis*, which contains high nutraceutical values, are employed to prepare green tea. EGCG was demonstrated to affect several carcinogenetic mechanisms such as mutation, cell proliferation, cell invasion, and apoptosis [137]. As some of these mechanisms are common to endometriosis, its effect on this condition has been studied both in vitro and in vivo.

Matsuzaki et al. assessed the in vitro effect of EGCG in endometriosis. Cell samples from 55 endometriosis patients were treated with EGCG and analyzed via RT-PCR, cell proliferation assays, in vitro migration and invasion assays. EGCG significantly reduced proliferation, cell migration, and invasion of endometriotic cells [138]. Although EGCG appears to beneficially affect endometriosis patients, its low bioavailability circumscribed to the ingestion of pure EGCG or green tea consumption limits its therapeutic use [138].

Table 3: The effect of antioxidative stress agent on endometriosis and the suggested mechanism.

Drugs	Authors	Study type	Effects	Mechanism
Vitamins C and E	Santanam et al. [130]	Human clinical study	Decreased pelvic pain, dysmenorrhea, dyspareunia, PF inflammatory markers, normal T-cell expression and secretion, interleukin-6, and monocyte chemotactic protein-1	↓ oxidatively modified lipoproteins
	Durak et al. [131]	Animal study	Reduced endometriotic cyst volume, weight, growth, and natural killer cell content	Antioxidant and immune stimulator, stimulation of leukocyte functions, enhanced NK function
	Mier-Cabrera et al. [132]	Human clinical study	Decreased OS markers: MDA, lipid hydroperoxides	Neutralizing ROS and RNS
Resveratrol	Amaya et al. [133]	Animal study	Reduced expression of ESR1 and proliferative activity (Ki67), agonist and antagonist of estrogen in low and high concentrations, respectively	↓ ESR1 in endometrial epithelium, action through nongenomic action of alternative estrogen receptors such as GPR30
Epigallocatechin-3-gallate	Matsuzaki and Darcha [138]	In vitro + in vivo	Inhibited cell proliferation, migration and invasion of endometrial tissue, decreased fibrotic markers, prevented progression of fibrosis	↓ transforming growth factor-b1-dependent increase in the mRNA expression of fibrotic markers, inhibited activation of MAPK and Smad signaling pathways in endometrial and endometriotic stromal cells
N-acetyl-L-cysteine	Pittaluga et al. [143]	In vitro + animal study	↓ endometrioma mass, reduced immunohistochemical staining of the inflammation-related COX-2 protein, and decreased MMP-9 expression and activity	Switching cell behavior from proliferation toward differentiation and decreased both tissue inflammation and cell invasiveness
	Porpora et al. [145]	Clinical study	↓ endometrioma size, pain reduction, decrease in cell invasive behavior, and ↓ in the inflammatory COX-2	↑ proteins of cell-cell junction complex such as E-cadherin and β-catenin

N-Acetylcysteine (NAC), the acetylated form of the amino acid cysteine, naturally present in some substances like garlic, exerts a marked antiproliferative action in vitro on cancer cells of epithelial origin [139]. The action of NAC has no role in cell death nor is it due to an unspecific toxic effect, rather it stems from a complex differentiation pathway, including the activation of several molecular mechanisms all converging toward a proliferation-to-differentiation switch that implies decreased cell proliferation and decreased cell locomotory behavior, particularly relevant in endometriosis. In addition, inflammatory protein activity and gene expression are also downregulated by NAC [140, 141]. Overall, NAC emerges as a thiol-containing compound working in the complex framework of redox signaling, and its effects go far beyond a generic antioxidant action [142].

In 2010, in an attempt to extrapolate these in vitro findings to a murine model of endometriosis, Pittalunga et al. provided evidence that NAC treatment helped reduce endometrioma size, at the same time decreasing tissue inflammation and cell invasiveness [143]. A similar effect of NAC in decreasing hydrogen peroxide production and cell proliferation was evidenced in cell and animal models of endometriosis, the result being attributed to the regulation of the extracellular regulated kinase ERK1/2 [144].

In 2013, these same authors carried out an observational cohort study on ovarian endometriosis, in an attempt to compare the evolution of ovarian endometriomas in NAC-treated and untreated control patients, with ultrasound measurements of mean lesion diameter and volume. NAC was administered per os for 3 months at a dosage of 600 mg three times daily on three consecutive days per week [145]. After three months, mean cyst diameter in NAC-treated patients was slightly reduced (−1.5 mm) whereas untreated patients experienced a significant increase (+6.6 mm; $P = 0.001$). Particularly, during NAC treatment, the percentage of cysts that were reduced in size outnumbered those whose size had increased. Twenty-four NAC-treated patients (versus 1 within controls) were able to avoid the scheduled laparoscopy as the cysts had decreased/disappeared and/or the relevant pain decreased (21 cases) or there was a pregnancy (1 case). There were eight pregnancies among NAC-treated patients versus six in untreated patients. The authors therefore concluded that NAC actually represents a simple and effective treatment for endometriosis, devoid of side effects, and suitable for women desiring a pregnancy [145] (Table 3).

4. Conclusions

Endometriosis is a chronic disease affecting nearly 10–15% of women of reproductive age, which leads to pain, irregular bleeding, and infertility [146, 147]. The etiology of endometriosis is not clear although general risk factors such as smoking, alcohol use, and low body mass index seem to have a role in its development [1]; in addition, recent studies have identified a possible role for OS and ROS in this condition. In particular, ROS seems to alter endothelial cell permeability and adhesion molecule expression, triggering an inflammatory process. OS substances may contribute to the pathogenesis of endometriosis through the activation of macrophages [47]. Activated macrophages can aggravate oxidative stress conditions through the production of lipid peroxides and other by-products of the reaction between apolipoproteins and peroxides. The sum of these events increases the concentrations of proinflammatory mediators, thus triggering inflammatory conditions in affected women [21–23]. Recent studies have described a cause–effect relationship between epigenetic mechanisms and endometriosis development. In particular, aberrant DNA methylation and histone modification have been associated with an increased risk of endometriosis. Overall, the available literature focuses on the efficacy of antioxidant therapy in the treatment and mitigation of endometriosis.

Conflicts of Interest

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

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References

[1] L. C. Giudice and L. C. Kao, "Endometriosis," *Lancet*, vol. 364, no. 9447, pp. 1789–1799, 2004.

[2] A. S. Laganà, V. L. La Rosa, A. M. C. Rapisarda et al., "Anxiety and depression in patients with endometriosis: impact and management challenges," *International Journal of Women's Health*, vol. 9, pp. 323–330, 2017.

[3] A. S. Laganà, I. Condemi, G. Retto et al., "Analysis of psychopathological comorbidity behind the common symptoms and signs of endometriosis," *European Journal of Obstetrics & Gynecology and Reproductive Biology*, vol. 194, pp. 30–33, 2015.

[4] A. S. Laganà, V. La Rosa, B. Petrosino, and S. G. Vitale, "Comment on "Risk of developing major depression and anxiety disorders among women with endometriosis: a longitudinal follow-up study"," *Journal of Affective Disorders*, vol. 208, pp. 672-673, 2017.

[5] S. G. Vitale, V. L. La Rosa, A. M. C. Rapisarda, and A. S. Laganà, "Comment on: "Impact of endometriosis on quality of life and mental health: pelvic pain makes the difference"," *Journal of Psychosomatic Obstetrics & Gynaecology*, vol. 38, no. 1, pp. 81-82, 2017.

[6] S. G. Vitale, V. L. La Rosa, A. M. C. Rapisarda, and A. S. Laganà, "Impact of endometriosis on quality of life and psychological well-being," *Journal of Psychosomatic Obstetrics & Gynaecology*, vol. 38, no. 4, pp. 317–319, 2017.

[7] S. G. Vitale, B. Petrosino, V. L. La Rosa, A. M. C. Rapisarda, and A. S. Laganà, "A systematic review of the association between psychiatric disturbances and endometriosis," *Journal of Obstetrics and Gynaecology Canada*, vol. 38, no. 12, pp. 1079-1080, 2016.

[8] O. Triolo, A. S. Laganà, and E. Sturlese, "Chronic pelvic pain in endometriosis: an overview," *Journal of Clinical Medicine Research*, vol. 5, no. 3, pp. 153–163, 2013.

[9] C. Chapron, A. Bourret, N. Chopin et al., "Surgery for bladder endometriosis: long-term results and concomitant management of associated posterior deep lesions," *Human Reproduction*, vol. 25, no. 4, pp. 884–889, 2010.

[10] C. Chapron, C. Souza, D. de Ziegler et al., "Smoking habits of 411 women with histologically proven endometriosis and 567 unaffected women," *Fertility and Sterility*, vol. 94, no. 6, pp. 2353–2355, 2010.

[11] S. Butticè, A. S. Laganà, G. Mucciardi et al., "Different patterns of pelvic ureteral endometriosis. What is the best treatment? Results of a retrospective analysis," *Archivio Italiano di Urologia, Andrologia*, vol. 88, no. 4, pp. 266–269, 2016.

[12] A. S. Laganà, S. G. Vitale, M. A. Trovato et al., "Full-thickness excision versus shaving by laparoscopy for intestinal deep infiltrating endometriosis: rationale and potential treatment options," *BioMed Research International*, vol. 2016, Article ID 3617179, 8 pages, 2016.

[13] A. S. Laganà, S. G. Vitale, F. M. Salmeri et al., "Unus pro omnibus, omnes pro uno: a novel, evidence-based, unifying theory for the pathogenesis of endometriosis," *Medical Hypotheses*, vol. 103, pp. 10–20, 2017.

[14] V. Sofo, M. Götte, A. S. Laganà et al., "Correlation between dioxin and endometriosis: an epigenetic route to unravel the pathogenesis of the disease," *Archives of Gynecology and Obstetrics*, vol. 292, no. 5, pp. 973–986, 2015.

[15] J. A. Sampson, "Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity," *American Journal of Obstetrics & Gynecology*, vol. 14, no. 4, pp. 422–469, 1927.

[16] J. Halme, M. G. Hammond, J. F. Hulka, S. G. Raj, and L. M. Talbert, "Retrograde menstruation in healthy women and in patients with endometriosis," *Obstetrics & Gynecology*, vol. 64, no. 2, pp. 151–154, 1984.

[17] P. Santulli, B. Borghese, S. Chouzenoux et al., "Serum and peritoneal interleukin-33 levels are elevated in deeply infiltrating endometriosis," *Human Reproduction*, vol. 27, no. 7, pp. 2001–2009, 2012.

[18] P. Santulli, L. Marcellin, J. C. Noel et al., "Sphingosine pathway deregulation in endometriotic tissues," *Fertility and Sterility*, vol. 97, no. 4, pp. 904–911.e5, 2012.

[19] A. S. Laganà, O. Triolo, F. M. Salmeri et al., "Natural killer T cell subsets in eutopic and ectopic endometrium: a fresh look to a busy corner," *Archives of Gynecology and Obstetrics*, vol. 293, no. 5, pp. 941–949, 2016.

[20] S. E. Bulun, "Endometriosis," *The New England Journal of Medicine*, vol. 360, no. 3, pp. 268–279, 2009.

[21] V. Vetvicka, A. S. Laganà, F. M. Salmeri et al., "Regulation of apoptotic pathways during endometriosis: from the molecular basis to the future perspectives," *Archives of Gynecology and Obstetrics*, vol. 294, no. 5, pp. 897–904, 2016.

[22] L. W. Jackson, E. F. Schisterman, R. Dey-Rao, R. Browne, and D. Armstrong, "Oxidative stress and endometriosis," *Human Reproduction*, vol. 20, no. 7, pp. 2014–2020, 2005.

[23] L. F. P. Carvalho, A. N. Samadder, A. Agarwal, L. F. C. Fernandes, and M. S. Abrão, "Oxidative stress biomarkers in patients with endometriosis: systematic review," *Archives of Gynecology and Obstetrics*, vol. 286, no. 4, pp. 1033–1040, 2012.

[24] A. S. Laganà, E. Sturlese, G. Retto, V. Sofo, and O. Triolo, "Interplay between misplaced Müllerian-derived stem cells and peritoneal immune dysregulation in the pathogenesis of endometriosis," *Obstetrics and Gynecology International*, vol. 2013, Article ID 527041, 20 pages, 2013.

[25] A. Agarwal, S. Gupta, and R. K. Sharma, "Role of oxidative stress in female reproduction," *Reproductive Biology and Endocrinology*, vol. 3, no. 1, p. 28, 2005.

[26] E. de Lamirande and C. Gagnon, "Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects," *Human Reproduction*, vol. 10, Supplement 1, pp. 15–21, 1995.

[27] A. S. Laganà, V. Sofo, F. M. Salmeri et al., "Oxidative stress during ovarian torsion in pediatric and adolescent patients: changing the perspective of the disease," *International Journal of Fertility & Sterility*, vol. 9, no. 4, pp. 416–423, 2016.

[28] R. H. Alul, M. Wood, J. Longo et al., "Vitamin C protects low-density lipoprotein from homocysteine-mediated oxidation," *Free Radical Biology & Medicine*, vol. 34, no. 7, pp. 881–891, 2003.

[29] R. A. Olek, W. Ziolkowski, J. J. Kaczor, L. Greci, J. Popinigis, and J. Antosiewicz, "Antioxidant activity of NADH and its analogue—an in vitro study," *Journal of Biochemistry and Molecular Biology*, vol. 37, no. 4, pp. 416–421, 2004.

[30] A. A. Murphy, N. Santanam, A. J. Morales, and S. Parthasarathy, "Lysophosphatidyl choline, a chemotactic factor for monocytes/T-lymphocytes is elevated in endometriosis," *The Journal of Clinical Endocrinology & Metabolism*, vol. 83, no. 6, pp. 2110–2113, 1998.

[31] A. Van Langendonckt, F. Casanas-Roux, and J. Donnez, "Oxidative stress and peritoneal endometriosis," *Fertility and Sterility*, vol. 77, no. 5, pp. 861–870, 2002.

[32] K. Yamaguchi, M. Mandai, S. Toyokuni et al., "Contents of endometriotic cysts, especially the high concentration of free iron, are a possible cause of carcinogenesis in the cysts through the iron-induced persistent oxidative stress," *Clinical Cancer Research*, vol. 14, no. 1, pp. 32–40, 2008.

[33] S. Defrère, J. C. Lousse, R. González-Ramos, S. Colette, J. Donnez, and A. Van Langendonckt, "Potential involvement of iron in the pathogenesis of peritoneal endometriosis," *Molecular Human Reproduction*, vol. 14, no. 7, pp. 377–385, 2008.

[34] A. Augoulea, A. Alexandrou, M. Creatsa, N. Vrachnis, and I. Lambrinoudaki, "Pathogenesis of endometriosis: the role of genetics, inflammation and oxidative stress," *Archives of Gynecology and Obstetrics*, vol. 286, no. 1, pp. 99–103, 2012.

[35] M. Alizadeh, S. Mahjoub, S. Esmaelzadeh, K. Hajian, Z. Basirat, and M. Ghasemi, "Evaluation of oxidative stress in endometriosis: a case-control study," *Caspian Journal of Internal Medicine*, vol. 6, no. 1, pp. 25–29, 2015.

[36] R. Gazvani and A. Templeton, "Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis," *Reproduction*, vol. 123, no. 2, pp. 217–226, 2002.

[37] T. Rahman, I. Hosen, M. M. T. Islam, and H. U. Shekhar, "Oxidative stress and human health," *Advances in Bioscience and Biotechnology*, vol. 3, no. 7, pp. 997–1019, 2012.

[38] L. Pirdel and M. Pirdel, "Role of iron overload-induced macrophage apoptosis in the pathogenesis of peritoneal endometriosis," *Reproduction*, vol. 147, no. 6, pp. R199–R207, 2014.

[39] A. S. Laganà, F. M. Salmeri, S. G. Vitale, O. Triolo, and M. Götte, "Stem cell trafficking during endometriosis," *Reproductive Sciences*, article 1933719116687661, 2017.

[40] R. Rong, S. Ramachandran, N. Santanam, A. A. Murphy, and S. Parthasarathy, "Induction of monocyte chemotactic protein-1 in peritoneal mesothelial and endometrial cells by oxidized low-density lipoprotein and peritoneal fluid from women with endometriosis," *Fertility and Sterility*, vol. 78, no. 4, pp. 843–848, 2002.

[41] J. Kao, B. S. Rosenstein, S. Peters, M. T. Milano, and S. J. Kron, "Cellular response to DNA damage," *Annals of the New York Academy of Sciences*, vol. 1066, no. 1, pp. 243–258, 2005.

[42] A. Agarwal, A. Aponte-Mellado, B. J. Premkumar, A. Shaman, and S. Gupta, "The effects of oxidative stress on female reproduction: a review," *Reproductive Biology and Endocrinology*, vol. 10, no. 1, p. 49, 2012.

[43] J. D. Morrow, K. E. Hill, R. F. Burk, T. M. Nammour, K. F. Badr, and L. J. Roberts, "A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 23, pp. 9383–9387, 1990.

[44] P. Montuschi, P. J. Barnes, and L. J. Roberts II, "Isoprostanes: markers and mediators of oxidative stress," *The FASEB Journal*, vol. 18, no. 15, pp. 1791–1800, 2004.

[45] J. D. Morrow, J. A. Awad, H. J. Boss, I. A. Blair, and L. J. Roberts, "Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 22, pp. 10721–10725, 1992.

[46] D. Pavlovic, V. Dordevic, and G. Kocic, "A "cross-talk" between oxidative stress and redox cell signaling," *Medical Biology*, vol. 9, pp. 131–137, 2002.

[47] S. Basu, "Isoprostanes: novel bioactive products of lipid peroxidation," *Free Radical Research*, vol. 38, no. 2, pp. 105–122, 2004.

[48] G. Polak, I. Wertel, B. Barczyński, W. Kwaśniewski, W. Bednarek, and J. Kotarski, "Increased levels of oxidative stress markers in the peritoneal fluid of women with endometriosis," *European Journal of Obstetrics & Gynecology and Reproductive Biology*, vol. 168, no. 2, pp. 187–190, 2013.

[49] P. Santulli, S. Chouzenoux, M. Fiorese et al., "Protein oxidative stress markers in peritoneal fluids of women with deep infiltrating endometriosis are increased," *Human Reproduction*, vol. 30, no. 1, pp. 49–60, 2015.

[50] A. Augoulea, G. Mastorakos, I. Lambrinoudaki, G. Christodoulakos, and G. Creatsas, "The role of the oxidative-stress in the endometriosis-related infertility," *Gynecological Endocrinology*, vol. 25, no. 2, pp. 75–81, 2009.

[51] D. Vinatier, M. Cosson, and P. Dufour, "Is endometriosis an endometrial disease?," *European Journal of Obstetrics & Gynecology and Reproductive Biology*, vol. 91, no. 2, pp. 113–125, 2000.

[52] D. J. Oosterlynck, F. J. Cornillie, M. Waer, M. Vandeputte, and P. R. Koninckx, "Women with endometriosis show a defect in natural killer activity resulting in a decreased cytotoxicity to autologous endometrium," *Fertility and Sterility*, vol. 56, no. 1, pp. 45–51, 1991.

[53] D. J. Oosterlynck, C. Meuleman, M. Waer, P. R. Koninckx, and M. Vandeputte, "Immunosuppressive activity of peritoneal fluid in women with endometriosis," *Obstetrics & Gynecology*, vol. 82, no. 2, pp. 206–212, 1993.

[54] H. Kanzaki, H. S. Wang, M. Kariya, and T. Mori, "Suppression of natural killer cell activity by sera from patients with endometriosis," *American Journal of Obstetrics & Gynecology*, vol. 167, no. 1, pp. 257–261, 1992.

[55] H. N. Ho, M. Y. Wu, and Y. S. Yang, "Peritoneal cellular immunity and endometriosis," *American Journal of Reproductive Immunology*, vol. 38, no. 6, pp. 400–412, 1997.

[56] K. N. Khan, H. Masuzaki, A. Fujishita, M. Kitajima, I. Sekine, and T. Ishimaru, "Differential macrophage infiltration in early and advanced endometriosis and adjacent peritoneum," *Fertility and Sterility*, vol. 81, no. 3, pp. 652–661, 2004.

[57] E. Seli and A. Arici, "Endometriosis: interaction of immune and endocrine systems," *Seminars in Reproductive Medicine*, vol. 21, no. 2, pp. 135–144, 2003.

[58] D. I. Lebovic, M. D. Mueller, and R. N. Taylor, "Immunobiology of endometriosis," *Fertility and Sterility*, vol. 75, no. 1, pp. 1–10, 2001.

[59] N. Sidell, S. W. Han, and S. Parthasarathy, "Regulation and modulation of abnormal immune responses in endometriosis," *Annals of the New York Academy of Sciences*, vol. 955, no. 1, pp. 159–173, 2002.

[60] Practice Committee of the American Society for Reproductive Medicine, "Endometriosis and infertility: a committee opinion," *Fertility and Sterility*, vol. 98, no. 3, pp. 591–598, 2012.

[61] N. G. Mahutte and A. Arici, "New advances in the understanding of endometriosis related infertility," *Journal of Reproductive Immunology*, vol. 55, no. 1-2, pp. 73–83, 2002.

[62] S. Marcoux, R. Maheux, S. Bérubé, and the Canadian Collaborative Group on Endometriosis, "Laparoscopic surgery in infertile women with minimal or mild endometriosis," *The New England Journal of Medicine*, vol. 337, no. 4, pp. 217–222, 1997.

[63] F. Parazzini, "Ablation of lesions or no treatment in minimal-mild endometriosis in infertile women: a randomized trial. Gruppo Italiano per lo Studio dell'Endometriosi," *Human Reproduction*, vol. 14, no. 5, pp. 1332–1334, 1999.

[64] C. Simon, A. Gutierrez, A. Vidal et al., "Outcome of patients with endometriosis in assisted reproduction: results from in-vitro fertilization and oocyte donation," *Human Reproduction*, vol. 9, no. 4, pp. 725–729, 1994.

[65] G. Mansour, A. Agarwal, E. Radwan, R. Sharma, J. Goldberg, and T. Falcone, "DNA damage in metaphase II oocytes is induced by peritoneal fluid from endometriosis patients," *Fertility and Sterility*, vol. 88, article S299, 2007.

[66] G. Mansour, H. Abdelrazik, R. K. Sharma, E. Radwan, T. Falcone, and A. Agarwal, "L-carnitine supplementation reduces oocyte cytoskeleton damage and embryo apoptosis induced by incubation in peritoneal fluid from patients with endometriosis," *Fertility and Sterility*, vol. 91, no. 5, pp. 2079–2086, 2009.

[67] I. D. Barcelos, R. C. Vieira, E. M. Ferreira, W. P. Martins, R. A. Ferriani, and P. A. Navarro, "Comparative analysis of the spindle and chromosome configurations of in vitro–matured oocytes from patients with endometriosis and from control subjects: a pilot study," *Fertility and Sterility*, vol. 92, no. 5, pp. 1749–1752, 2009.

[68] S. Assou, T. Anahory, V. Pantesco et al., "The human cumulus–oocyte complex gene-expression profile," *Human Reproduction*, vol. 21, no. 7, pp. 1705–1719, 2006.

[69] S. Assou, D. Haouzi, K. Mahmoud et al., "A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study," *Molecular Human Reproduction*, vol. 14, no. 12, pp. 711–719, 2008.

[70] S. Assou, D. Haouzi, J. De Vos, and S. Hamamah, "Human cumulus cells as biomarkers for embryo and pregnancy outcomes," *Molecular Human Reproduction*, vol. 16, no. 8, pp. 531–538, 2010.

[71] S. Gasca, F. Pellestor, S. Assou et al., "Identifying new human oocyte marker genes: a microarray approach," *Reproductive BioMedicine Online*, vol. 14, no. 2, pp. 175–183, 2007.

[72] Z. G. Ouandaogo, D. Haouzi, S. Assou et al., "Human cumulus cells molecular signature in relation to oocyte nuclear maturity stage," *PLoS One*, vol. 6, no. 11, article e27179, 2011.

[73] Z. G. Ouandaogo, N. Frydman, L. Hesters et al., "Differences in transcriptomic profiles of human cumulus cells isolated from oocytes at GV, MI and MII stages after in vivo and in vitro oocyte maturation," *Human Reproduction*, vol. 27, no. 8, pp. 2438–2447, 2012.

[74] E. Y. Adashi, "Endocrinology of the ovary," *Human Reproduction*, vol. 9, no. 5, pp. 815–827, 1994.

[75] R. Buccione, A. C. Schroeder, and J. J. Eppig, "Interactions between somatic cells and germ cells throughout mammalian oogenesis," *Biology of Reproduction*, vol. 43, no. 4, pp. 543–547, 1990.

[76] J. J. Eppig, K. Wigglesworth, and F. L. Pendola, "The mammalian oocyte orchestrates the rate of ovarian follicular development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 5, pp. 2890–2894, 2002.

[77] R. B. Gilchrist, L. J. Ritter, and D. T. Armstrong, "Oocyte–somatic cell interactions during follicle development in mammals," *Animal Reproduction Science*, vol. 82-83, pp. 431–446, 2004.

[78] S. Makabe, T. Naguro, and T. Stallone, "Oocyte–follicle cell interactions during ovarian follicle development, as seen by high resolution scanning and transmission electron microscopy in humans," *Microscopy Research & Technique*, vol. 69, no. 6, pp. 436–449, 2006.

[79] S. Senbon, Y. Hirao, and T. Miyano, "Interactions between the oocyte and surrounding somatic cells in follicular development: lessons from in vitro culture," *The Journal of Reproduction and Development*, vol. 49, no. 4, pp. 259–269, 2003.

[80] M. A. Sirard, F. Richard, P. Blondin, and C. Robert, "Contribution of the oocyte to embryo quality," *Theriogenology*, vol. 65, no. 1, pp. 126–136, 2006.

[81] F. A. M. de Loos, M. M. Bevers, S. J. Dieleman, and T. A. M. Kruip, "Morphology of preovulatory bovine follicles as related to oocyte maturation," *Theriogenology*, vol. 35, no. 3, pp. 527–535, 1991.

[82] T. Fair, "Follicular oocyte growth and acquisition of developmental competence," *Animal Reproduction Science*, vol. 78, no. 3-4, pp. 203–216, 2003.

[83] R. J. Webb, H. Bains, C. Cruttwell, and J. Carroll, "Gap-junctional communication in mouse cumulus-oocyte complexes: implications for the mechanism of meiotic maturation," *Reproduction*, vol. 123, no. 1, pp. 41–52, 2002.

[84] C. R. Harlow, D. J. Cahill, L. A. Maile et al., "Reduced preovulatory granulosa cell steroidogenesis in women with endometriosis," *The Journal of Clinical Endocrinology & Metabolism*, vol. 81, no. 1, pp. 426–429, 1996.

[85] I. D. E. S. Barcelos, F. C. Donabella, C. P. Ribas et al., "Down-regulation of the *CYP19A1* gene in cumulus cells of infertile women with endometriosis," *Reproductive BioMedicine Online*, vol. 30, no. 5, pp. 532–541, 2015.

[86] A. M. Sanchez, V. S. Vanni, L. Bartiromo et al., "Is the oocyte quality affected by endometriosis? A review of the literature," *Journal of Ovarian Research*, vol. 10, no. 1, p. 43, 2017.

[87] A. K. Singh, R. Chattopadhyay, B. Chakravarty, and K. Chaudhury, "Markers of oxidative stress in follicular fluid of women with endometriosis and tubal infertility undergoing IVF," *Reproductive Toxicology*, vol. 42, pp. 116–124, 2013.

[88] F. Liu, L. He, Y. Liu, Y. Shi, and H. Du, "The expression and role of oxidative stress markers in the serum and follicular fluid of patients with endometriosis," *Clinical and Experimental Obstetrics & Gynecology*, vol. 40, no. 3, pp. 372–376, 2013.

[89] L. Prieto, J. F. Quesada, O. Cambero et al., "Analysis of follicular fluid and serum markers of oxidative stress in women with infertility related to endometriosis," *Fertility and Sterility*, vol. 98, no. 1, pp. 126–130, 2012.

[90] M. Vignali, M. Infantino, R. Matrone et al., "Endometriosis: novel etiopathogenetic concepts and clinical perspectives," *Fertility and Sterility*, vol. 78, no. 4, pp. 665–678, 2002.

[91] A. A. Murphy, W. Palinski, S. Rankin, A. J. Morales, and S. Parthasarathy, "Evidence for oxidatively modified lipid-protein complexes in endometrium and endometriosis," *Fertility and Sterility*, vol. 69, no. 6, pp. 1092–1094, 1998.

[92] E. Somigliana, P. Viganò, G. B. La Sala et al., "Follicular fluid as a favourable environment for endometrial and endometriotic cell growth in vitro," *Human Reproduction*, vol. 16, no. 6, pp. 1076–1080, 2001.

[93] T. Regiani, F. B. Cordeiro, L. V. T. da Costa et al., "Follicular fluid alterations in endometriosis: label-free proteomics by MS^E as a functional tool for endometriosis," *Systems Biology in Reproductive Medicine*, vol. 61, no. 5, pp. 263–276, 2015.

[94] M. G. Da Broi, F. O. de Albuquerque, A. Z. de Andrade, R. L. Cardoso, A. A. Jordão Junior, and P. A. Navarro, "Increased concentration of 8-hydroxy-2′-deoxyguanosine in follicular fluid of infertile women with endometriosis," *Cell and Tissue Research*, vol. 366, no. 1, pp. 231–242, 2016.

[95] M. G. Da Broi and P. A. Navarro, "Oxidative stress and oocyte quality: ethiopathogenic mechanisms of minimal/mild endometriosis-related infertility," *Cell and Tissue Research*, vol. 364, no. 1, pp. 1–7, 2016.

[96] H. Zhao, Z. Han, X. Ji, and Y. Luo, "Epigenetic regulation of oxidative stress in ischemic stroke," *Aging and Disease*, vol. 7, no. 3, pp. 295–306, 2016.

[97] Å. K. Hedman, M. Zilmer, J. Sundström, L. Lind, and E. Ingelsson, "DNA methylation patterns associated with oxidative stress in an ageing population," *BMC Medical Genomics*, vol. 9, no. 1, p. 72, 2016.

[98] H. Kobayashi, S. Imanaka, H. Nakamura, and A. Tsuji, "Understanding the role of epigenomic, genomic and genetic alterations in the development of endometriosis (Review)," *Molecular Medicine Reports*, vol. 9, no. 5, pp. 1483–1505, 2014.

[99] F. Ito, Y. Yamada, A. Shigemitsu et al., "Role of oxidative stress in epigenetic modification in endometriosis," *Reproductive Sciences*, vol. 24, no. 11, pp. 1493–1502, 2017.

[100] S. Houshdaran, C. R. Nezhat, K. C. Vo, Z. Zelenko, J. C. Irwin, and L. C. Giudice, "Aberrant endometrial DNA methylome

and associated gene expression in women with endometriosis," *Biology of Reproduction*, vol. 95, no. 5, p. 93, 2016.

[101] Y. Niu, T. L. DesMarais, Z. Tong, Y. Yao, and M. Costa, "Oxidative stress alters global histone modification and DNA methylation," *Free Radical Biology & Medicine*, vol. 82, pp. 22–28, 2015.

[102] Y. Li, G. Gorelik, F. M. Strickland, and B. C. Richardson, "Oxidative stress, T cell DNA methylation, and lupus," *Arthritis & Rhematology*, vol. 66, no. 6, pp. 1574–1582, 2014.

[103] F. J. Roca, H. A. Loomans, A. T. Wittman, C. J. Creighton, and S. M. Hawkins, "Ten-eleven translocation genes are downregulated in endometriosis," *Current Molecular Medicine*, vol. 16, no. 3, pp. 288–298, 2016.

[104] Y. J. Xin, B. Yuan, B. Yu et al., "Tet1-mediated DNA demethylation regulates neuronal cell death induced by oxidative stress," *Scientific Reports*, vol. 5, no. 1, p. 7645, 2015.

[105] National Cancer Institute, "SEER cancer statistics review (CSR) 1975–2014," April 2017, https://seer.cancer.gov/csr/1975_2014.

[106] H. S. Kim, T. H. Kim, H. H. Chung, and Y. S. Song, "Risk and prognosis of ovarian cancer in women with endometriosis: a meta-analysis," *British Journal of Cancer*, vol. 110, no. 7, pp. 1878–1890, 2014.

[107] C. L. Pearce, C. Templeman, M. A. Rossing et al., "Association between endometriosis and risk of histological subtypes of ovarian cancer: a pooled analysis of case–control studies," *The Lancet Oncology*, vol. 13, no. 4, pp. 385–394, 2012.

[108] A. Kondi-Pafiti, E. Papakonstantinou, C. Iavazzo, C. Grigoriadis, N. Salakos, and O. Gregoriou, "Clinicopathological characteristics of ovarian carcinomas associated with endometriosis," *Archives of Gynecology and Obstetrics*, vol. 285, no. 2, pp. 479–483, 2012.

[109] L. A. Brinton, L. C. Sakoda, M. E. Sherman et al., "Relationship of benign gynecologic diseases to subsequent risk of ovarian and uterine tumors," *Cancer Epidemiology Biomarkers & Prevention*, vol. 14, no. 12, pp. 2929–2935, 2005.

[110] S. Komiyama, D. Aoki, E. Tominaga, N. Susumu, Y. Udagawa, and S. Nozawa, "Prognosis of Japanese patients with ovarian clear cell carcinoma associated with pelvic endometriosis: clinicopathologic evaluation," *Gynecologic Oncology*, vol. 72, no. 3, pp. 342–346, 1999.

[111] S. Wang, L. Qiu, J. H. Lang et al., "Clinical analysis of ovarian epithelial carcinoma with coexisting pelvic endometriosis," *American Journal of Obstetrics & Gynecology*, vol. 208, no. 5, pp. 413.e1–413.e5, 2013.

[112] S. Noli, S. Cipriani, G. Scarfone et al., "Long term survival of ovarian endometriosis associated clear cell and endometrioid ovarian cancers," *International Journal of Gynecological Cancer*, vol. 23, no. 2, pp. 244–248, 2013.

[113] L. Qiu, S. Wang, J. H. Lang et al., "The occurrence of endometriosis with ovarian carcinomas is not purely coincidental," *European Journal of Obstetrics & Gynecology and Reproductive Biology*, vol. 170, no. 1, pp. 225–228, 2013.

[114] L. N. Heidemann, D. Hartwell, C. H. Heidemann, and K. M. Jochumsen, "The relation between endometriosis and ovarian cancer—a review," *Acta Obstetricia et Gynecologica Scandinavica*, vol. 93, no. 1, pp. 20–31, 2014.

[115] H. Kobayashi, "Potential scenarios leading to ovarian cancer arising from endometriosis," *Redox Report*, vol. 21, no. 3, pp. 119–126, 2016.

[116] N. Sato, H. Tsunoda, M. Nishida et al., "Loss of heterozygosity on 10q23.3 and mutation of the tumor suppressor gene *PTEN* in benign endometrial cyst of the ovary: possible sequence progression from benign endometrial cyst to endometrioid carcinoma and clear cell carcinoma of the ovary," *Cancer Research*, vol. 60, no. 24, pp. 7052–7056, 2000.

[117] M. Martini, M. Ciccarone, G. Garganese et al., "Possible involvement of *hMLH1*, $p16^{INK4a}$ and *PTEN* in the malignant transformation of endometriosis," *International Journal of Cancer*, vol. 102, no. 4, pp. 398–406, 2002.

[118] D. M. Dinulescu, T. A. Ince, B. J. Quade, S. A. Shafer, D. Crowley, and T. Jacks, "Role of *K-ras* and *Pten* in the development of mouse models of endometriosis and endometrioid ovarian cancer," *Nature Medicine*, vol. 11, no. 1, pp. 63–70, 2005.

[119] K. C. Wiegand, S. P. Shah, O. M. al-Agha et al., "*ARID1A* mutations in endometriosis-associated ovarian carcinomas," *The New England Journal of Medicine*, vol. 363, no. 16, pp. 1532–1543, 2010.

[120] S. Jones, T. L. Wang, I. M. Shih et al., "Frequent mutations of chromatin remodeling gene *ARID1A* in ovarian clear cell carcinoma," *Science*, vol. 330, no. 6001, pp. 228–231, 2010.

[121] D. Reisman, S. Glaros, and E. A. Thompson, "The SWI/SNF complex and cancer," *Oncogene*, vol. 28, no. 14, pp. 1653–1668, 2009.

[122] T. L. Mao, L. Ardighieri, A. Ayhan et al., "Loss of ARID1A expression correlates with stages of tumor progression in uterine endometrioid carcinoma," *The American Journal of Surgical Pathology*, vol. 37, no. 9, pp. 1342–1348, 2013.

[123] M. S. Anglesio, A. Bashashati, Y. K. Wang et al., "Multifocal endometriotic lesions associated with cancer are clonal and carry a high mutation burden," *The Journal of Pathology*, vol. 236, no. 2, pp. 201–209, 2015.

[124] S. Yamamoto, H. Tsuda, M. Takano, K. Iwaya, S. Tamai, and O. Matsubara, "*PIK3CA* mutation is an early event in the development of endometriosis-associated ovarian clear cell adenocarcinoma," *The Journal of Pathology*, vol. 225, no. 2, pp. 189–194, 2011.

[125] S. Ye, J. Yang, Y. You et al., "Clinicopathologic significance of HNF-1ß, AIRD1A, and PIK3CA expression in ovarian clear cell carcinoma: a tissue microarray study of 130 cases," *Medicine*, vol. 95, no. 9, article e3003, 2016.

[126] Y. Matsuo, H. Tashiro, H. Yanai, T. Moriya, and H. Katabuchi, "Clinicopathological heterogeneity in ovarian clear cell adenocarcinoma: a study on individual therapy practice," *Medical Molecular Morphology*, vol. 48, no. 3, pp. 146–154, 2015.

[127] T. Matsumoto, M. Yamazaki, H. Takahashi et al., "Distinct β-*catenin* and *PIK3CA* mutation profiles in endometriosis-associated ovarian endometrioid and clear cell carcinomas," *American Journal of Clinical Pathology*, vol. 144, no. 3, pp. 452–463, 2015.

[128] R. Wu, N. Hendrix-Lucas, R. Kuick et al., "Mouse model of human ovarian endometrioid adenocarcinoma based on somatic defects in the Wnt/β-catenin and PI3K/Pten signaling pathways," *Cancer Cell*, vol. 11, no. 4, pp. 321–333, 2007.

[129] H. Winarto, M. I. Tan, M. Sadikin, and S. I. Wanandi, "*ARID1A* Expression is down-regulated by oxidative stress in endometriosis and endometriosis-associated ovarian cancer," *Translational Oncogenomics*, vol. 9, 2017.

[130] N. Santanam, N. Kavtaradze, A. Murphy, C. Dominguez, and S. Parthasarathy, "Antioxidant supplementation reduces endometriosis-related pelvic pain in humans," *Translational Research*, vol. 161, no. 3, pp. 189–195, 2013.

[131] Y. Durak, A. Kokcu, M. Kefeli, D. Bildircin, H. Çelik, and T. Alper, "Effect of vitamin C on the growth of experimentally induced endometriotic cysts," *The Journal of Obstetrics and Gynaecology Research*, vol. 39, no. 7, pp. 1253–1258, 2013.

[132] J. Mier-Cabrera, M. Genera-García, J. de la Jara-Díaz, O. Perichart-Perera, F. Vadillo-Ortega, and C. Hernández-Guerrero, "Effect of vitamins C and E supplementation on peripheral oxidative stress markers and pregnancy rate in women with endometriosis," *International Journal of Gynaecology & Obstetrics*, vol. 100, no. 3, pp. 252–256, 2008.

[133] S. C. Amaya, R. F. Savaris, C. J. Filipovic et al., "Resveratrol and endometrium: a closer look at an active ingredient of red wine using in vivo and in vitro models," *Reproductive Sciences*, vol. 21, no. 11, pp. 1362–1369, 2014.

[134] R. J. Reiter, D. X. Tan, J. C. Mayo, R. M. Sainz, J. Leon, and Z. Czarnocki, "Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans," *Acta Biochimica Polonica*, vol. 50, no. 4, pp. 1129–1146, 2003.

[135] B. Yilmaz, S. Kilic, O. Aksakal et al., "Melatonin causes regression of endometriotic implants in rats by modulating angiogenesis, tissue levels of antioxidants and matrix metalloproteinases," *Archives of Gynecology and Obstetrics*, vol. 292, no. 1, pp. 209–216, 2015.

[136] D. G. Nagle, D. Ferreira, and Y. D. Zhou, "Epigallocatechin-3-gallate (EGCG): chemical and biomedical perspectives," *Phytochemistry*, vol. 67, no. 17, pp. 1849–1855, 2006.

[137] L. A. Beltz, D. K. Bayer, A. L. Moss, and I. M. Simet, "Mechanisms of cancer prevention by green and black tea polyphenols," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 6, no. 5, pp. 389–406, 2006.

[138] S. Matsuzaki and C. Darcha, "Antifibrotic properties of epigallocatechin-3-gallate in endometriosis," *Human Reproduction*, vol. 29, no. 8, pp. 1677–1687, 2014.

[139] T. Parasassi, R. Brunelli, L. Bracci-Laudiero et al., "Differentiation of normal and cancer cells induced by sulfhydryl reduction: biochemical and molecular mechanisms," *Cell Death & Differentiation*, vol. 12, no. 10, pp. 1285–1296, 2005.

[140] A. C. Gustafsson, I. Kupershmidt, E. Edlundh-Rose et al., "Global gene expression analysis in time series following *N*-acetyl L-cysteine induced epithelial differentiation of human normal and cancer cells in vitro," *BMC Cancer*, vol. 5, no. 1, p. 75, 2005.

[141] E. Edlundh-Rose, I. Kupershmidt, A. C. Gustafsson et al., "Gene expression analysis of human epidermal keratinocytes after *N*-acetyl L-cysteine treatment demonstrates cell cycle arrest and increased differentiation," *Pathobiology*, vol. 72, no. 4, pp. 203–212, 2005.

[142] T. Parasassi, R. Brunelli, G. Costa et al., "Thiol redox transitions in cell signaling: a lesson from *N*-acetylcysteine," *The Scientific World Journal*, vol. 10, pp. 1192–1202, 2010.

[143] E. Pittaluga, G. Costa, E. Krasnowska et al., "More than antioxidant: *N*-acetyl-L-cysteine in a murine model of endometriosis," *Fertility and Sterility*, vol. 94, no. 7, pp. 2905–2908, 2010.

[144] C. Ngô, C. Chéreau, C. Nicco, B. Weill, C. Chapron, and F. Batteux, "Reactive oxygen species controls endometriosis progression," *The American Journal of Pathology*, vol. 175, no. 1, pp. 225–234, 2009.

[145] M. G. Porpora, R. Brunelli, G. Costa et al., "A promise in the treatment of endometriosis: an observational cohort study on ovarian endometrioma reduction by *N*-acetylcysteine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 240702, 7 pages, 2013.

[146] A. S. Laganà, S. G. Vitale, R. Granese et al., "Clinical dynamics of Dienogest for the treatment of endometriosis: from bench to bedside," *Expert Opinion on Drug Metabolism & Toxicology*, vol. 13, no. 6, pp. 593–596, 2017.

[147] S. G. Vitale, V. L. La Rosa, A. M. C. Rapisarda, and A. S. Laganà, "Endometriosis and infertility: the impact on quality of life and mental health," *Journal of Endometriosis and Pelvic Pain Disorders*, vol. 9, no. 2, pp. 112–115, 2017.

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Review Article

The Role of Stress-Induced O-GlcNAc Protein Modification in the Regulation of Membrane Transport

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O-linked N-acetylglucosamine (O-GlcNAc) is a posttranslational modification that is increasingly recognized as a signal transduction mechanism. Unlike other glycans, O-GlcNAc is a highly dynamic and reversible process that involves the addition and removal of a single N-acetylglucosamine molecule to Ser/Thr residues of proteins. UDP-GlcNAc—the direct substrate for O-GlcNAc modification—is controlled by the rate of cellular metabolism, and thus O-GlcNAc is dependent on substrate availability. Serving as a feedback mechanism, O-GlcNAc influences the regulation of insulin signaling and glucose transport. Besides nutrient sensing, O-GlcNAc was also implicated in the regulation of various physiological and pathophysiological processes. Due to improvements of mass spectrometry techniques, more than one thousand proteins were detected to carry the O-GlcNAc moiety; many of them are known to participate in the regulation of metabolites, ions, or protein transport across biological membranes. Recent studies also indicated that O-GlcNAc is involved in stress adaptation; overwhelming evidences suggest that O-GlcNAc levels increase upon stress. O-GlcNAc elevation is generally considered to be beneficial during stress, although the exact nature of its protective effect is not understood. In this review, we summarize the current data regarding the oxidative stress-related changes of O-GlcNAc levels and discuss the implications related to membrane trafficking.

1. Introduction

The function and impact of protein O-linked N-acetylglucosamine (O-GlcNAc) modification are very complex and only partially discovered despite almost 1300 scientific studies were published in the last 30 years. Although a relatively simple molecular mechanism (the addition and removal of N-acetylglucosamine on Ser/Thr residues), it is a focal point of numerous converging and diverging cellular events. One of the most profound properties of O-GlcNAc is that it is directly embedded in the metabolic regulation of the cells [1]. Since metabolism is basically required for and influences every other cellular function, the role of O-GlcNAc to mediate signals to and from metabolic systems seems to be an obvious choice for nature. Indeed, several studies showed that both increased (e.g., in diabetes) and decreased (fasting) glucose metabolism have an impact on O-GlcNAc [2–6]. Consequently, O-GlcNAc directly influences various regulatory systems, such as the transcriptional machinery, protein synthesis, trafficking and degradation, and regulation of glucose uptake [7, 8]. Glucose metabolism was the most studied element in this respect, but the involvement of other metabolic pathways (nucleotide synthesis, amino acid, and lipid metabolism) was also proposed [1].

Despite difficulties in discerning cause and effect (e.g., malignant cells may develop altered metabolic rate and/or O-GlcNAc levels independently), it appears that besides metabolic challenges, regular cellular events such as mitosis, cell differentiation, and response to a hormonal signal or cell-cell adhesion may also directly influence O-GlcNAc modifications on proteins [9–13]. Moreover, a wide variety of stressors, including osmotic challenge, hyperthermia, heavy ion toxicity, hypoxia, and oxidative stress, was also shown to impact O-GlcNAc [14–17]. The most comprehensive data available were provided by studies done on cardiomyocytes under ischemic or oxidative conditions. The majority of these studies showed that elevation of O-GlcNAc prevented or at

least ameliorated the damage caused by the stress. Several mechanisms were proposed to explain the protective effect of O-GlcNAc under stress situation, such as increased heat shock protein synthesis, inhibition of protein degradation, inhibition of apoptosis, and modulation of calcium homeostasis [18]. Although membrane transport is involved in many of these mechanisms and several studies demonstrated evidences concerning O-GlcNAc's influence on membrane trafficking (Table 1), a comprehensive understanding of this interaction is missing. In this review, we summarize our current understanding of the intracellular process called O-GlcNAc modification, its adaptive response regarding oxidative stress, and its influence on membrane traffic, including glucose and ion transport and also synaptic, nuclear, and mitochondrial transport.

2. Regulation of Proteins by O-GlcNAc

O-linked N-acetylglucosamine (or O-GlcNAc) is a reversible, dynamic posttranslational modification (PTM) affecting serine and threonine residues of proteins. It was first discovered in 1984 by Torres and Hart [19]. The set of O-GlcNAc targets includes around 1500 proteins which are located both in the nucleus, the cytoplasm, and the mitochondria of the cells [20]. Likewise, several membrane proteins were found to be O-GlcNAcylated on their intracellular domain, for example, inositol 1,4,5-trisphosphate (InsP3) receptor type I, beta-amyloid precursor protein (APP), or epidermal growth factor receptor (EGFR) [21–23]. Interestingly, recent discoveries showed that even extracellular domains can carry the O-GlcNAc modification [24]; however, the latter seems to be irreversible and controlled by a different enzyme (EGF repeat-specific O-GlcNAc transferase termed EOGT) than cytoplasmic O-GlcNAc modification.

O-GlcNAc cycling is controlled by the action of O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), the enzymes that add and remove O-GlcNAc, respectively. The substrate of the transferase reaction is the uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), the product of the nutrient-sensitive hexosamine biosynthetic pathway (HBP). Approximately, 1–3% of total glucose is entering this pathway which integrates glucose, amino acid, fatty acid, and nucleotide metabolism [1]. Using UDP-GlcNAc, OGT attaches a single O-linked N-acetylglucosamine (O-GlcNAc) moiety to Ser or Thr residues of proteins (Figure 1).

O-GlcNAc modification may influence the proteins' functions [7, 25, 26], protect from degradation [27, 28], influence protein-protein interaction or localization [29–32], and possibly alter protein hydrophobicity [17]. The most studied effect is its competition with phosphorylation, since O-GlcNAc can occupy the same residues as phosphorylation. However, other interactions such as proximal site competition and proximal site occupation were also proposed [25]. O-GlcNAc also influences protein synthesis via modulation of the action of transcription factors such as c-myc, NFκB, and p53 [33–37]. It is estimated that around 25% of the O-GlcNAc-modified proteins are involved in transcriptional regulation [38]. Mechanistically, O-GlcNAcylation can affect the translocation, DNA binding, transactivation and stability of transcription factors. Moreover, O-GlcNAcylation regulates protein synthesis also by cotranslational glycosylation which protects nascent polypeptide chains from ubiquitination [28]. This interplay with ubiquitination also has a general impact on protein stability and turnover by reducing proteasome degradation [27, 39]. O-GlcNAc plays a role in protein folding and unfolded protein response as well [40, 41].

O-GlcNAc modification is abundantly present in higher eukaryotes, and it is required for the normal functions of the cells [42, 43]. It influences several cellular processes, including nutrient sensing, cell cycle regulation, transcriptional regulation, Ca^{2+} handling, cytoskeletal organization, or nuclear translocation [1, 7, 25, 44–48]. Naturally, O-GlcNAc's involvement in pathophysiological processes was soon proposed. Its reciprocal relationship with phosphorylation was best characterized in Alzheimer's models; abnormal low level of O-GlcNAc may give a way for hyperphosphorylation on tau proteins which are prone to form neuron-damaging neurofibrillary tangles [49, 50]. Elevated level of O-GlcNAc can also cause deleterious effects: in diabetes, long-term hyperglycemia will inevitably lead to increased flux through the HBP and increased O-GlcNAc [51, 52]. Chronic imbalance of O-GlcNAc could lead to disturbed transcriptional factor activation, reactive oxygen species production, altered signal transductions, or inhibition of eNOS activity [53, 54]. Surprisingly, a few studies found that short-term hypoglycemia also elevated O-GlcNAc levels [6]. This paradox may be resolved if O-GlcNAc is considered as a stress adaptation mechanism that is triggered by acute challenges, such as hypoglycemia. Indeed, a large number of data suggest that protein O-GlcNAc modification dynamically increases after the cells are exposed to various type of environmental challenges [15, 16].

3. Oxidative Stress and O-GlcNAc

Disruption of redox regulation has been implicated in many conditions, such as aging, neurodegenerative diseases, ischemic events, arterial hypertension, and diabetes. Recent advances in O-GlcNAc-related studies suggest that disturbed O-GlcNAc regulation is involved in the development of these conditions. It also seems to be that stress response and O-GlcNAc are connected [14, 55]. Increasing number of evidences suggest that oxidative stress may stimulate the hexosamine biosynthetic pathway and consequently O-GlcNAcylation [16, 51]. Reactive oxygen species (ROS) can modify protein functions by oxidation of cysteine residues [56]. Within the glycolytic metabolic pathway, the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is particularly sensitive to inhibition by ROS [57]. GAPDH is a key enzyme controlling the metabolic flux through glycolysis and Krebs cycle; thus, its inhibition results in diverting glucose to bypass pathways, such as the pentose phosphate shunt and the HBP [14, 51]. It has also been shown that inhibition of GAPDH by mitochondrial superoxide is an important factor in increased O-GlcNAcylation associated with hyperglycemia [54, 58]. Jones et al. have shown in myocytes that incubation with hydrogen peroxide

Table 1: O-GlcNAc-influenced membrane or membrane-associated transport proteins.

Protein	Function	Evidence for direct influence by O-GlcNAc	Evidence for indirect influence by O-GlcNAc	Reference
Potassium voltage-gated channel subfamily KQT member 3 (KCNQ3)	Voltage-dependent K^+ channel	+	–	[97]
Potassium voltage-gated channel subfamily KQT member 2 and 5 (KCNQ2, KCNQ5)	Voltage-dependent K^+ channel	MS hit	Form complex with KCNQ3	[93, 98]
Small conductance calcium-activated potassium channel protein 2 and 3 (KCNN2, KCNN3)	Voltage-independent calcium-activated K^+ channel	MS hit	–	[93]
Calcium-activated potassium channel subunit alpha-1 (KCNMA1)	Calcium-activated K^+ channel	MS hit	–	[93]
Potassium voltage-gated channel subfamily A member 4 (KCNA4)	Voltage-dependent K^+ channel	MS hit	–	[93]
Voltage-gated sodium channels	Sodium transport	MS hit	Ankyrin G (link the cytoplasmic domains of integral proteins to cytoskeletal proteins)	[93, 100]
Sodium/potassium-transporting ATPase subunit alpha-2 and alpha-3	Plasma membrane sodium-potassium exchanger	MS hit	–	[93]
Stromal interaction molecule 1 (STIM1)	Ca^{2+} store depletion triggered Ca^{2+} influx in nonexcitable cells	+	–	[96]
Voltage-dependent calcium channels (CACNB3, CACNG3, CACNA1B, CACNA1A, CACNA1G)	Voltage-dependent Ca^{2+} channels in murine synapses	MS hit	–	[93]
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA)	Endoplasmic Ca^{2+} reuptake. Playing a role in cardiac muscle contractility	+	Phospholamban (repressor of SERCA)	[94, 95]
Voltage-dependent anion-selective channel protein 1 and 3 (VDAC1, VDAC3)	Voltage-dependent anion channels in the outer mitochondrial and cell membrane	+	Bcl-2 (inhibits mPTP opening possibly by interaction with VDAC)	[59, 93, 151, 152]
Chloride channel-2 (ClC-2)	Cl^- channel	–	Sp1 (transcription factor)	[102]
Synapsin I	Regulation of synaptic vesicle release	+	–	[32, 114]
Alpha-synuclein	Regulation of neurotransmitter release	+	Identified O-GlcNAc sites influence its aggregation properties	[118]
Adaptor protein-1 (AP-1)	Endocytosis	–	Interaction with synergin gamma that has been identified as O-GlcNAc target	[37, 119, 120]
Piccolo (PCLO)	Cycling of synaptic vesicles	+	–	[116]
Amyloid-β precursor protein (APP)	Synapse formation	+	–	[22]
Solute carrier family 2, facilitated glucose transporter member 4 (GLUT4)	Glucose transporter	+	Munc18c (GLUT4 vesicle protein, influencing its translocation)	[109, 110]
Solute carrier family 2, facilitated glucose transporter member 1 (GLUT1)	Glucose transporter	–	HIF-1α (induces the transcription of GLUT1. O-GlcNAc affects the degradation of HIF-1α.)	[111]

Table 1: Continued.

Protein	Function	Evidence for direct influence by O-GlcNAc	Evidence for indirect influence by O-GlcNAc	Reference
Nucleoporins (Nups)	Nuclear transport	+	−	[26, 143–146]
Nup153	Nuclear transport for proteins and RNA	+	−	[160]
Importin α5	Nuclear transport receptor for proteins	−	Mediates the nuclear import of OGT	[37, 161]
Beta-catenin	Intracellular adhesion and transcriptional coactivator	+	−	[31]
Nuclear factor-κB (NFκB)	Transcriptional factor	+	−	[33, 48, 140, 141]

Specific proteins are selected and included in the table based on either direct, published evidence for O-GlcNAc modification, or indirect evidence (e.g., a molecular partner of the protein is known to be O-GlcNAcylated, or O-GlcNAc influences the expression of the protein). We considered finding O-GlcNAc protein only by mass spectrometry screening combined with various enrichment techniques (e.g., wheat germ agglutinin binding) as direct—albeit weak—evidence and indicated in the table as "MS hit."

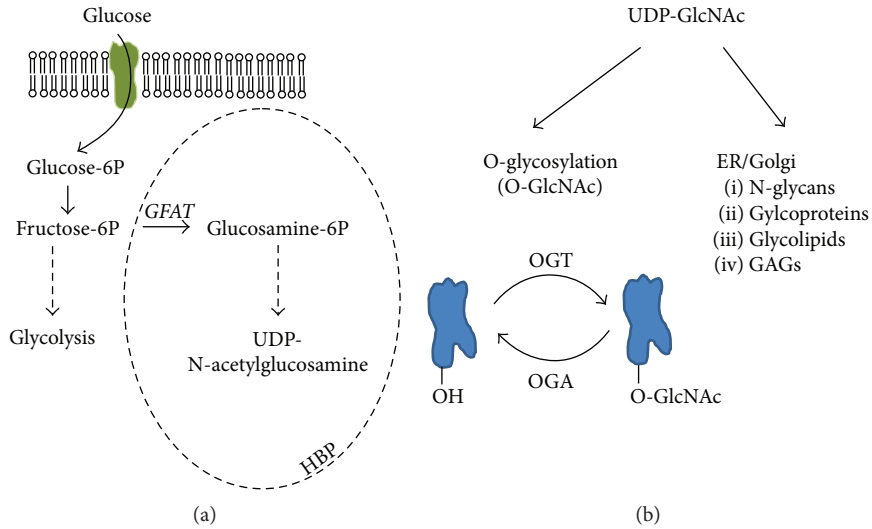

Figure 1: The hexosamine biosynthesis pathway (HBP) and the O-GlcNAc posttranslational modification. (a) An estimated 1–3% of the total glucose enters HBP. The key enzymatic reaction of this pathway is the addition of an amino group from glutamine to fructose-6-phosphate by the rate-limiting enzyme glutamine-fructose-6-phosphate amidotransferase (GFAT). Following subsequent steps (addition of an acetyl group, converting 6-phosphate to 1-phosphate and finally the transfer to UDP), the end product of HBP is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). (b) The majority of UDP-GlcNAc is utilized in the endoplasmic reticulum and the Golgi apparatus for various glycolipid, glycoprotein, and glycan synthesis. A small, but significant, percentage of UDP-GlcNAc serves as a substrate pool for the dynamic, reversible posttranslational modification termed O-GlcNAc. A single N-acetylglucosamine group is attached to the Ser/Thr residues of target proteins by O-GlcNAc transferase, while the removal of this group is managed by the enzyme O-GlcNAcase. O-GlcNAc modification occurs predominantly in the cytoplasm and in the nucleus, and it is strongly dependent on substrate availability (i.e., the metabolic flux through HBP).

caused an early increase of O-GlcNAc levels followed by a gradual decrease after 45 min [59]. Similarly, ROS-induced O-GlcNAcylation has been reported in cultured 3T3-L1 adipocytes. In these cells, urea induced ROS production which increased O-GlcNAc modification of insulin signaling molecule IRS-1 [60]. Kátai et al. demonstrated that protein O-GlcNAcylation was transiently elevated in a neuroblastoma cell line following oxidative stress [16]. The expressional level or the activity of OGT after various stresses, including hypoxia/reperfusion and oxidative stress, has been also found to be increased [15, 16, 61]. Interestingly, in some of the studies, the activity and expression of OGA also increased following stress [62].

O-GlcNAc can in turn modulate the response to oxidative stress: however, data are contradictory in this field [14]. It has been shown that increased O-glycosylation by either elevated OGT activity or OGA inhibition attenuated ROS generation induced by H_2O_2 or hypoxia [63–65]. However, Goldberg et al. demonstrated that high-glucose-induced ROS production was prevented by O-GlcNAc depletion in mesangial cells, which was speculated to be caused by the influence of O-GlcNAc on NADPH oxidase phosphorylation [66]. Nevertheless, the majority of the data shows that O-GlcNAc either directly or indirectly influences enzymes participating in the redox regulation [67]. For example, in various tissues, hyperglycemia induces an increase in mitochondrial superoxide production in association with elevated O-GlcNAcylation and a reciprocal decrease in phosphorylation of eNOS at the primary positive regulatory site, Ser-1177 [54, 68, 69]. Inducible NOS (iNOS) production is mediated by the NFκB pathway, of which stimulation by TNFα or LPS has been reported to be affected by O-GlcNAc. TNFα-induced iNOS expression was shown to be drastically decreased by high O-GlcNAc [70, 71], while LPS-induced NF-κB activation is suggested to be inhibited by OGT [72]. Expression of endogenous enzymatic antioxidants like superoxide dismutase, glutathione peroxidase, and catalase is increased in the case of OGA inhibition, while reduction of O-GlcNAc decreases expressional level of these genes [63, 73].

An important mediator of response to oxidative stress is the heat shock protein (HSP) family. HSP expression is at a low level under normal physiological conditions, but in response to stress such as heat, oxidative damage, or heavy metal poisoning, it dramatically increases [74]. Zachara et al. found that elevating O-GlcNAc by blocking OGA prior to heat shock increased the thermotolerance and sped up the increase of HSP70 and HSP40 [15]. In a later article, the same research group also showed that O-GlcNAc modification might compete with glycogen synthase kinase 3β-dependent phosphorylation (GSK3β) on HSP90β proteins [75]. Using an inducible OGT null cell line, Kazemi et al. have screened the expression of 84 molecular chaperones and have shown that 18 HSP proteins have reduced mRNA expression. The authors found that O-GlcNAc may also influence HSP expression by promoting the phosphorylation and inhibition of GSK-3β [76].

In general, augmentation of O-glycosylation seems to attenuate oxidative damage. The connection between O-GlcNAc and oxidative stress was extensively studied in neural, retinal cells and cardiomyocytes due to its clinical

significance in neurodegenerative disorders and ischemic organ damages [16, 63, 77, 78]. In response to ischemia-reperfusion injury, the amount of O-GlcNAc dramatically increases [64, 79, 80]. The role of O-GlcNAc in myocardial protection against oxidative stress is associated with calcium paradox. It is suggested that elevated HBP flux and O-GlcNAc inhibit Ca^{2+} influx [45]. Changes in intracellular Ca^{2+} play a critical role in initiating cardiomyocyte apoptosis and necrosis resulting from Ca^{2+} overload [79].

4. O-GlcNAc and Ion Channels

4.1. Ca^{2+} Channels. While intracellular free calcium ($[Ca^{2+}]_i$) concentration remains usually below 100 nM [81], in extracellular space and in compartmentalized intracellular stores, the calcium concentration is 10000 times higher, up in the millimolar range. $[Ca^{2+}]_i$ is regulated by a multitude of mechanisms, specific channels allowing Ca^{2+} to enter the cells from the extracellular space such as voltage-gated channels, ligand-gated channels, and the elusive store-operated channels. Ryanodine receptors and IP3 receptors present on the endoplasmic reticulum or sarcoplasmic reticulum can also quickly release a large amount of Ca^{2+}. The removal of $[Ca^{2+}]_i$ is managed mostly by SERCA, the plasma membrane Ca^{2+}-ATPase (PMCA), and Na^+/Ca^{2+} exchanger (NCX) [82, 83]. Calcium-binding proteins, such as calmodulin, calcineurin, and also the actin-myosin-troponin complex, are not only downstream elements of the $[Ca^{2+}]_i$ signaling but also serve as intracellular buffer to bind Ca^{2+} [84]. Intracellular calcium signaling is similarly versatile compared to O-GlcNAc regulation; $[Ca^{2+}]_i$ is a central messenger in several signaling pathways, influencing transcriptional factors, calcium-dependent phospholipase A2, protein kinase C, and various proteases [85]. It is a significant contributor to the deleterious effects of ischemia/reperfusion injuries, that is, abnormal elevation of $[Ca^{2+}]_i$ will lead to apoptosis and hypertrophy [63, 86, 87].

O-GlcNAc and $[Ca^{2+}]_i$ regulation can intercept each other at several levels. Many of the downstream elements of $[Ca^{2+}]_i$ signaling are influenced by O-GlcNAc modification, including calmodulin-dependent kinase IV, myosin, actin, and PKC [88–90]. More importantly, O-GlcNAc seems to be influencing $[Ca^{2+}]_i$ as well [45]. The most studied models in this respect were cardiac ischemia/reperfusion and calcium paradox experiments [65, 79]. Based on these studies, it seems to be that artificially elevating O-GlcNAc levels are a prosurvival mechanism [91]. Moreover, it was found that stress itself will elevate O-GlcNAc and that preconditioning protects the cells at least partially via increased O-GlcNAc levels [92]. One of the mediators of this protection was $[Ca^{2+}]_i$. O-GlcNAc was shown to suppress calcium elevation and calcium overload elicited by agonists, oxidative stress. Liu et al. showed that increased O-GlcNAc is also effective to decrease calcium overload in calcium-paradox experiments when a short perfusion with Ca^{2+}-free medium followed by perfusion with a normal amount of Ca^{2+} would lead to rapid calcium overload and cellular damage [79].

Which of the $[Ca^{2+}]_i$ regulatory elements are affected by O-GlcNAc? Regarding voltage-dependent calcium channels, detailed functional information is not yet available but Trinidad et al. identified calcium voltage-gated channel subunit beta 3 (CACNB3), gamma 3 (CACNG3), alpha 1B (CACNA1B), alpha 1A subunit (CACNA1A), and alpha 1G subunit (CACNA1G) as O-GlcNAc modified proteins of murine synapse [93]. SERCA itself has been described to be O-GlcNAc modified [94], while O-GlcNAcylation of phospholamban (SERCA repressor) also modulates its inhibitory effects on SERCA, correlating with reduced cardiac function in diabetic cardiomyopathy [95]. A frequently studied nonvoltage-gated calcium entry pathway is the store-operated calcium entry (SOCE) which is an extracellular Ca^{2+} influx into the cytoplasm in response to intracellular Ca^{2+} store depletion. Nagy et al. demonstrated that the latter process is blocked by O-GlcNAc elevation in cardiomyocytes [45]. Although the exact mechanism of SOCE still needs to be clarified, another important mediator protein of SOCE, stromal interaction molecule 1 (STIM1), has been proved to be influenced by O-GlcNAc [96]. During ER Ca^{2+} depletion induced by thapsigargin or EGTA, STIM1 proteins form puncta in ER/SR membrane which was inhibited in a dose-dependent manner by elevating O-GlcNAc levels. Moreover, it has been shown that STIM1 itself is a target for O-GlcNAc and that increasing STIM1 O-GlcNAcylation significantly modified its phosphorylation [96].

4.2. Other Ion Channels. Given that calcium is an important intracellular messenger, it is no surprise that its interaction with O-GlcNAc modification attracted significant research interest. In contrast to calcium, the influence of O-GlcNAc on the regulation of other ions and ion channels is less documented yet. Nevertheless, the data available at present does suggest that O-GlcNAc—and consequently O-GlcNAc-related cellular mechanisms such as carbohydrate metabolism and/or stress response—might have an impact on several elements of sodium, potassium, and chloride transport. For example, Ruan et al. have shown that voltage-dependent K^+ channel KCNQ3 (Kv7.3) interacts with OGT and it is probably O-GlcNAc modified at threonine 655 in neurons [97]. This protein functions in the regulation of neuronal excitability by associating with the related KCNQ2 or KCNQ5 thus forming an M-channel [98]. Defects in this gene are a cause of benign familial neonatal convulsions (BFNC) [99]. KCNN2, KCNN3, KCNMA1, and KCNA4 were also identified by mass spectrometry screening as potential O-GlcNAc proteins [93].

To our knowledge, no direct evidence was published yet on the potential O-GlcNAc modification of sodium channels. However, indirect data shows that sodium pumps still might be influenced by O-GlcNAc. The same study that probed murine synapses for O-GlcNAc-modified proteins and identified potassium channels also found HexNAc peptide characteristic for voltage-gated sodium channels and for sodium/potassium-transporting ATPases [93]. Indirect influence by O-GlcNAc on sodium transport has been speculated by other authors. Namely, isoforms of ankyrin G at nodes of Ranvier have been shown to be modified by O-GlcNAc. Ankyrins are spectrin-binding proteins that link the cytoplasmic domains of membrane proteins to the

spectrin/actin network, in particular, ankyrin G binds voltage-gated sodium channels. The authors speculate that O-GlcNAcylated serine-rich domain of ankyrin G may be involved in targeting voltage-dependent sodium channels to specific locations [100].

Among voltage-dependent anion channels (VDACs), VDAC1 seems to be O-GlcNAc modified [59, 93]. VDAC1 is extensively studied because of its clinical significance. It was shown to be overexpressed in many cancer types, and silencing of its gene inhibits tumor growth [101]. VDAC plays a critical role in the transport of small, negatively charged molecules across the mitochondrial membrane. Considering that this function is an important part of the cellular metabolism, it is plausible that O-GlcNAc modification could regulate mitochondrial activity through VDAC. Indirect evidence shows that Cl^- channels might be also regulated by O-GlcNAc. For example, the expression of chloride channel-2 (ClC-2) is dependent on the Sp1 transcription factor. Vij and Zeitlin published that O-GlcNAcylation of Sp1 is required for proper ClC-2 gene expression [102]. On the other hand, our recent publication [17] showed that osmotic resistance and volume regulation are influenced by O-GlcNAc. Since osmotic regulation (under hypotonic conditions) is controlled in a large part by a mechanism called regulatory volume decrease and carried out by the activation of a chloride conductance upon cell swelling (IClswell). Based on bioinformatics analysis of the ICln protein, which is responsible for IClswell, the presence of various O-GlcNAcylation sites on ICln is likely [103].

4.3. Glucose Transport. There is plenty of evidence that O-GlcNAc may regulate glucose transport. In fact, one of the first functions proposed for O-GlcNAc was nutrient sensing and O-GlcNAc is considered a key participant in insulin resistance [1]. As mentioned earlier, substrate production for O-GlcNAc is provided by the HBP. Since O-GlcNAc formation is dependent on the metabolic flux through HBP, which in turn is dependent on glucose (and subsequent fructose-6-phosphate) availability, metabolic changes can influence O-GlcNAc levels. Increased O-GlcNAc is thought to regulate glycogen synthesis, glucose metabolism, and glucose transport [5, 75, 104]. Several intermediate messengers of the insulin receptor signaling cascade have been identified as target for O-GlcNAc modulation such as IRS-1 and Akt [105–107].

The principal glucose transporter protein that mediates glucose uptake is glucose transporter type 4 (GLUT4), which plays a key role in regulating glucose homeostasis. GLUT4 is one of 13 sugar transporter proteins (GLUT1 to GLUT12 and HMIT) in humans. It is mainly expressed in skeletal muscle and adipose tissues. In an unstimulated state, it is mostly located in intracellular vesicles but a rapid translocation into the plasma membrane occurs after insulin stimulation to increase glucose uptake [108]. There is a growing evidence that increased O-GlcNAcylation of GLUT4 vesicle proteins such as Munc18c and others has a role in the inhibition of glucose transport in diabetes [109] and GLUT4 itself is suspected to be an O-GlcNAc target [110]. In cancer cells, the transcription factor HIF-1α (hypoxia-inducible factor 1α) induces a metabolic shift to aerobic glycolysis through the upregulation of various glycolytic proteins, including GLUT1 [111]. Ferrer et al. showed that OGT and O-GlcNAc modification are required to prevent HIF-1α proteasomal degradation in breast cancer cells thus enabling GLUT1 expression, glucose uptake, and survival in breast cancer cells [111].

4.4. Synaptic Transport. Nerve terminals are especially enriched in O-GlcNAcylation [112]. According to the recent data of Lagerlöf et al., OGT is present not only in presynaptic, but also in postsynaptic density and they proposed that O-GlcNAc is an important regulator of the synaptic maturation and plasticity [113]. One of the more abundant phosphoproteins in the brain is synapsin I. It belongs to the synapsin family that anchors synaptic vesicles to the cytoskeleton thus playing a role in neurotransmitter release control [114]. Synapsin I controls the size and release of residual pool of synaptic vesicles, and disruption of synapsin I function causes reduced size of the synaptic vesicle pool, defects in synaptic plasticity, memory deficits, and epileptic seizures. Synapsin I was among the first proteins that were found to be heavily O-GlcNAc modified [115]. Skorobogatko et al. have also found that synapsin I is O-GlcNAcylated during hippocampal synaptogenesis in rats. The authors identified three novel O-GlcNAc sites on the protein; two of them are also known as Ca^{2+}/calmodulin-dependent protein kinase II phosphorylation sites. They also showed that the O-GlcNAc site of Thr-87—which is located within an amphipathic lipid-packing sensor motif—interferes with the binding of synapsin I to synaptic vesicles. When O-GlcNAc modification is impossible due to mutation of Thr-87, synapsin I tends to localize to synapses. Lacking O-GlcNAc on Thr-87 also led to increased density and size of synaptic vesicles [32]. Among with synapsin I, the protein Piccolo was also found to be heavily O-GlcNAc modified [116]. Both proteins are involved in the regulation of synaptic vesicles, and both are known to be phosphorylated, suggesting that interaction between O-GlcNAc and phosphorylation might have a mutual regulatory role.

Alpha-synuclein, a small protein consisting of 140 amino acids, is also implicated to take part in regulating neurotransmitter release. The protein is specifically enriched in presynaptic nerve terminals and is likely to play a role in the development of Parkinson's disease [117]. Its potential implication is suggested in exocytic processes and in the recycling of synaptic vesicles through association with the cell membrane [118]. In vivo, endogenous O-GlcNAcylation of alpha-synuclein at threonine 64 and 72 in mice and serine 87 in humans has been identified. Available data suggest that the presence of these modifications reduces the chance for aggregation and the toxicity of the protein but likely has no or little effect on its binding or remodeling membranes [118].

In a proteome-wide identification of O-GlcNAc-modified proteins, synergin gamma has also been detected as a target [37]. Through interaction with adaptor protein 1 (AP-1) complex, synergin gamma is involved in the trafficking of clathrin-coated vesicles to different directions like the trans-Golgi network or the plasma membrane

[119, 120]. Perez-Cervera et al. have shown a connection between O-GlcNAc and lipid rafts. They demonstrated that OGT is present in lipid microdomains and that its localization at the raft is regulated by insulin signaling [121]. Although the role of O-GlcNAc is not clarified yet, it is noteworthy to mention that lipid rafts seem to play an important role in synaptic signaling and plasticity and, moreover, are involved in endocytic and exocytic transport routes [122, 123].

The O-GlcNAc modification of amyloid precursor protein (APP) carries special importance; inadequate proteolysis of APP produces amyloid-beta, which is a hydrophobic peptide and the major hallmark of Alzheimer's disease (AD) [22, 124]. APP is an integral membrane protein involved in synaptic formation and repair. While it is not known whether O-GlcNAc directly regulates the normal function of APP, its role in the normal processing and trafficking to the plasma membrane has been studied by several researchers [125–127]. Apparently, increased O-GlcNAc modification on APP will facilitate its traffic to the membrane and decrease its endocytosis, resulting in reduced formation of the pathological amyloid-beta products. This process could be a promising therapeutic target to be exploited. On the other hand, O-GlcNAc modification of APP has interesting implications regarding AD and carbohydrate metabolism. Type 2 diabetes is associated with a higher risk of AD, some even use the term "type 3 diabetes" for AD [128]. It seems to be that similarly to other tissues, neuronal cells develop insulin resistance and decrease glucose uptake—or in a more severe case almost completely switch to keton and fatty acid metabolism [129]. Amyloid-beta accumulation contributes to this metabolic switch by causing mitochondrial dysfunction and oxidative stress. Taken together, decreased O-GlcNAc modification on APP, intracellular hypoglycemia, and oxidative stress via continuous amyloid-beta deposition leads to neuronal degeneration [52, 130, 131].

4.5. Nuclear Transport. In eukaryotic cells, an important location of transport is at the double nuclear membrane. Bidirectional exchanges through this membrane are carried out by 125×10^6 Da supramolecular complexes, called nuclear pore complexes (NPC) [132]. NPCs consist of various copies of ~30 different subunits called nucleoporins (Nups). Proteins below 40 kDa can pass through NPCs via passive diffusion, but protein transport above 40 kDa is an energy-dependent process. It requires the presence of nuclear localization signal (NLS) that is a sequence with high basic amino acid content exposed on the protein surface or a leucine-rich nuclear export signal (NES) [133]. O-GlcNAc has been suggested to interfere at two levels in the nuclear transport; it can modify proteins designated to nuclear translocation but may also alter the nucleoporins of nuclear pore complex [134].

First studies in 1989 assumed sugar residues can act as nuclear targeting signals [135]. Later, Duverger et al. performed experiments with fluorescein-coupled bovine serum albumin (BSA) in either electroporated or digitonin-permeabilized cells. According to their results, sugar-substituted BSA was able to enter the nucleus while control, unsubstituted albumin, stayed in the cytosol [136]. Subsequent studies also suggested the presence of an NLS-independent, sugar-mediated nuclear import of proteins [137, 138]. O-GlcNAc is likely to have remarkable influence on the nuclear transport and activity of beta-catenin. Besides participating in cell-cell adhesion, this protein also has a role in expression regulation as a transcriptional coactivator mediating *wnt* signaling. The latter function is involved in cell proliferation and invasion. O-GlcNAcylation of beta-catenin was demonstrated to have an inverse relationship with the protein's nuclear localization and transcriptional activity. Minimal O-GlcNAcylation of beta-catenin has been shown in tumor cells together with an elevated transcriptional state, while in normal cells, significantly, O-GlcNAcylated beta-catenin is associated with decreased transcriptional activity [31]. A central element in stress-related transcriptional regulation is NFκB, which normally stays in the cytoplasm due to the inhibitory action of IκBα which masks the NLS sequence of NFκB [139]. Several studies showed that O-GlcNAc had a positive influence on NFκB activation and nuclear translocation [48, 140, 141]. Multiple sites were found in the sequence of the p65 subunit to be directly O-GlcNAc modified, and data suggest that O-GlcNAc might disrupt/prevent the masking effect of IκBα [48, 140]. Interestingly, Xing et al. found that O-GlcNAc modification has an opposite effect on NFκB activation [33]. Other studies also elaborated on the inhibitory role of O-GlcNAc on NFκB [72, 142]. This contradiction of results is difficult to resolve without more experimental data; however, the most plausible explanation would be that the interplay between several O-GlcNAc and phosphorylation sites on NFκB has many variations. Depending on the cell type, the duration, type and severity of stress, and different post-translational patterns may develop on NFκB and produce different outcomes.

Nups were some of the first described OGT substrates, and they are among proteins with the highest O-GlcNAc density [143, 144]. As of now, 18 Nups have been identified as potential candidates for O-GlcNAc-modified proteins [145]. Despite emerging knowledge, the specific function of O-GlcNAc on Nups remains to be elucidated [26]. Recently, O-GlcNAc has been suggested to alter several structural and biophysical properties of NPCs and influence the interactions between soluble nuclear transport receptors (NTR) and Nups located at the central channel of NPCs, the so-called FG-Nups (phenylalanine-glycine-rich Nups) [146]. This way, O-GlcNAc may alter protein-protein interactions at the NPC thus modulating its permeability. Moreover, stability of FG Nups is also influenced by O-glycosylation via protection from ubiquitination and subsequent proteasomal degradation [145]. Thus, cross-talk between O-GlcNAcylation and ubiquitination also plays a role in stabilizing the NPC and maintaining the integrity of the selectivity filter.

4.6. Mitochondrial Transport. O-GlcNAcylation affects not only nucleocytoplasmic but also mitochondrial proteins as well by the help of mitochondrial (mOGT) and

nucleocytoplasmic OGT (ncOGT) isoforms. The substrate for the modification, UDP-GlcNAc, is transferred into mitochondria via pyrimidine nucleotide carrier 1 (PNC1) [147, 148]. Only a few data is available regarding O-GlcNAc modification of specific mitochondrial carrier proteins. Mitochondrial permeability transition pore (mPTP) represents a nonspecific pore located in both the outer and inner mitochondrial membranes and allows molecules below 1.5 kDa to enter and exit the mitochondrial matrix. mPTP is activated by calcium overload and oxidative stress; its opening is a critical step in the initiation of apoptosis and cell death [63]. Reports demonstrated that elevated O-GlcNAc attenuates the mPTP opening [59, 149]. A central element of mPTP, voltage-dependent anion channel (VDAC), was revealed to be O-GlcNAc modified in cultured cardiac myocytes [59]. It was also demonstrated that cardiac mitochondria isolated from selective OGA-inhibited mice and OGT-overexpressing rat cardiomyocytes were resistant to the mPTP induction, while OGT inhibition increased sensitivity to Ca^{2+}-induced mitochondrial swelling [59]. Hirose et al. also found supporting evidence for the protective effect of O-GlcNAc due to inhibition of mPTP opening; in their study, they used the anesthetic isoflurane for the preconditioning of cardiac myocytes and revealed that isoflurane increased O-GlcNAc modification of VDAC [150]. Another important player of mitochondrial permeabilization and apoptosis is Bcl-2, an antiapoptotic protein that inhibits mPTP opening possibly by direct interaction with VDAC. Bcl-2 is upregulated in association with hypoxic injury-induced cell death [151, 152]. Glucosamine treatment and OGT overexpression both significantly increased mitochondrial Bcl-2 levels under normoxic conditions and augmented the response to ischemia/reperfusion thus mediating the hyperglycemia-induced protective effect against hypoxic injury [64].

5. Conclusion and Perspectives

The number of proteins found to be O-GlcNAcylated is still rapidly increasing as of today. This is partly fueled by improvements in methodology, for example, by recent developments of mass spectrometry techniques (such as native mass spectrometry) or the availability of more potent and specific OGA and OGT inhibitors [153–155]. On the other hand, O-GlcNAc also received heightened interest in recent years; it is an excellent candidate for a direct signaling link between diverse cellular functions. A more practical reason of why O-GlcNAc deserves special attention is that it has a potential in human medicine. As mentioned above, O-GlcNAc plays a significant role in the development of AD and in diabetes, but disturbances of O-GlcNAc regulation are now considered in other syndromes such as malignant disorders or inflammatory diseases [33, 70, 156]. Recently, substantial research work has been dedicated to clarify its significance in hypoxia-induced or oxidative stress-related pathophysiological events [14, 63, 92]. Measurement of O-GlcNAc levels in human patients could report about important information. A few studies already attempted to use O-GlcNAc analysis to predict the extent of metabolic dysfunction and the complications of diabetes [157, 158]. On the other hand, intervening in O-GlcNAc regulation by specific OGT and OGA inhibitors could significantly improve the outcome of some diseases. For example, the protective effect of increased O-GlcNAc modification in ischemia/reperfusion experiments could be translated and utilized in human medicine in the future [79]. In malignant diseases, specific OGT inhibitors might support chemotherapy efforts by hindering insulin-independent glucose uptake or tilting the balance toward apoptosis in malignant cells [111].

What makes O-GlcNAc such a promising research subject—that is, its versatile nature—also makes it a difficult scientific endeavor. Since it influences so many different proteins and a wide variety of protein functions, general experimental approaches such as interfering with overall O-GlcNAc by altering the HBP metabolism or even by specific OGA/OGT inhibitors might lead to false results. The complexity that underlies this relatively simple mechanism is revealed by apparent paradoxes; for example, in acute stress situations, O-GlcNAc seems to be protective whereas chronic hyperglycemia-induced O-GlcNAc elevation clearly has a negative effect. In contrast, permanently decreased O-GlcNAc levels have been associated with AD, despite the fact that diabetic patients are more prone to the disease and that increased oxidative stress (which is supposed to elevate O-GlcNAc) is thought to contribute to the development of AD [159]. It seems to be that the analysis of both the spatial and temporal distribution of O-GlcNAc on individual proteins will be required for a complete understanding. Thus, studying O-GlcNAc on individual proteins is not only an option but it is also a necessity to locate specific regulatory events. In this review, our aim was to sum up the growing number of evidences supporting the idea that O-GlcNAc—directly or indirectly—influences membrane traffic elements. These data, taken together with the fact that O-GlcNAc is an important part of the cellular stress-adaptation mechanism, provides a firm basis for further studies to elucidate O-GlcNAc's role in the regulation of membrane transport under normal and pathological conditions as well.

Conflicts of Interest

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

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References

[1] L. Wells, K. Vosseller, and G. W. Hart, "A role for *N*-acetylglucosamine as a nutrient sensor and mediator of insulin resistance," *Cellular and Molecular Life Sciences*, vol. 60, no. 2, pp. 222–228, 2003.

[2] J. L. McLarty, S. A. Marsh, and J. C. Chatham, "Post-translational protein modification by *O*-linked N-acetyl-glucosamine: its role in mediating the adverse effects of diabetes on the heart," *Life Sciences*, vol. 92, no. 11, pp. 621–627, 2013.

[3] C. Springhorn, T. E. Matsha, R. T. Erasmus, and M. F. Essop, "Exploring leukocyte *O*-GlcNAcylation as a novel diagnostic tool for the earlier detection of type 2 diabetes mellitus," *The Journal of Clinical Endocrinology & Metabolism*, vol. 97, no. 12, pp. 4640–4649, 2012.

[4] R. J. Clark, P. M. McDonough, E. Swanson et al., "Diabetes and the accompanying hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear *O*-GlcNAcylation," *Journal of Biological Chemistry*, vol. 278, no. 45, pp. 44230–44237, 2003.

[5] J. G. Kang, S. Y. Park, S. Ji et al., "*O*-GlcNAc protein modification in cancer cells increases in response to glucose deprivation through glycogen degradation," *Journal of Biological Chemistry*, vol. 284, no. 50, pp. 34777–34784, 2009.

[6] L. Zou, X. Zhu-Mauldin, R. B. Marchase et al., "Glucose deprivation-induced increase in protein *O*-GlcNAcylation in cardiomyocytes is calcium-dependent," *Journal of Biological Chemistry*, vol. 287, no. 41, pp. 34419–34431, 2012.

[7] T. Nagy, A. Miseta, and L. Kovacs, "Protein-associated O-GlcNAc, a multifunctional mechanism in cell signaling and its role in the pathogenesis of diabetes, stress and malignant diseases," *Biochemia Medica*, vol. 17, no. 2, pp. 162–177, 2007.

[8] J. A. Hanover, M. W. Krause, and D. C. Love, "The hexosamine signaling pathway: O-GlcNAc cycling in feast or famine," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1800, no. 2, pp. 80–95, 2010.

[9] E. P. Tan, F. E. Duncan, and C. Slawson, "The sweet side of the cell cycle," *Biochemical Society Transactions*, vol. 45, no. 2, pp. 313–322, 2017.

[10] V. Dehennaut, T. Lefebvre, Y. Leroy, J.-P. Vilain, J.-C. Michalski, and J.-F. Bodart, "Survey of *O*-GlcNAc level variations in *Xenopus laevis* from oogenesis to early development," *Glycoconjugate Journal*, vol. 26, no. 3, pp. 301–311, 2009.

[11] H. Jang, T. W. Kim, S. Yoon et al., "*O*-GlcNAc regulates pluripotency and reprogramming by directly acting on core components of the pluripotency network," *Cell Stem Cell*, vol. 11, no. 1, pp. 62–74, 2012.

[12] M.-D. Li, H.-B. Ruan, J. P. Singh et al., "*O*-GlcNAc transferase is involved in glucocorticoid receptor-mediated transrepression," *Journal of Biological Chemistry*, vol. 287, no. 16, pp. 12904–12912, 2012.

[13] M. Bektas and D. S. Rubenstein, "The role of intracellular protein *O*-glycosylation in cell adhesion and disease," *Journal of Biomedical Research*, vol. 25, no. 4, pp. 227–236, 2011.

[14] J. Chatham and R. Marchase, "Protein O-GlcNAcylation: a critical regulator of the cellular response to stress," *Current Signal Transduction Therapy*, vol. 5, no. 1, pp. 49–59, 2010.

[15] N. E. Zachara, N. O'Donnell, W. D. Cheung, J. J. Mercer, J. D. Marth, and G. W. Hart, "Dynamic *O*-GlcNAc modification of nucleocytoplasmic proteins in response to stress: a survival response of mammalian cells," *Journal of Biological Chemistry*, vol. 279, no. 29, pp. 30133–30142, 2004.

[16] E. Kátai, J. Pál, V. S. Poór, R. Purewal, A. Miseta, and T. Nagy, "Oxidative stress induces transient O-GlcNAc elevation and tau dephosphorylation in SH-SY5Y cells," *Journal of Cellular and Molecular Medicine*, vol. 20, no. 12, pp. 2269–2277, 2016.

[17] T. Nagy, A. Balasa, D. Frank et al., "O-GlcNAc modification of proteins affects volume regulation in Jurkat cells," *European Biophysics Journal*, vol. 39, no. 8, pp. 1207–1217, 2010.

[18] M. R. Martinez, T. B. Dias, P. S. Natov, and N. E. Zachara, "Stress-induced O-GlcNAcylation: an adaptive process of injured cells," *Biochemical Society Transactions*, vol. 45, no. 1, pp. 237–249, 2017.

[19] C. R. Torres and G. W. Hart, "Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc," *Journal of Biological Chemistry*, vol. 259, no. 5, pp. 3308–3317, 1984.

[20] G. W. Hart, C. Slawson, G. Ramirez-Correa, and O. Lagerlof, "Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease," *Annual Review of Biochemistry*, vol. 80, no. 1, pp. 825–858, 2011.

[21] J. Rengifo, C. J. Gibson, E. Winkler, T. Collin, and B. E. Ehrlich, "Regulation of the inositol 1,4,5-trisphosphate receptor type I by *O*-GlcNAc glycosylation," *The Journal of Neuroscience*, vol. 27, no. 50, pp. 13813–13821, 2007.

[22] L. S. Griffith, M. Mathes, and B. Schmitz, "β-amyloid precursor protein is modified with O-linked N-acetylglucosamine," *Journal of Neuroscience Research*, vol. 41, no. 2, pp. 270–278, 1995.

[23] R. S. Haltiwanger and G. A. Philipsberg, "Mitotic arrest with nocodazole induces selective changes in the level of *O*-linked *N*-acetylglucosamine and accumulation of incompletely processed *N*-glycans on proteins from HT29 cells," *Journal of Biological Chemistry*, vol. 272, no. 13, pp. 8752–8758, 1997.

[24] S. Varshney and P. Stanley, "EOGT and *O*-GlcNAc on secreted and membrane proteins," *Biochemical Society Transactions*, vol. 45, no. 2, pp. 401–408, 2017.

[25] C. Butkinaree, K. Park, and G. W. Hart, "O-linked β-*N*-acetylglucosamine (*O*-GlcNAc): extensive crosstalk with phosphorylation to regulate signaling and transcription in response to nutrients and stress," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1800, no. 2, pp. 96–106, 2010.

[26] M. Eustice, M. R. Bond, and J. A. Hanover, "*O*-GlcNAc cycling and the regulation of nucleocytoplasmic dynamics," *Biochemical Society Transactions*, vol. 45, no. 2, pp. 427–436, 2017.

[27] H.-B. Ruan, Y. Nie, and X. Yang, "Regulation of protein degradation by O-GlcNAcylation: crosstalk with ubiquitination," *Molecular & Cellular Proteomics*, vol. 12, no. 12, pp. 3489–3497, 2013.

[28] Y. Zhu, T.-W. Liu, S. Cecioni, R. Eskandari, W. F. Zandberg, and D. J. Vocadlo, "O-GlcNAc occurs cotranslationally to stabilize nascent polypeptide chains," *Nature Chemical Biology*, vol. 11, no. 5, pp. 319–325, 2015.

[29] M. D. Roos, K. Su, J. R. Baker, and J. E. Kudlow, "O glycosylation of an Sp1-derived peptide blocks known Sp1 protein interactions," *Molecular and Cellular Biology*, vol. 17, no. 11, pp. 6472–6480, 1997.

[30] X. Yang and K. Qian, "Protein O-GlcNAcylation: emerging mechanisms and functions," *Nature Reviews Molecular Cell Biology*, vol. 18, no. 7, pp. 452–465, 2017.

[31] R. Sayat, B. Leber, V. Grubac, L. Wiltshire, and S. Persad, "*O*-GlcNAc-glycosylation of β-catenin regulates its nuclear localization and transcriptional activity," *Experimental Cell Research*, vol. 314, no. 15, pp. 2774–2787, 2008.

[32] Y. Skorobogatko, A. Landicho, R. J. Chalkley, A. V. Kossenkov, G. Gallo, and K. Vosseller, "*O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) site thr-87 regulates synapsin I localization to synapses and size of the reserve pool of synaptic vesicles," *Journal of Biological Chemistry*, vol. 289, no. 6, pp. 3602–3612, 2014.

[33] D. Xing, K. Gong, W. Feng et al., "*O*-GlcNAc modification of NFκB p65 inhibits TNF-α-induced inflammatory mediator expression in rat aortic smooth muscle cells," *PLoS One*, vol. 6, no. 8, article e24021, 2011.

[34] W. H. Yang, J. E. Kim, H. W. Nam et al., "Modification of p53 with *O*-linked *N*-acetylglucosamine regulates p53 activity and stability," *Nature Cell Biology*, vol. 8, no. 10, pp. 1074–1083, 2006.

[35] T. Y. Chou, C. V. Dang, and G. W. Hart, "Glycosylation of the c-Myc transactivation domain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 10, pp. 4417–4421, 1995.

[36] L. Wells, S. A. Whelan, and G. W. Hart, "O-GlcNAc: a regulatory post-translational modification," *Biochemical and Biophysical Research Communications*, vol. 302, no. 3, pp. 435–441, 2003.

[37] H. Hahne, N. Sobotzki, T. Nyberg et al., "Proteome wide purification and identification of O-GlcNAc-modified proteins using click chemistry and mass spectrometry," *Journal of Proteome Research*, vol. 12, no. 2, pp. 927–936, 2013.

[38] S. Ozcan, S. S. Andrali, and J. E. L. Cantrell, "Modulation of transcription factor function by O-GlcNAc modification," *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, vol. 1799, no. 5-6, pp. 353–364, 2010.

[39] F. Zhang, K. Su, X. Yang, D. B. Bowe, A. J. Paterson, and J. E. Kudlow, "O-GlcNAc modification is an endogenous inhibitor of the proteasome," *Cell*, vol. 115, no. 6, pp. 715–725, 2003.

[40] Z. V. Wang, Y. Deng, N. Gao et al., "Spliced X-box binding protein 1 couples the unfolded protein response to hexosamine biosynthetic pathway," *Cell*, vol. 156, no. 6, pp. 1179–1192, 2014.

[41] G. A. Ngoh, T. Hamid, S. D. Prabhu, and S. P. Jones, "*O*-GlcNAc signaling attenuates ER stress-induced cardiomyocyte death," *American Journal of Physiology - Heart and Circulatory Physiology*, vol. 297, no. 5, pp. H1711–H1719, 2009.

[42] D. C. Love, M. W. Krause, and J. A. Hanover, "O-GlcNAc cycling: Emerging roles in development and epigenetics," *Seminars in Cell & Developmental Biology*, vol. 21, no. 6, pp. 646–654, 2010.

[43] Y. R. Yang, M. Song, H. Lee et al., "O-GlcNAcase is essential for embryonic development and maintenance of genomic stability," *Aging Cell*, vol. 11, no. 3, pp. 439–448, 2012.

[44] V. Dehennaut, T. Lefebvre, C. Sellier et al., "*O*-linked *N*-acetylglucosaminyltransferase inhibition prevents G_2/M transition in *Xenopus laevis* oocytes," *Journal of Biological Chemistry*, vol. 282, no. 17, pp. 12527–12536, 2007.

[45] T. Nagy, V. Champattanachai, R. B. Marchase, and J. C. Chatham, "Glucosamine inhibits angiotensin II-induced cytoplasmic Ca^{2+} elevation in neonatal cardiomyocytes via protein-associated *O*-linked *N*-acetylglucosamine," *American Journal of Physiology - Cell Physiology*, vol. 290, no. 1, pp. C57–C65, 2006.

[46] F. Liu, K. Iqbal, I. Grundke-Iqbal, G. W. Hart, and C.-X. Gong, "O-GlcNAcylation regulates phosphorylation of tau: a mechanism involved in Alzheimer's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 29, pp. 10804–10809, 2004.

[47] J. L. E. Walgren, T. S. Vincent, K. L. Schey, and M. G. Buse, "High glucose and insulin promote *O*-GlcNAc modification of proteins, including α-tubulin," *American Journal of Physiology - Endocrinology and Metabolism*, vol. 284, no. 2, pp. E424–E434, 2003.

[48] W. H. Yang, S. Y. Park, H. W. Nam et al., "NFκB activation is associated with its *O*-GlcNAcylation state under hyperglycemic conditions," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 45, pp. 17345–17350, 2008.

[49] C.-X. Gong, F. Liu, I. Grundke-Iqbal, and K. Iqbal, "Post-translational modifications of tau protein in Alzheimer's disease," *Journal of Neural Transmission*, vol. 112, no. 6, pp. 813–838, 2005.

[50] Y. Liu, F. Liu, I. Grundke-Iqbal, K. Iqbal, and C. X. Gong, "Brain glucose transporters, *O*-GlcNAcylation and phosphorylation of tau in diabetes and Alzheimer's disease," *Journal of Neurochemistry*, vol. 111, no. 1, pp. 242–249, 2009.

[51] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[52] W. B. Dias and G. W. Hart, "*O*-GlcNAc modification in diabetes and Alzheimer's disease," *Molecular BioSystems*, vol. 3, no. 11, pp. 766–772, 2007.

[53] U. Karunakaran and N. H. Jeoung, "O-GlcNAc modification: friend or foe in diabetic cardiovascular disease," *Korean Diabetes Journal*, vol. 34, no. 4, pp. 211–219, 2010.

[54] X. L. Du, D. Edelstein, S. Dimmeler, Q. Ju, C. Sui, and M. Brownlee, "Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site," *The Journal of Clinical Investigation*, vol. 108, no. 9, pp. 1341–1348, 2001.

[55] P. S. Banerjee, O. Lagerlöf, and G. W. Hart, "Roles of O-GlcNAc in chronic diseases of aging," *Molecular Aspects of Medicine*, vol. 51, pp. 1–15, 2016.

[56] V. Adler, Z. Yin, K. D. Tew, and Z. Ronai, "Role of redox potential and reactive oxygen species in stress signaling," *Oncogene*, vol. 18, no. 45, pp. 6104–6111, 1999.

[57] D.-M. Chuang, C. Hough, and V. V. Senatorov, "Glyceraldehyde-3-phosphate dehydrogenase, apoptosis, and neurodegenerative diseases," *Annual Review of Pharmacology and Toxicology*, vol. 45, no. 1, pp. 269–290, 2005.

[58] X. L. Du, D. Edelstein, L. Rossetti et al., "Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 22, pp. 12222–12226, 2000.

[59] S. P. Jones, N. E. Zachara, G. A. Ngoh et al., "Cardioprotection by *N*-acetylglucosamine linkage to cellular proteins," *Circulation*, vol. 117, no. 9, pp. 1172–1182, 2008.

[60] M. D'Apolito, X. Du, H. Zong et al., "Urea-induced ROS generation causes insulin resistance in mice with chronic renal

failure," *The Journal of Clinical Investigation*, vol. 120, no. 1, pp. 203–213, 2010.

[61] W. D. Cheung and G. W. Hart, "AMP-activated protein kinase and p38 MAPK activate *O*-GlcNAcylation of neuronal proteins during glucose deprivation," *Journal of Biological Chemistry*, vol. 283, no. 19, pp. 13009–13020, 2008.

[62] J. A. Groves, A. O. Maduka, R. N. O'Meally, R. N. Cole, and N. E. Zachara, "Fatty acid synthase inhibits the *O*-GlcNAcase during oxidative stress," *Journal of Biological Chemistry*, vol. 292, no. 16, pp. 6493–6511, 2017.

[63] G. A. Ngoh, L. J. Watson, H. T. Facundo, and S. P. Jones, "Augmented O-GlcNAc signaling attenuates oxidative stress and calcium overload in cardiomyocytes," *Amino Acids*, vol. 40, no. 3, pp. 895–911, 2011.

[64] V. Champattanachai, R. B. Marchase, and J. C. Chatham, "Glucosamine protects neonatal cardiomyocytes from ischemia-reperfusion injury via increased protein *O*-GlcNAc and increased mitochondrial Bcl-2," *American Journal of Physiology - Cell Physiology*, vol. 294, no. 6, pp. C1509–C1520, 2008.

[65] G. A. Ngoh, H. T. Facundo, T. Hamid, W. Dillmann, N. E. Zachara, and S. P. Jones, "Unique hexosaminidase reduces metabolic survival signal and sensitizes cardiac myocytes to hypoxia/reoxygenation injury," *Circulation Research*, vol. 104, no. 1, pp. 41–49, 2009.

[66] H. Goldberg, C. Whiteside, and I. G. Fantus, "*O*-linked *β*-*N*-acetylglucosamine supports p38 MAPK activation by high glucose in glomerular mesangial cells," *American Journal of Physiology - Endocrinology and Metabolism*, vol. 301, no. 4, pp. E713–E726, 2011.

[67] V. V. Lima, K. Spitler, H. Choi, R. C. Webb, and R. C. Tostes, "O-GlcNAcylation and oxidation of proteins: is signalling in the cardiovascular system becoming sweeter?," *Clinical Science*, vol. 123, no. 8, pp. 473–486, 2012.

[68] B. Musicki, M. F. Kramer, R. E. Becker, and A. L. Burnett, "Inactivation of phosphorylated endothelial nitric oxide synthase (Ser-1177) by *O*-GlcNAc in diabetes-associated erectile dysfunction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 33, pp. 11870–11875, 2005.

[69] M. Federici, R. Menghini, A. Mauriello et al., "Insulin-dependent activation of endothelial nitric oxide synthase is impaired by O-linked glycosylation modification of signaling proteins in human coronary endothelial cells," *Circulation*, vol. 106, no. 4, pp. 466–472, 2002.

[70] R. H. P. Hilgers, D. Xing, K. Gong, Y.-F. Chen, J. C. Chatham, and S. Oparil, "Acute *O*-GlcNAcylation prevents inflammation-induced vascular dysfunction," *American Journal of Physiology - Heart and Circulatory Physiology*, vol. 303, no. 5, pp. H513–H522, 2012.

[71] L. G. Nöt, C. A. Brocks, L. Vámhidy, R. B. Marchase, and J. C. Chatham, "Increased *O*-linked ß-N-acetylglucosamine levels on proteins improves survival, reduces inflammation and organ damage 24 hours after trauma-hemorrhage in rats," *Critical Care Medicine*, vol. 38, no. 2, pp. 562–571, 2010.

[72] S.-Y. Hwang, J.-S. Hwang, S.-Y. Kim, and I.-O. Han, "*O*-GlcNAc transferase inhibits LPS-mediated expression of inducible nitric oxide synthase through an increased interaction with mSin3A in RAW264.7 cells," *American Journal of Physiology - Cell Physiology*, vol. 305, no. 6, pp. C601–C608, 2013.

[73] G. D. Liu, C. Xu, L. Feng, and F. Wang, "The augmentation of O-GlcNAcylation reduces glyoxal-induced cell injury by attenuating oxidative stress in human retinal microvascular endothelial cells," *International Journal of Molecular Medicine*, vol. 36, no. 4, pp. 1019–1027, 2015.

[74] B. Kalmar and L. Greensmith, "Induction of heat shock proteins for protection against oxidative stress," *Advanced Drug Delivery Reviews*, vol. 61, no. 4, pp. 310–318, 2009.

[75] Z. Wang, A. Pandey, and G. W. Hart, "Dynamic interplay between *O*-linked *N*-acetylglucosaminylation and glycogen synthase kinase-3-dependent phosphorylation," *Molecular & Cellular Proteomics*, vol. 6, no. 8, pp. 1365–1379, 2007.

[76] Z. Kazemi, H. Chang, S. Haserodt, C. McKen, and N. E. Zachara, "*O*-linked *β*-*N*-acetylglucosamine (*O*-GlcNAc) regulates stress-induced heat shock protein expression in a GSK-3*β*-dependent manner," *Journal of Biological Chemistry*, vol. 285, no. 50, pp. 39096–39107, 2010.

[77] A. C. Wang, E. H. Jensen, J. E. Rexach, H. V. Vinters, and L. C. Hsieh-Wilson, "Loss of *O*-GlcNAc glycosylation in forebrain excitatory neurons induces neurodegeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 52, pp. 15120–15125, 2016.

[78] Y.-J. Chen, Y.-S. Huang, J.-T. Chen et al., "Protective effects of glucosamine on oxidative-stress and ischemia/reperfusion-induced retinal injury," *Investigative Ophthalmology & Visual Science*, vol. 56, no. 3, pp. 1506–1516, 2015.

[79] J. Liu, Y. Pang, T. Chang, P. Bounelis, J. Chatham, and R. Marchase, "Increased hexosamine biosynthesis and protein *O*-GlcNAc levels associated with myocardial protection against calcium paradox and ischemia," *Journal of Molecular and Cellular Cardiology*, vol. 40, no. 2, pp. 303–312, 2006.

[80] V. Champattanachai, R. B. Marchase, and J. C. Chatham, "Glucosamine protects neonatal cardiomyocytes from ischemia-reperfusion injury via increased protein-associated *O*-GlcNAc," *American Journal of Physiology - Cell Physiology*, vol. 292, no. 1, pp. C178–C187, 2007.

[81] J. M. Weinberg, J. A. Davis, and M. A. Venkatachalam, "Cytosolic-free calcium increases to greater than 100 micromolar in ATP-depleted proximal tubules," *The Journal of Clinical Investigation*, vol. 100, no. 3, pp. 713–722, 1997.

[82] T. A. Stewart, K. T. D. S. Yapa, and G. R. Monteith, "Altered calcium signaling in cancer cells," *Biochimica et Biophysica Acta (BBA) - Biomembranes*, vol. 1848, no. 10, Part B, pp. 2502–2511, 2015.

[83] R. S. Lewis, "Calcium signaling mechanisms in T lymphocytes," *Annual Review of Immunology*, vol. 19, no. 1, pp. 497–521, 2001.

[84] E. Carafoli and J. Krebs, "Why calcium? How calcium became the best communicator," *Journal of Biological Chemistry*, vol. 291, no. 40, pp. 20849–20857, 2016.

[85] D. E. Clapham, "Calcium signaling," *Cell*, vol. 131, no. 6, pp. 1047–1058, 2007.

[86] B. Zhivotovsky and S. Orrenius, "Calcium and cell death mechanisms: a perspective from the cell death community," *Cell Calcium*, vol. 50, no. 3, pp. 211–221, 2011.

[87] M. A. H. Talukder, J. L. Zweier, and M. Periasamy, "Targeting calcium transport in ischaemic heart disease," *Cardiovascular Research*, vol. 84, no. 3, pp. 345–352, 2009.

[88] W. B. Dias, W. D. Cheung, Z. Wang, and G. W. Hart, "Regulation of calcium/calmodulin-dependent kinase IV

by *O*-GlcNAc modification," *Journal of Biological Chemistry*, vol. 284, no. 32, pp. 21327–21337, 2009.

[89] C. Cieniewski-Bernard, M. Lambert, E. Dupont, V. Montel, L. Stevens, and B. Bastide, "O-GlcNAcylation, contractile protein modifications and calcium affinity in skeletal muscle," *Frontiers in Physiology*, vol. 5, p. 421, 2014.

[90] J. A. Matthews, M. Acevedo-Duncan, and R. L. Potter, "Selective decrease of membrane-associated PKC-α and PKC-ε in response to elevated intracellular *O*-GlcNAc levels in transformed human glial cells," *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1743, no. 3, pp. 305–315, 2005.

[91] J. C. Chatham, L. G. Nöt, N. Fülöp, and R. B. Marchase, "Hexosamine biosynthesis and protein O-glycosylation: the first line of defense against stress, ischemia, and trauma," *Shock*, vol. 29, no. 4, pp. 431–440, 2008.

[92] J. C. Chatham and R. B. Marchase, "The role of protein *O*-linked β-*N*-acetylglucosamine in mediating cardiac stress responses," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1800, no. 2, pp. 57–66, 2010.

[93] J. C. Trinidad, D. T. Barkan, B. F. Gulledge et al., "Global identification and characterization of both *O*-GlcNAcylation and phosphorylation at the murine synapse," *Molecular & Cellular Proteomics*, vol. 11, no. 8, pp. 215–229, 2012.

[94] V. L. Johnsen, D. D. Belke, C. C. Hughey et al., "Enhanced cardiac protein glycosylation (O-GlcNAc) of selected mitochondrial proteins in rats artificially selected for low running capacity," *Physiological Genomics*, vol. 45, no. 1, pp. 17–25, 2013.

[95] S. Yokoe, M. Asahi, T. Takeda et al., "Inhibition of phospholamban phosphorylation by *O*-GlcNAcylation: implications for diabetic cardiomyopathy," *Glycobiology*, vol. 20, no. 10, pp. 1217–1226, 2010.

[96] X. Zhu-Mauldin, S. A. Marsh, L. Zou, R. B. Marchase, and J. C. Chatham, "Modification of STIM1 by *O*-linked *N*-Acetylglucosamine (*O*-GlcNAc) attenuates store-operated calcium entry in neonatal cardiomyocytes," *Journal of Biological Chemistry*, vol. 287, no. 46, pp. 39094–39106, 2012.

[97] H.-B. Ruan, M. O. Dietrich, Z.-W. Liu et al., "O-GlcNAc transferase enables AgRP neurons to suppress browning of white fat," *Cell*, vol. 159, no. 2, pp. 306–317, 2014.

[98] C. L. Wladyka and D. L. Kunze, "KCNQ/M-currents contribute to the resting membrane potential in rat visceral sensory neurons," *The Journal of Physiology*, vol. 575, no. 1, pp. 175–189, 2006.

[99] B. C. Schroeder, C. Kubisch, V. Stein, and T. J. Jentsch, "Moderate loss of function of cyclic-AMP-modulated KCNQ2/KCNQ3 K^+ channels causes epilepsy," *Nature*, vol. 396, no. 6712, pp. 687–690, 1998.

[100] X. Zhang and V. Bennett, "Identification of *O*-linked *N*-acetylglucosamine modification of ankyrin$_G$ isoforms targeted to nodes of Ranvier," *Journal of Biological Chemistry*, vol. 271, no. 49, pp. 31391–31398, 1996.

[101] V. Shoshan-Barmatz and D. Mizrachi, "VDAC1: from structure to cancer therapy," *Frontiers in Oncology*, vol. 2, p. 164, 2012.

[102] N. Vij and P. L. Zeitlin, "Regulation of the ClC-2 lung epithelial chloride channel by glycosylation of SP1," *American Journal of Respiratory Cell and Molecular Biology*, vol. 34, no. 6, pp. 754–759, 2006.

[103] R. Gupta and S. Brunak, "Prediction of glycosylation across the human proteome and the correlation to protein function," *Pacific Symposium on Biocomputing*, vol. 322, pp. 310–322, 2002.

[104] W. Yi, P. M. Clark, D. E. Mason et al., "Phosphofructokinase 1 glycosylation regulates cell growth and metabolism," *Science*, vol. 337, no. 6097, pp. 975–980, 2012.

[105] S. Y. Park, J. Ryu, and W. Lee, "O-GlcNAc modification on IRS-1 and Akt2 by PUGNAc inhibits their phosphorylation and induces insulin resistance in rat primary adipocytes," *Experimental & Molecular Medicine*, vol. 37, no. 3, pp. 220–229, 2005.

[106] S. Wang, X. Huang, D. Sun et al., "Extensive crosstalk between *O*-GlcNAcylation and phosphorylation regulates Akt signaling," *PLoS One*, vol. 7, no. 5, article e37427, 2012.

[107] M. G. Buse, "Hexosamines, insulin resistance, and the complications of diabetes: current status," *American Journal of Physiology - Endocrinology and Metabolism*, vol. 290, no. 1, pp. E1–E8, 2006.

[108] S. Huang and M. P. Czech, "The GLUT4 glucose transporter," *Cell Metabolism*, vol. 5, no. 4, pp. 237–252, 2007.

[109] G. W. Hart and Y. Akimoto, "The O-GlcNAc modification," in *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY, USA, 2nd edition, 2009.

[110] M. G. Buse, K. A. Robinson, B. A. Marshall, R. C. Hresko, and M. M. Mueckler, "Enhanced *O*-GlcNAc protein modification is associated with insulin resistance in GLUT1-overexpressing muscles," *American Journal of Physiology - Endocrinology and Metabolism*, vol. 283, no. 2, pp. E241–E250, 2002.

[111] C. M. Ferrer, T. P. Lynch, V. L. Sodi et al., "O-GlcNAcylation regulates cancer metabolism and survival stress signaling via regulation of the HIF-1 pathway," *Molecular Cell*, vol. 54, no. 5, pp. 820–831, 2014.

[112] R. N. Cole and G. W. Hart, "Cytosolic O-glycosylation is abundant in nerve terminals," *Journal of Neurochemistry*, vol. 79, no. 5, pp. 1080–1089, 2001.

[113] O. Lagerlöf, G. W. Hart, and R. L. Huganir, "O-GlcNAc transferase regulates excitatory synapse maturity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 7, pp. 1684–1689, 2017.

[114] P. E. Ceccaldi, F. Grohovaz, F. Benfenati, E. Chieregatti, P. Greengard, and F. Valtorta, "Dephosphorylated synapsin I anchors synaptic vesicles to actin cytoskeleton: an analysis by videomicroscopy," *Journal of Cell Biology*, vol. 128, no. 5, pp. 905–912, 1995.

[115] R. N. Cole and G. W. Hart, "Glycosylation sites flank phosphorylation sites on synapsin I: O-linked *N*-acetylglucosamine residues are localized within domains mediating synapsin I interactions," *Journal of Neurochemistry*, vol. 73, no. 1, pp. 418–428, 1999.

[116] K. Vosseller, J. C. Trinidad, R. J. Chalkley et al., "*O*-linked *N*-acetylglucosamine proteomics of postsynaptic density preparations using lectin weak affinity chromatography and mass spectrometry," *Molecular & Cellular Proteomics*, vol. 5, no. 5, pp. 923–934, 2006.

[117] M. G. Spillantini, M. L. Schmidt, V. M.-Y. Lee, J. Q. Trojanowski, R. Jakes, and M. Goedert, "α-Synuclein in Lewy bodies," *Nature*, vol. 388, no. 6645, pp. 839-840, 1997.

[118] N. P. Marotta, Y. H. Lin, Y. E. Lewis et al., "O-GlcNAc modification blocks the aggregation and toxicity of the

protein α-synuclein associated with Parkinson's disease," *Nature Chemistry*, vol. 7, no. 11, pp. 913–920, 2015.

[119] J. Hirst, G. H. H. Borner, M. Harbour, and M. S. Robinson, "The aftiphilin/p200/γ-synergin complex," *Molecular Biology of the Cell*, vol. 16, no. 5, pp. 2554–2565, 2005.

[120] M. S. Robinson and J. S. Bonifacino, "Adaptor-related proteins," *Current Opinion in Cell Biology*, vol. 13, no. 4, pp. 444–453, 2001.

[121] Y. Perez-Cervera, V. Dehennaut, M. Aquino Gil et al., "Insulin signaling controls the expression of *O*-GlcNAc transferase and its interaction with lipid microdomains," *The FASEB Journal*, vol. 27, no. 9, pp. 3478–3486, 2013.

[122] B. Zonta and L. Minichiello, "Synaptic membrane rafts: traffic lights for local neurotrophin signaling?," *Frontiers in Synaptic Neuroscience*, vol. 5, p. 9, 2013.

[123] E. Ikonen, "Roles of lipid rafts in membrane transport," *Current Opinion in Cell Biology*, vol. 13, no. 4, pp. 470–477, 2001.

[124] R. L. Neve, D. L. McPhie, and Y. Chen, "Alzheimer's disease: a dysfunction of the amyloid precursor protein," *Brain Research*, vol. 886, no. 1-2, pp. 54–66, 2000.

[125] K. T. Jacobsen and K. Iverfeldt, "O-GlcNAcylation increases non-amyloidogenic processing of the amyloid-β precursor protein (APP)," *Biochemical and Biophysical Research Communications*, vol. 404, no. 3, pp. 882–886, 2011.

[126] S. A. Yuzwa, X. Shan, B. A. Jones et al., "Pharmacological inhibition of *O*-GlcNAcase (OGA) prevents cognitive decline and amyloid plaque formation in bigenic tau/APP mutant mice," *Molecular Neurodegeneration*, vol. 9, no. 1, p. 42, 2014.

[127] Y. S. Chun, O.-H. Kwon, H. G. Oh et al., "Threonine 576 residue of amyloid-β precursor protein regulates its trafficking and processing," *Biochemical and Biophysical Research Communications*, vol. 467, no. 4, pp. 955–960, 2015.

[128] E. Blázquez, E. Velázquez, V. Hurtado-Carneiro, and J. M. Ruiz-Albusac, "Insulin in the brain: its pathophysiological implications for States related with central insulin resistance, type 2 diabetes and Alzheimer's disease," *Frontiers in Endocrinology*, vol. 5, p. 161, 2014.

[129] J. Yao, J. R. Rettberg, L. P. Klosinski, E. Cadenas, and R. D. Brinton, "Shift in brain metabolism in late onset Alzheimer's disease: implications for biomarkers and therapeutic interventions," *Molecular Aspects of Medicine*, vol. 32, no. 4–6, pp. 247–257, 2011.

[130] Y. Zhu, X. Shan, S. A. Yuzwa, and D. J. Vocadlo, "The emerging link between *O*-GlcNAc and Alzheimer disease," *Journal of Biological Chemistry*, vol. 289, no. 50, pp. 34472–34481, 2014.

[131] G. Verdile, S. J. Fuller, and R. N. Martins, "The role of type 2 diabetes in neurodegeneration," *Neurobiology of Disease*, vol. 84, pp. 22–38, 2015.

[132] T. D. Allen, J. M. Cronshaw, S. Bagley, E. Kiseleva, and M. W. Goldberg, "The nuclear pore complex: mediator of translocation between nucleus and cytoplasm," *Journal of Cell Science*, vol. 113, Part 10, pp. 1651–1659, 2000.

[133] G. Kabachinski and T. U. Schwartz, "The nuclear pore complex – structure and function at a glance," *Journal of Cell Science*, vol. 128, no. 3, pp. 423–429, 2015.

[134] C. Guinez, W. Morelle, J.-C. Michalski, and T. Lefebvre, "*O*-GlcNAc glycosylation: a signal for the nuclear transport of cytosolic proteins?," *The International Journal of Biochemistry & Cell Biology*, vol. 37, no. 4, pp. 765–774, 2005.

[135] J. Hubert, A. P. Sève, P. Facy, and M. Monsigny, "Are nuclear lectins and nuclear glycoproteins involved in the modulation of nuclear functions?," *Cell Differentiation and Development*, vol. 27, no. 2, pp. 69–81, 1989.

[136] E. Duverger, V. Carpentier, A. C. Roche, and M. Monsigny, "Sugar-dependent nuclear import of glycoconjugates from the cytosol," *Experimental Cell Research*, vol. 207, no. 1, pp. 197–201, 1993.

[137] C. Rondanino, M.-T. Bousser, M. Monsigny, and A.-C. Roche, "Sugar-dependent nuclear import of glycosylated proteins in living cells," *Glycobiology*, vol. 13, no. 7, pp. 509–519, 2003.

[138] E. Duverger, C. Pellerin-Mendes, R. Mayer, A. C. Roche, and M. Monsigny, "Nuclear import of glycoconjugates is distinct from the classical NLS pathway," *Journal of Cell Science*, vol. 108, Part 4, pp. 1325–1332, 1995.

[139] M. D. Jacobs and S. C. Harrison, "Structure of an IκBα/NF-κB complex," *Cell*, vol. 95, no. 6, pp. 749–758, 1998.

[140] D. Zhang, Y. Cai, M. Chen, L. Gao, Y. Shen, and Z. Huang, "OGT-mediated O-GlcNAcylation promotes NF-κB activation and inflammation in acute pancreatitis," *Inflammation Research*, vol. 64, no. 12, pp. 943–952, 2015.

[141] A. Golks, T.-T. T. Tran, J. F. Goetschy, and D. Guerini, "Requirement for O-linked *N*-acetylglucosaminyltransferase in lymphocytes activation," *The EMBO Journal*, vol. 26, no. 20, pp. 4368–4379, 2007.

[142] L. Zou, S. Yang, V. Champattanachai et al., "Glucosamine improves cardiac function following trauma-hemorrhage by increased protein *O*-GlcNAcylation and attenuation of NF-κB signaling," *American Journal of Physiology - Heart and Circulatory Physiology*, vol. 296, no. 2, pp. H515–H523, 2009.

[143] J. A. Hanover, C. K. Cohen, M. C. Willingham, and M. K. Park, "O-linked N-acetylglucosamine is attached to proteins of the nuclear pore. Evidence for cytoplasmic and nucleoplasmic glycoproteins," *Journal of Biological Chemistry*, vol. 262, no. 20, pp. 9887–9894, 1987.

[144] G. D. Holt, C. M. Snow, A. Senior, R. S. Haltiwanger, L. Gerace, and G. W. Hart, "Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetylglucosamine," *The Journal of Cell Biology*, vol. 104, no. 5, pp. 1157–1164, 1987.

[145] Y. Zhu, T.-W. Liu, Z. Madden et al., "Post-translational *O*-GlcNAcylation is essential for nuclear pore integrity and maintenance of the pore selectivity filter," *Journal of Molecular Cell Biology*, vol. 8, no. 1, pp. 2–16, 2016.

[146] A. A. Labokha, S. Gradmann, S. Frey et al., "Systematic analysis of barrier-forming FG hydrogels from *Xenopus* nuclear pore complexes," *The EMBO Journal*, vol. 32, no. 2, pp. 204–218, 2013.

[147] R. Trapannone, D. Mariappa, A. T. Ferenbach, and D. M. F. van Aalten, "Nucleocytoplasmic human O-GlcNAc transferase is sufficient for O-GlcNAcylation of mitochondrial proteins," *Biochemical Journal*, vol. 473, no. 12, pp. 1693–1702, 2016.

[148] J. L. Sacoman, R. Y. Dagda, A. R. Burnham-Marusich, R. K. Dagda, and P. M. Berninsone, "Mitochondrial *O*-GlcNAc transferase (mOGT) regulates mitochondrial structure, function, and survival in HeLa cells," *Journal of Biological Chemistry*, vol. 292, no. 11, pp. 4499–4518, 2017.

[149] G. A. Ngoh, L. J. Watson, H. T. Facundo, W. Dillmann, and S. P. Jones, "Non-canonical glycosyltransferase modulates post-hypoxic cardiac myocyte death and mitochondrial

permeability transition," *Journal of Molecular and Cellular Cardiology*, vol. 45, no. 2, pp. 313–325, 2008.

[150] K. Hirose, Y. M. Tsutsumi, R. Tsutsumi et al., "Role of the O-linked *β*-*N*-acetylglucosamine in the cardioprotection induced by isoflurane," *Anesthesiology*, vol. 115, no. 5, pp. 955–962, 2011.

[151] R. W. Birkinshaw and P. E. Czabotar, "The BCL-2 family of proteins and mitochondrial outer membrane permeabilisation," *Seminars in Cell & Developmental Biology*, vol. 72, pp. 152–162, 2017.

[152] Y. Tsujimoto and S. Shimizu, "VDAC regulation by the Bcl-2 family of proteins," *Cell Death and Differentiation*, vol. 7, no. 12, pp. 1174–1181, 2000.

[153] A. C. Leney, K. Rafie, D. M. F. van Aalten, and A. J. R. Heck, "Direct monitoring of protein O-GlcNAcylation by high-resolution native mass spectrometry," *ACS Chemical Biology*, vol. 12, no. 8, pp. 2078–2084, 2017.

[154] A. Ostrowski and D. M. F. van Aalten, "Chemical tools to probe cellular *O*-GlcNAc signalling," *Biochemical Journal*, vol. 456, no. 1, pp. 1–12, 2013.

[155] H. C. Dorfmueller, V. S. Borodkin, M. Schimpl, S. M. Shepherd, N. A. Shpiro, and D. M. F. van Aalten, "GlcNAcstatin: a picomolar, selective O-GlcNAcase inhibitor that modulates intracellular O-glcNAcylation levels," *Journal of the American Chemical Society*, vol. 128, no. 51, pp. 16484-16485, 2006.

[156] Y. Fardini, V. Dehennaut, T. Lefebvre, and T. Issad, "*O*-GlcNAcylation: a new cancer hallmark?," *Frontiers in Endocrinology*, vol. 4, p. 99, 2013.

[157] J. P. Myslicki, J. Shearer, D. S. Hittel, C. C. Hughey, and D. D. Belke, "O-GlcNAc modification is associated with insulin sensitivity in the whole blood of healthy young adult males," *Diabetology & Metabolic Syndrome*, vol. 6, no. 1, p. 96, 2014.

[158] P. Degrell, J. Cseh, M. Mohás et al., "Evidence of *O*-linked *N*-acetylglucosamine in diabetic nephropathy," *Life Sciences*, vol. 84, no. 13-14, pp. 389–393, 2009.

[159] D. Luque-Contreras, K. Carvajal, D. Toral-Rios, D. Franco-Bocanegra, and V. Campos-Peña, "Oxidative stress and metabolic syndrome: cause or consequence of Alzheimer's disease?," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 497802, 11 pages, 2014.

[160] M. Kodiha, D. Tran, A. Morogan, C. Qian, and U. Stochaj, "Dissecting the signaling events that impact classical nuclear import and target nuclear transport factors," *PLoS One*, vol. 4, no. 12, article e8420, 2009.

[161] H. G. Seo, H. B. Kim, M. J. Kang, J. H. Ryum, E. C. Yi, and J. W. Cho, "Identification of the nuclear localisation signal of O-GlcNAc transferase and its nuclear import regulation," *Scientific Reports*, vol. 6, no. 1, article 34614, 2016.

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Clinical Study

Effect of Vitamin D Receptor Activation on the AGE/RAGE System and Myeloperoxidase in Chronic Kidney Disease Patients

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Vitamin D receptor (VDR) activation has been reported to increase circulating levels of the advanced glycation end products (AGE) and their decoy receptor (RAGE). However, until now, the effect of VDR activation on AGE and RAGE has not been tested in the setting of a randomized, double-blind clinical trial. We have therefore analyzed the effect of VDR activation by paricalcitol on pentosidine, S100A12/ENRAGE, and RAGE and on established biomarkers of oxidative stress like myeloperoxidase in CKD patients in the PENNY trial. At baseline, human S100A12/ENRAGE, RAGE, and myeloperoxidase, but not pentosidine, were intercorrelated, and the association between S100A12/ENRAGE and myeloperoxidase ($r = 0.71$, $P < 0.001$) was the strongest among these correlations. Paricalcitol failed to modify biomarkers of the AGE/RAGE system and myeloperoxidase in unadjusted and adjusted analyses by the generalized linear model (GLM). No effect modification by other risk factors was registered. Paricalcitol does not modify biomarkers of the AGE/RAGE system and myeloperoxidase in CKD patients. The apparent increase in RAGE levels by VDR activation reported in previous uncontrolled studies is most likely due to confounding factors rather than to VDR activation per se. This trial is registered with NCT01680198.

1. Introduction

The vitamin D receptor (VDR) is part of the superfamily of nuclear receptors that regulate several genes containing a vitamin D-responsive gene promoter element. VDR-responsive genes are involved in cell proliferation and differentiation, membrane transport, cell adhesion, matrix mineralization, inflammation, and oxidative stress [1]. Mitigation of oxidative stress is considered a major pathway implicated in the renal and cardiovascular protective effects of VDR activation [2]. Several biological mechanisms may lead to oxidative stress, and multiple biomarkers of oxidative stress exist [3]. Among these mechanisms, stimulation of the advanced glycation end product (AGE) receptor by a low-molecular weight AGEs like pentosidine or by compounds of the S100/calgranulin family like S100A12/ENRAGE is a relevant pathway leading to cardiovascular disease and renal damage in CKD patients [4]. On the other hand, the circulating receptor of AGE (RAGE) acts as a decoy receptor and affords protection from cardiovascular disease in the same patients [5, 6]. As to myeloperoxidase, oxidants derived from the activity of this enzyme such as hypochlorous acid may critically interfere with several cell functions thereby engendering tissue and organ damage. Myeloperoxidase gene ablation [7] prevents renal injury after surgical removal of the 4/5 of renal mass in the rat, and high myeloperoxidase levels are considered relevant for the progression of renal disease and cardiovascular complications in the CKD population [8]. We have previously shown that pentosidine, a major AGE, is a marker of concentric remodeling in dialysis patients [9] and that circulating soluble RAGE correlates inversely with atherosclerosis [5] and left ventricular hypertrophy [6] in patients with chronic kidney disease (CKD). Furthermore, in a secondary analysis in the paricalcitol and endothelial function in chronic kidney disease (PENNY) trial [10], we have recently observed that pentosidine modifies the sclerostin response to

Table 1: Demographic, clinical, and biochemical characteristics of the two study arms at baseline.

	Active group ($n = 44$)	Placebo group ($n = 44$)	P
Age (years)	63 ± 11	62 ± 12	0.65
Male sex (%)	59%	70%	0.27
Current smokers (%)	12%	19%	0.37
Past smokers (%)	45%	41%	0.66
Diabetes (%)	34%	36%	0.82
BMI (kg/m^2)	29 ± 5	29 ± 5	0.66
Systolic/diastolic BP (mmHg)	123 ± 16/73 ± 9	129 ± 21/73 ± 11	0.16/0.81
Heart rate (beats/min)	67 ± 8	68 ± 10	0.64
Cholesterol (mg/dL)	164 ± 41	162 ± 43	0.84
HDL cholesterol (mg/dL)	47 ± 11	50 ± 13	0.18
LDL cholesterol (mg/dL)	88 ± 34	88 ± 36	0.91
$eGFR_{Cyst}$ (mL/min/1.73m^2)	34 ± 12	29 ± 13	0.06
Hemoglobin (g/dL)	12 ± 2	12 ± 2	0.49
Calcium (mmol/L)	2.25 ± 0.12	2.21 ± 0.10	0.16
Phosphate (mmol/L)	1.20 ± 0.19	1.23 ± 0.16	0.29
Parathormone (pg/mL)	102 (81–146)	102 (85–154)	0.70
FGF-23 (pg/mL)	64.7 (52.7–81.2)	78.0 (53.7–103.1)	0.07
1,25-OH vitamin D (pmol/L)	101.4 ± 41.6	93.6 ± 41.8	0.32
25-OH vitamin D (nmol/L)	33 ± 16	38 ± 16	0.19
C-reactive protein (mg/L)	1.18 (0.68–3.02)	2.49 (0.99–3.74)	0.11
S100A12/ENRAGE (ng/mL)	165 (103–469)	175 (88–272)	0.39
Pentosidine (pmol/mL)	43.6 (31.2–108.9)	44.1 (31.2–99.5)	0.87
Human RAGE (pg/mL)	2072 (1571–2984)	2027 (1481–2794)	0.81
Myeloperoxidase (ng/mL)	128.5 (71.5–204.0)	127.8 (91.5–176.8)	0.90

Data are expressed as mean ± SD, median and interquartile range, or percent frequency as appropriate. BMI: body mass index; BP: blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; eGFR: estimated glomerular filtration rate; FGF-23: fibroblast growth factor-23.

VDR activation by paricalcitol [11]. However, until now, there is no randomized clinical trial that tested the effect of VDR activation on AGE and RAGE and on myeloperoxidase in CKD population. With this background in mind, we have now made a thorough analysis of the effect of paricalcitol treatment on pentosidine, S100A12/ENRAGE, and RAGE circulating levels in the PENNY trial.

2. Materials and Methods

The study protocol was approved by the ethics committee of our institution. A written informed consent was obtained from each participant.

2.1. Patients. The protocol of the PENNY trial and the corresponding CONSORT flow diagram are detailed in the previous paper describing the main results of the study [10]. Briefly, the PENNY trial is a double-blind, randomized, parallel-group trial (ClinicalTrials.gov identifier: NCT01680198) which enrolled 88 patients with CKD stages 3 to 4. The inclusion criteria were age ranging between 18 and 80 years, parathormone ≥ 65 pg/mL, serum total Ca between 2.2 and 2.5 mmol/L, and phosphate levels between 2.9 mg/dL and 4.5 mg/dL. The exclusion criteria were treatment with vitamin D compounds or antiepileptic drugs and the presence of neoplasia, symptomatic cardiovascular disease, or liver disease. Patients who met the inclusion criteria were randomized (1 : 1) to receive 2 μg paricalcitol once daily or matching placebo for 12 weeks after a 2-week run-in. Measurement of relevant variables in the PENNY trial was made at baseline, after 12 weeks of treatment with paricalcitol or placebo, and again 2 weeks after stopping these treatments. The dose of paricalcitol was adjusted on the basis of serum parathormone and Ca, and the maximum dose allowed was 2 μg daily. No vitamin D compounds were allowed during the trial. Demographic, clinical, and biochemical data of the two study arms are listed in Table 1.

2.2. Laboratory Measurements. Serum calcium, phosphate, glucose, and lipids were measured in the routine clinical pathology laboratory at our institution. Serum creatinine was measured by the Roche enzymatic, IDMS-calibrated method and serum cystatin C by the Siemens Dade Behring kit, and the GFR was calculated by the CKD-Epi creatinine-cystatin formula [12]. Plasma parathormone was measured by an immunoradiometric assay (DiaSorin, Stillwater, MN, USA) and 25-OH VD and 1,25-OH VD by a radioimmunoassay (Immunodiagnostic Systems, Boldon, UK). Serum human RAGE, myeloperoxidase, S100A12/ENRAGE, and plasma pentosidine were measured by validated ELISA

Table 2: Intercorrelations of biomarkers of oxidative stress.

	Human RAGE	S100A12/ENRAGE	Myeloperoxidase	Pentosidine
Human RAGE	1	$r = -0.252$, $P = 0.02$	$r = -0.270$, $P = 0.01$	$r = -0.009$, $P = 0.93$
S100A12/ENRAGE	$r = -0.252$, $P = 0.02$	1	$r = 0.777$, $P < 0.001$	$r = -0.091$, $P = 0.40$
Myeloperoxidase	$r = -0.270$, $P = 0.01$	$r = 0.777$, $P < 0.001$	1	$r = -0.171$, $P = 0.11$
Pentosidine	$r = -0.009$, $P = 0.93$	$r = -0.091$, $P = 0.40$	$r = -0.171$, $P = 0.11$	1

methods by using commercially available kits by R&D Systems (Minneapolis, MN) (human RAGE and myeloperoxidase), MBL International (Woburn, MA) (S100A12/ENRAGE), and Cusabio (College Park, MD) (pentosidine). The intra- and interassay coefficients of variation (CV) for each kit are the following: human RAGE: 5.7%–7.7%; myeloperoxidase: 2.1%–9.0%; S100A12/ENRAGE: 4.3%–5.4%; and pentosidine: <8%–<10%. Serum and plasma samples were kept frozen at −80° degrees, without freeze-thaw cycles, until analysis, and biomarker measurements were performed in a single assay.

2.3. Statistical Analysis. Data are reported as mean ± standard deviation (normally distributed data), median and interquartile range (nonnormally distributed data), or percent frequency, and comparisons between groups were made by independent *t*-test, Mann–Whitney test, or chi-square test. Correlates of markers of oxidative stress (human RAGE, S100A12/ENRAGE, myeloperoxidase, and pentosidine) were analyzed by using Pearson's correlation coefficient (on $\log_{10}$-transformed data, when appropriate) and linear regression analyses. The effect of paricalcitol on these biomarkers after 12 weeks of treatment was analyzed by applying the generalized linear model (GLM). Differences in risk factors at baseline not controlled by randomization and due to chance were accounted for by introducing the same risk factors in the GLM. The effect sizes of paricalcitol on the outcome measures in this study were summarized by the generalized eta squared (η^2), as recommended by Bakeman [13]. The changes in biomarkers of oxidative stress in paricalcitol-treated and untreated patients after stopping the interventions (paricalcitol and placebo) were investigated by using the paired *t*-test applied to the measurements made at the 12th week (end of the trial) and to those made 2 weeks after the end of the trial. The potential effect modification by demographic (age and gender) and bone mineral disorder biomarkers at baseline (calcium, phosphate, 25-OH vitamin D, 1,25-OH vitamin D, PTH, and FGF23) on the relationship between allocation arm and markers of oxidative stress was investigated by standard interaction analyses by introducing into the models' appropriate multiplicative terms [14]. Data analysis was performed by SPSS for Windows (version 24.0, Chicago, Illinois, USA).

3. Results

At baseline, patients randomized to paricalcitol and placebo did not differ for demographic, clinical, and biochemical characteristics, except for the eGFR which tended to be higher ($P = 0.06$) in patients receiving paricalcitol and FGF-23 which tended to be lower ($P = 0.07$) in the same patients (Table 1). No patient had vitamin D deficiency (25-OH VD levels < 10 ng/mL), whereas vitamin D insufficiency (25-OH VD levels > 10 ng/mL to <30 ng/mL) was noticed in 26 patients in the PCT group and in 19 patients in the placebo group ($P = 0.20$). Alongside comparable plasma levels of bone disorder biomarkers—including serum calcium and phosphate, 25-OH vitamin D, 1,25-OH vitamin D, PTH, and FGF23—the average values of S100A12/ENRAGE, pentosidine, RAGE, and myeloperoxidase at baseline were very similar in the two study arms (Table 1). As detailed in the source study [10], drug treatments, including ACE inhibitors, sartans, hypoglycemic agents, statins, and proton pump inhibitors, were similar between the two groups except for calcium carbonate, more frequently administered in patients on placebo (22.7%) than in those on the paricalcitol arm (0%) ($P = 0.003$).

3.1. Intercorrelations of AGE/RAGE and Myeloperoxidase in CKD Patients and Other Functional Relationships of These Biomarkers. At baseline, human S100A12/ENRAGE, RAGE, and myeloperoxidase, but not pentosidine, were mutually correlated (Table 2) and the correlation between S100A12/ENRAGE and myeloperoxidase was the strongest among these correlations (Figure 1).

S100A12/ENRAGE, RAGE, and myeloperoxidase coherently associated with body weight (S100A12/ENRAGE: $r = 0.274$, $P = 0.01$; human RAGE: $r = -0.276$, $P = 0.009$; and myeloperoxidase: $r = 0.331$, $P = 0.002$) while pentosidine did not ($r = 0.176$, $P = 0.10$). Human RAGE ($r = -0.295$, $P = 0.008$) and myeloperoxidase ($r = 0.245$, $P = 0.03$) correlated also with waist circumference while S100A12/ENRAGE ($r = 0.202$, $P = 0.07$) and pentosidine ($r = 0.17$, $P = 0.30$) did not. Finally, among these biomarkers, RAGE was the sole to correlate with C-reactive protein ($r = -0.263$, $P = 0.01$). Apart from the direct link between 25-OH vitamin D and pentosidine ($r = 0.254$, $P = 0.02$), no correlation was found between the same biomarkers and biomarkers of bone mineral disorder (PTH, 1,25-OH vitamin D, and FGF23).

3.2. Effect of Paricalcitol on Biomarkers of Oxidative Stress. After a 12-week treatment, paricalcitol suppressed PTH and 1,25-OH_2 vitamin D, producing a modest rise in serum calcium and phosphate, a marked rise in FGF23, and no change in 25-OH VD (see Supplementary Figure 1 and [10]). However, vitamin D receptor activation by this drug largely failed to modify S100A12/ENRAGE, pentosidine, RAGE, and myeloperoxidase (Figure 2 and Table 3). These results did not

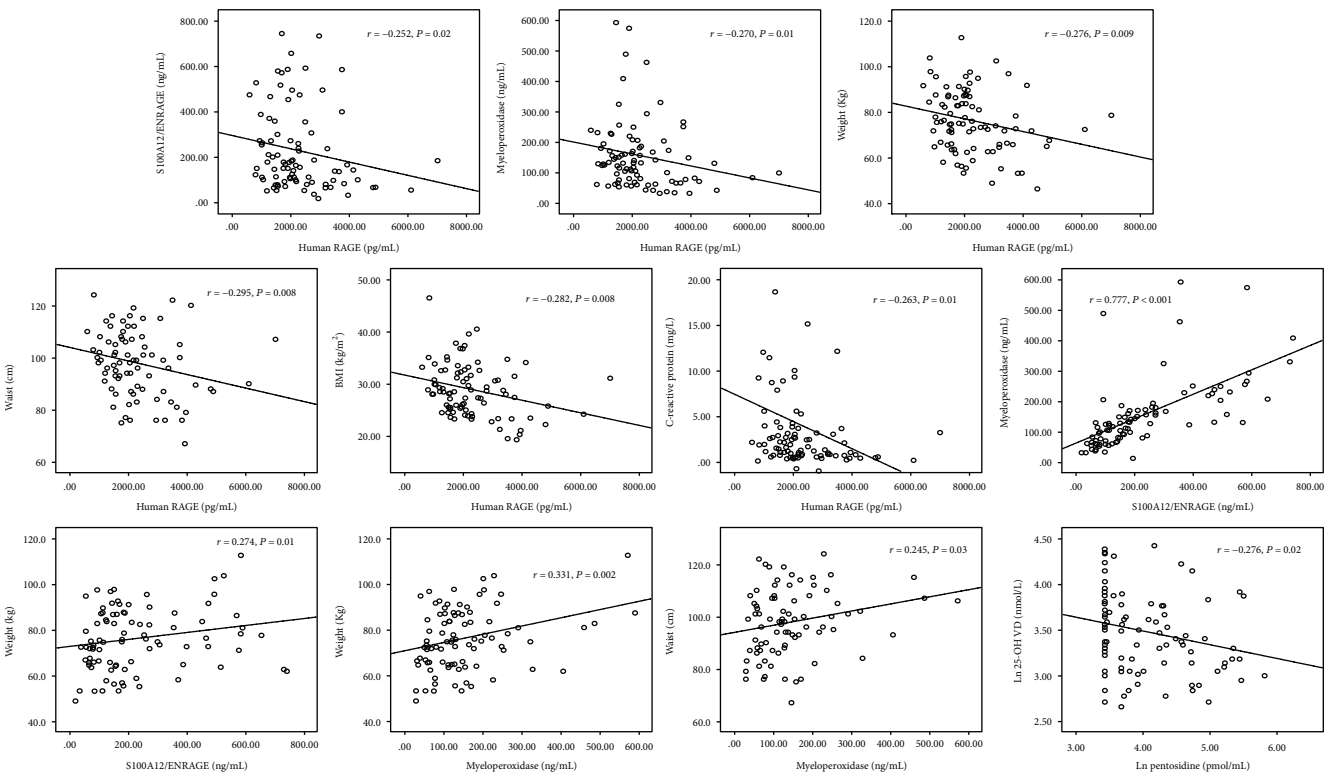

Figure 1: Main correlates of human RAGE, S100A12/ENRAGE, myeloperoxidase, and pentosidine.

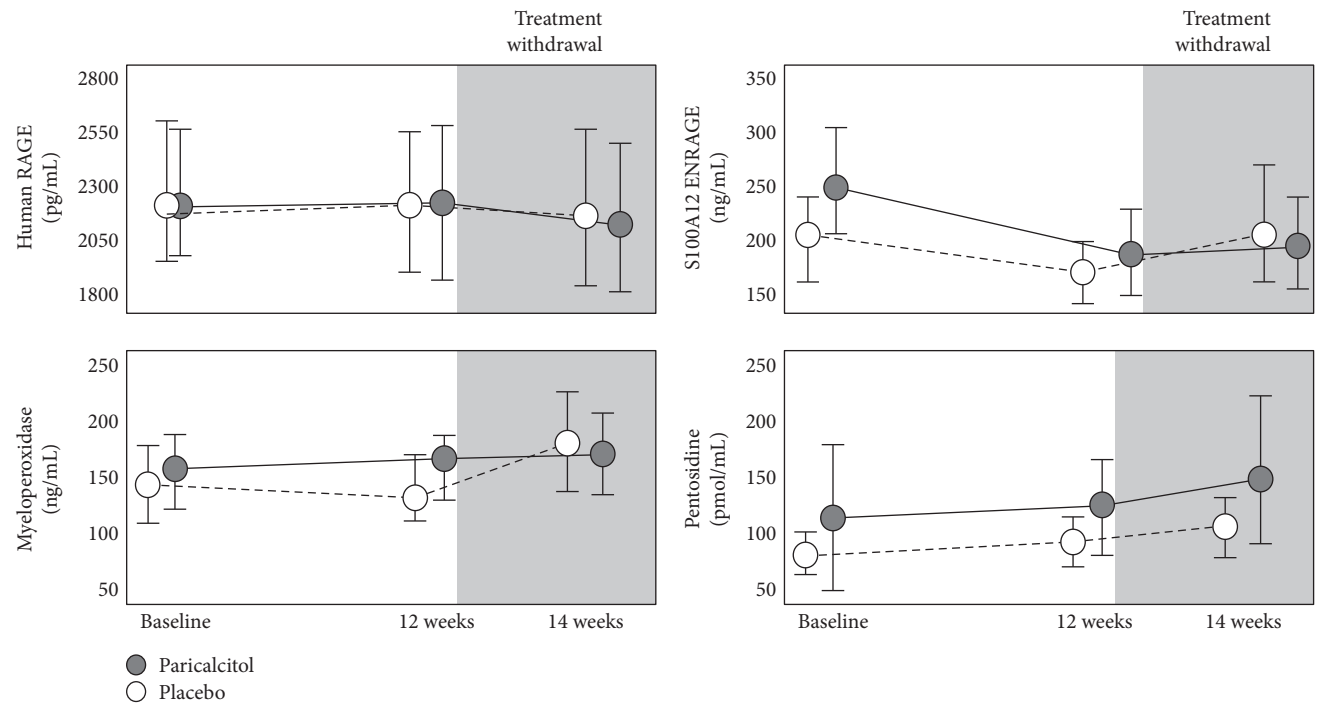

Figure 2: Effects of paricalcitol on human RAGE, S100A12/ENRAGE, myeloperoxidase, and pentosidine after 12 weeks of treatment and 2 weeks after stopping paricalcitol. Data are expressed as mean and 95% CI.

change after adjustment for the variables that differed at baseline between the study arms, that is, eGFR, calcium carbonate treatment, and FGF23 (Table 3). Diabetes did not modify the effect of paricalcitol treatment on AGE and RAGE (all P for effect modification ≥ 0.173). Effect modification analyses did not show any interaction with age, gender,

Table 3: Generalized linear models showing no effect of paricalcitol on serum human RAGE, myeloperoxidase, S100A12/ENRAGE, and plasma pentosidine after 12 weeks of treatment.

	Univariate	Adjusted for eGFR, calcium carbonate treatment, and FGF23
Human RAGE	$\eta^2 = 0.000$, $P = 0.91$	$\eta^2 = 0.005$, $P = 0.54$
S100A12/ENRAGE	$\eta^2 = 0.005$, $P = 0.51$	$\eta^2 = 0.001$, $P = 0.83$
Myeloperoxidase	$\eta^2 = 0.016$, $P = 0.24$	$\eta^2 = 0.022$, $P = 0.18$
Pentosidine	$\eta^2 = 0.001$, $P = 0.74$	$\eta^2 = 0.005$, $P = 0.52$

baseline 25-OH vitamin D, 1,25-OH vitamin D, calcium, phosphate, PTH, and FGF23 (all $P > 0.05$). The levels of these biomarkers remained unchanged after stopping the treatment with paricalcitol/placebo (Figure 2).

4. Discussion

This study performed within the framework of the randomized clinical trial [10] shows that paricalcitol largely fails to modify biomarkers of the AGE/RAGE system and major biomarkers of oxidative stress like myeloperoxidase in CKD patients.

Protein glycation is a complex series of reactions occurring in all tissues and fluids where glucose reacts with proteins giving rise to a series of advanced glycation end products (AGE) [15]. Incomplete digestion of AGE-modified protein results in the formation of low-molecular weight degradation products incorporating AGE modifications including pentosidine, N(epsilon)-(carboxymethyl)lysine (CML), and free-imidazole AGEs [16]. Low-molecular weight (LMW) AGEs activate AGE-specific receptors (RAGE) while high-molecular weight AGEs do not activate this pathway and induce tissue and organ damage by a different mechanism [17]. LMW AGEs bind to RAGE in various tissues including the endothelium and tubule tissues in the kidney and induce vascular and renal damage via activation of the nuclear factor κB, a major inflammatory pathway, and via the mitogen-activated protein kinase pathway [18]. Among LMW AGEs, pentosidine is seen as a powerful biomarker of AGE-dependent damage in disparate conditions including diabetes, aging, and CKD, particularly so in kidney failure [19]. Apart from pentosidine and LMW AGEs, the AGE receptor is also activated by S100A12/ENRAGE, an important ligand for this receptor that has been implicated in vascular inflammation, coronary and aortic atherosclerosis, and plaque vulnerability and in human cardiovascular disease [20].

In theory, stimulation of the VDR appears to be a relevant pathway whereby alterations in the AGE/RAGE pathway may be favorably affected in patients with CKD. Indeed, vitamin D supplementation mitigates the accumulation of AGEs in the vascular system in rats with streptozotocin-induced diabetes [21], and treatment with 1,25-OH vitamin D increased serum RAGE in an uncontrolled, sequential study in CKD patients on chronic dialysis [22]. Furthermore, in a nonrandomized study in vitamin-deficient women with ovary polycystic disease, treatment with 1,25-OH vitamin D increased RAGE levels, an effect that went along with a parallel decline in serum anti-Mullerian hormone levels, a critical alteration implicated in impaired folliculogenesis in these patients [23]. In a previous analysis in the PENNY trial, we observed that paricalcitol treatment, while not affecting circulating levels of pentosidine, modified the relationship between this AGE and sclerostin, a bone hormone which increases after treatment with both inactive vitamin D forms like cholecalciferol [24] and activated vitamin D compounds like paricalcitol [11]. With this background in mind, we set out to test the hypothesis that the AGE/RAGE system and myeloperoxidase levels in CKD patients may be favorably affected by treatment with paricalcitol. In this respect, the PENNY trial [10], a double-blind, randomized trial testing the effects of paricalcitol on CKD patients, offered the ideal setting for investigating this hypothesis. Indeed, serum samples for the measurement of the key biomarkers considered in the present study were available in all patients (no missing sample). Pentosidine, S100A12/ENRAGE, RAGE, and myeloperoxidase were measured by well-validated methods (see Materials and Methods) with very good intra- and interassay variability (<10%), and thorough analysis of the mutual correlations among these compounds showed consistent internal relationships suggesting that these biomarkers reflect AGE/RAGE status in CKD patients. Notably, myeloperoxidase was strongly associated with RAGE and AGE ligands, and RAGE associated with both body weight and waist circumference, a well-recognized metric of abdominal adiposity in CKD patients [25]. However, contrarily to our hypothesis, paricalcitol treatment failed to affect the circulating levels of RAGE ligands investigated in this study as well as myeloperoxidase levels. Notably, this was true both in unadjusted analyses by the generalized linear model as well as in analyses by the same model adjusted for variables that marginally differed in the study arms like the eGFR and treatment with calcium carbonate and FGF23.

Even though results in this study robustly negate that paricalcitol treatment may favorably affect the circulating levels of biomarkers of the AGE/RAGE system and of myeloperoxidase, circulating levels of these compounds may not adequately reflect levels of the same biomarkers at tissue level. Therefore, the fact that we did not measure the tissue levels of these biomarkers is a limitation of our study. Another limitation of this study is the small sample size, calculated on the primary outcome of the trial (i.e., modification in endothelial function) rather than on the (hypothetic) effect of paricalcitol on AGE-RAGE. Furthermore, all patients enrolled in this study were Caucasian and followed up in a single Nephrology Unit, and this might impair the generalizability of results. However, circulating levels of pentosidine [26], S100A12/ENRAGE [27], RAGE [5, 6], and myeloperoxidase [7] have already been associated with evidence of tissue damage and clinical events in CKD patients. Thus, failure of

paricalcitol to modify these biomarkers implies that this drug is unlikely to meaningfully modify the potential risk for adverse clinical outcomes related to AGE accumulation in CKD. The strength of our study is that it is based on a randomized, double-blind trial with no missing blood sample throughout the trial.

5. Conclusions

In conclusion, paricalcitol does not modify biomarkers of the AGE/RAGE system and major biomarkers of oxidative stress like myeloperoxidase in CKD patients. Our data suggest that the apparent increase in RAGE levels during treatment with 1,25-OH vitamin D in previous studies in hemodialysis patients [22] and in women with polycystic ovary [23] is most likely due to uncontrolled confounding factors rather than to 1,25-OH vitamin D treatment.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

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

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Supplementary Materials

Effects of Paricalcitol on biomarkers of mineral-bone disorder after 12 weeks of treatment and 2 weeks after stopping Paricalcitol. Data are expressed as mean and 95% CI. (*Supplementary materials*)

References

[1] R. Bouillon, G. Carmeliet, L. Verlinden et al., "Vitamin D and human health: lessons from vitamin D receptor null mice," *Endocrine Reviews*, vol. 29, no. 6, pp. 726–776, 2008.

[2] J. Dong, S. L. Wong, C. W. Lau et al., "Calcitriol protects renovascular function in hypertension by down-regulating angiotensin II type 1 receptors and reducing oxidative stress," *European Heart Journal*, vol. 33, no. 23, pp. 2980–2990, 2012.

[3] B. Palmieri and V. Sblendorio, "Oxidative stress tests: overview on reliability and use. Part I," *European Review for Medical and Pharmacological Sciences*, vol. 11, no. 5, pp. 309–342, 2007.

[4] A. Gugliucci and T. Menini, "The axis AGE-RAGE-soluble RAGE and oxidative stress in chronic kidney disease," *Advances in Experimental Medicine and Biology*, vol. 824, pp. 191–208, 2014.

[5] G. Basta, D. Leonardis, F. Mallamaci et al., "Circulating soluble receptor of advanced glycation end product inversely correlates with atherosclerosis in patients with chronic kidney disease," *Kidney International*, vol. 77, no. 3, pp. 225–231, 2010.

[6] D. Leonardis, G. Basta, F. Mallamaci et al., "Circulating soluble receptor for advanced glycation end product (sRAGE) and left ventricular hypertrophy in patients with chronic kidney disease (CKD)," *Nutrition, Metabolism, & Cardiovascular Diseases*, vol. 22, no. 9, pp. 748–755, 2012.

[7] A. Lehners, S. Lange, G. Niemann et al., "Myeloperoxidase deficiency ameliorates progression of chronic kidney disease in mice," *American Journal of Physiology - Renal Physiology*, vol. 307, no. 4, pp. F407–F417, 2014.

[8] B. Kisic, D. Miric, I. Dragojevic, J. Rasic, and L. Popovic, "Role of myeloperoxidase in patients with chronic kidney disease," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 1069743, 10 pages, 2016.

[9] C. Zoccali, F. Mallamaci, K. Asahia et al., "Pentosidine, carotid atherosclerosis and alterations in left ventricular geometry in hemodialysis patients," *Journal of Nephrology*, vol. 14, no. 4, pp. 293–298, 2001.

[10] C. Zoccali, G. Curatola, V. Panuccio et al., "Paricalcitol and endothelial function in chronic kidney disease trial," *Hypertension*, vol. 64, no. 5, pp. 1005–1011, 2014.

[11] C. Torino, P. Pizzini, S. Cutrupi et al., "Active vitamin D treatment in CKD patients raises serum sclerostin and this effect is modified by circulating pentosidine levels," *Nutrition, Metabolism & Cardiovascular Diseases*, vol. 27, no. 3, pp. 260–266, 2017.

[12] A. S. Levey, J. P. Bosch, J. B. Lewis et al., "A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation," *Annals of Internal Medicine*, vol. 130, no. 6, pp. 461–470, 1999.

[13] R. Bakeman, "Recommended effect size statistics for repeated measures designs," *Behavior Research Methods*, vol. 37, no. 3, pp. 379–384, 2005.

[14] R. de Mutsert, K. J. Jager, C. Zoccali, and F. W. Dekker, "The effect of joint exposures: examining the presence of interaction," *Kidney International*, vol. 75, no. 7, pp. 677–681, 2009.

[15] M. Brownlee, A. Cerami, H. Vlassara, A. Cerami, and H. Vlassara, "Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications," *The New England Journal of Medicine*, vol. 318, no. 20, pp. 1315–1321, 1988.

[16] M. C. Thomas, J. M. Forbes, R. MacIsaac, G. Jerums, and M. E. Cooper, "Low-molecular weight advanced glycation end products: markers of tissue AGE accumulation and more?," *Annals of the New York Academy of Sciences*, vol. 1043, no. 1, pp. 644–654, 2005.

[17] D. Deluyker, V. Ferferieva, J.-P. Noben et al., "Cross-linking *versus* RAGE: how do high molecular weight advanced glycation products induce cardiac dysfunction?," *International Journal of Cardiology*, vol. 210, pp. 100–108, 2016.

[18] J. Xie, J. D. Méndez, V. Méndez-Valenzuela, and M. M. Aguilar-Hernández, "Cellular signalling of the receptor for advanced glycation end products (RAGE)," *Cellular Signalling*, vol. 25, no. 11, pp. 2185–2197, 2013.

[19] D. R. Sell, R. H. Nagaraj, S. K. Grandhee et al., "Pentosidine: a molecular marker for the cumulative damage to proteins in

diabetes, aging, and uremia," *Diabetes/Metabolism Reviews*, vol. 7, no. 4, pp. 239–251, 1991.

[20] A. Oesterle and M. A. H. Bowman, "S100A12 and the S100/calgranulins: emerging biomarkers for atherosclerosis and possibly therapeutic targets," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 35, no. 12, pp. 2496–2507, 2015.

[21] E. Salum, J. Kals, P. Kampus et al., "Vitamin D reduces deposition of advanced glycation end-products in the aortic wall and systemic oxidative stress in diabetic rats," *Diabetes Research and Clinical Practice*, vol. 100, no. 2, pp. 243–249, 2013.

[22] J. Y. Sung, W. Chung, A. J. Kim et al., "Calcitriol treatment increases serum levels of the soluble receptor of advanced glycation end products in hemodialysis patients with secondary hyperparathyroidism," *The Tohoku Journal of Experimental Medicine*, vol. 230, no. 1, pp. 59–66, 2013.

[23] M. Irani, H. Minkoff, D. B. Seifer, and Z. Merhi, "Vitamin D increases serum levels of the soluble receptor for advanced glycation end products in women with PCOS," *The Journal of Clinical Endocrinology and Metabolism*, vol. 99, no. 5, pp. E886–E890, 2014.

[24] B. Dawson-Hughes, S. S. Harris, L. Ceglia, and N. J. Palermo, "Effect of supplemental vitamin D and calcium on serum sclerostin levels," *European Journal of Endocrinology*, vol. 170, no. 4, pp. 645–650, 2014.

[25] C. Zoccali, C. Torino, G. Tripepiand, and F. Mallamaci, "Assessment of obesity in chronic kidney disease: what is the best measure?," *Current Opinion in Nephrology and Hypertension*, vol. 21, no. 6, pp. 641–646, 2012.

[26] A. Machowska, J. Sun, A. R. Qureshi et al., "Plasma pentosidine and its association with mortality in patients with chronic kidney disease," *PLoS One*, vol. 11, no. 10, article e0163826, 2016.

[27] N. Isoyama, P. Leurs, A. R. Qureshi et al., "Plasma S100A12 and soluble receptor of advanced glycation end product levels and mortality in chronic kidney disease stage 5 patients," *Nephrology, Dialysis, Transplantation*, vol. 30, no. 1, pp. 84–91, 2015.

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Review Article

P-glycoprotein (ABCB1) and Oxidative Stress: Focus on Alzheimer's Disease

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ATP-binding cassette (ABC) transporters, in particular P-glycoprotein (encoded by ABCB1), are important and selective elements of the blood-brain barrier (BBB), and they actively contribute to brain homeostasis. Changes in ABCB1 expression and/or function at the BBB may not only alter the expression and function of other molecules at the BBB but also affect brain environment. Over the last decade, a number of reports have shown that ABCB1 actively mediates the transport of beta amyloid (Aβ) peptide. This finding has opened up an entirely new line of research in the field of Alzheimer's disease (AD). Indeed, despite intense research efforts, AD remains an unsolved pathology and effective therapies are still unavailable. Here, we review the crucial role of ABCB1 in the Aβ transport and how oxidative stress may interfere with this process. A detailed understanding of ABCB1 regulation can provide the basis for improved neuroprotection in AD and also enhanced therapeutic drug delivery to the brain.

1. Introduction

The rise in life expectancy, with the relative aging of the population, involves serious demographic, ethical, social, economic, and medical problems. In particular, the incidence of neurodegenerative diseases has increased considerably by ten or fifteen years to this part. Common to all of the neurodegenerative disorders is the irreversible degeneration of distinct subsets of neurons, the accumulation of aggregated peptides, and the imbalance of cellular oxidative state.

Alzheimer's disease (AD) is the most common cause of dementia and one of the most important causes of morbidity and mortality among the aging population. The appearance of beta amyloid (Aβ) plaques in the extracellular compartment of the brain parenchyma is a hallmark of AD, and biochemical and genetic findings highlight the crucial role of the Aβ peptide in the pathogenesis of AD [1]. In addition to the recognized pathological signs of senile plaques and neurofibrillary tangles, the presence of extensive oxidative stress (OS) is a contributing factor in the progression of AD. The accumulation of free radical damage and alterations in the activities of antioxidant enzymes are also present in AD patients [2]. However, the exact mechanisms by which the redox balance is altered and the sources of free radicals in the AD brain are still unknown. It has been demonstrated that Aβ is capable of promoting the formation of ROS through a mechanism that involves the PI3K/Akt/GSK3 and MAPK/ERK1/2 pathways [3] and that OS may increase Aβ production and aggregation as well facilitate tau phosphorylation, forming a vicious cycle that promotes the progression of AD [4].

The question to ask is why the Aβ peptide accumulates in the brain. There are two possible explanations: (i) the overproduction of Aβ in the brain and (ii) the reduced clearance of Aβ from the brain [5, 6]. Only familial AD (5% of cases) is due to the overproduction of Aβ because of mutations in the amyloid precursor protein (APP) gene or in the APP processing enzymes [7, 8], while the greater part (95%) of the so-called sporadic AD cases are probably caused by dysfunctions in Aβ aggregation, degradation, and removal [5, 9]. It has been proposed that the underlying cause of Aβ accumulation in AD is a reduced clearance of Aβ from the brain via the blood-brain barrier (BBB) [10, 11].

ATP-binding cassette (ABC) transporters are multidomain integral membrane proteins that use the energy of ATP hydrolysis to translocate solutes across cellular membranes in all mammalian species [12]. In the last decade, a number of reports have shown that members of the ABC superfamily of membrane proteins, in particular P-glycoprotein (encoded by ABCB1), actively mediate the transport of Aβ [13]. Cirrito et al. [14] demonstrated that the deficiency of ABCB1 at the BBB increased Aβ deposition in an AD mouse model, suggesting that Aβ is transported out of the brain or periarterial interstitial fluid through this transport system.

Although many studies on ABCB1 and AD are present in the literature, the link between OS and ABC membrane transport systems, during aging and in OS-related diseases, as AD, is still unclear, thus providing an urgent need for a deeper understanding of mechanisms through which such processes and diseases develop. In this review, we discuss the possible role of ABCB1 and OS in AD and consider how a fuller understanding of these aspects might promote the development of more effective treatment strategies.

2. Blood-Brain Barrier and Oxidative Stress

In the human body, the brain represents the most sensitive organ to OS, not only because its own proper function requires the precise control of the extracellular environment but also because of the huge demand for nutrients by the brain itself. Indeed, the oxygen requirements of the brain tissue accounts for approximately 20% of the total human oxygen consumption [15]. The BBB is an essential biochemical and physical barrier that separates the central nervous system (CNS) from the bloodstream and plays a fundamental role in the balance of the brain microenvironment. Indeed, it maintains the ion balance and the low gradient of excitatory neurotransmitters, regulates the transport of specific nutrients, and limits the entry of toxic substances both endogenous and exogenous [13]. This is essential for a reliable synaptic transmission and an effective neuroregulation activity. In this view, it promotes the longevity of the SNC and prevents premature death and cellular neurodegeneration [16].

This barrier is mainly formed by a monolayer of tightly junctioned endothelial cells. Anyway, this is not enough to form a functionally BBB per se, which requires the presence of interaction with adjacent glial cells as well as neurons, pericytes, and collagen extracellular matrix [17, 18]. This intricate relationship between both vascular and neuronal cells is called implied neurovascular unit (NVU). The NVU avoids the entry of compounds from the circulating blood to the brain via paracellular or transcellular diffusion. For this reason, the brain homeostasis is maintained through specific transporters or passive diffusion mechanisms [19]. In fact, oxygen, carbon dioxide, glucose, nucleosides, vitamins, and part of liposoluble drugs can reach the SNC, but it has been reported that the BBB is responsible for blocking the delivery of more than 98% of drugs [20–22].

Several neurodegenerative diseases are characterized by increased inflammation. Indeed, neuroinflammation exacerbates the pathology by generating inflammatory mediators, as well as by activating microglia, and by the production of reactive oxygen species (ROS). All together, these events contribute to spread OS [23, 24]. The resulting condition is that the BBB tight junctions are wrecked, causing a consistent variation in brain microenvironment [25].

Microglia, as the first and primary active immune defense in the CNS, express multiple subfamilies of ABC transporters and are particularly sensitive to brain injury or disease and switch their morphology and phenotype to an "activated" state in response to brain insults [26, 27].

Among ROS, the superoxide anion, a by-product of physiological processes, contribute to BBB endothelial dysfunction [28–30]. In normal conditions, superoxide dismutase (SOD) enzyme regulates biological activity of superoxide, but under oxidative conditions, the anion is produced at high levels that overcome the metabolic capacity of SOD. BBB damage can be intensified by conjugation of superoxide and nitric oxide (NO) to form peroxynitrite, a cytotoxic and proinflammatory molecule. Peroxynitrite causes significant injury to microvessels through lipid peroxidation, consumption of endogenous antioxidants, and induction of mitochondrial failure [31, 32]. Overall, OS contributes to disruption of endothelial cell-cell interactions and to BBB injury by promoting redistribution or downregulation of critical tight junction proteins such as claudin-5, occludin, zonula occludens-1, and junctional adhesion molecule-1 [33–36].

In particular, the importance of brain-to-blood transport of brain-derived metabolites across the BBB has gained increasing attention as a potential mechanism in the pathogenesis of neurodegenerative disorders.

3. ABC Transporters

The cells forming the NVU achieve the control of brain homeostasis and microenvironment by the expression of complex active transport systems, such as ion channels, pumps, receptors, and transporters on the luminal or abluminal side of the BBB [37]. Despite the presence of BBB, small molecules and macromolecules could be transported into the brain to maintain its homeostasis. There are three main classes of BBB transporters: carrier-mediated transporters (CMT), active efflux transporters (AET), and receptor-mediated transporter (RMT). The CMT and AET systems are mainly responsible for the transport of small molecules, while the RMT systems are reserved for large molecules and involve endocytic transport. AET transporters include ABC family members, which form one of the largest of all protein families and are central to many important biomedical phenomena, including resistance of cancers and pathogenic microbes to drugs [38]. In fact, ABC transport system regulates drug bioavailability, metabolism, and distribution in cells and in the extracellular matrix, limiting substrate cellular influx and retention [39–42]. ABC proteins share a common molecular structure composed by nucleotide-binding domains, which conserved peptide motifs, and transmembrane domains, usually composed of six transmembrane helices [43]. In the BBB, ABC transporters are localized on the blood-facing plasma membrane where they allow unidirectional transport from the cytoplasm to the

extracellular space. This localization can be considered as a strategy to protect the brain against the numerous lipophilic xenobiotics which, because of their chemical structure, should rapidly diffuse across endothelial cell membranes [42, 44–46].

The human ABC superfamily includes 48 multidrug transporters, belonging to one of the seven ABC A to ABC G subfamilies. These subfamilies include the ABCA member 1 (ABCA1) that acts as cholesterol efflux regulatory protein (CERP) and extrudes phospholipids from cell membranes to Apolipoprotein E (ApoE); the ABCB subfamily member 1 (ABCB1), which mediates multidrug resistance (MDR); the ABCC subfamily of multidrug resistance-associated proteins (MRP); and the ABCG2 subfamily of the breast cancer resistance protein (BRCP) [40]. Depending on their subfamily, these transporters act either as "protectors" or as "vehicle" for bioactive molecules produced by cells [45].

The first transporter to be identified and studied was the ABCB1, a phosphorylated glycoprotein of 170 kDa usually localized on the luminal side of the brain capillary endothelial cells. In humans, it is coded from the mdr1 gene, of which there are more than 50 polymorphisms at the level of single nucleotide. Because of its highly polymorphic nature, this gene is responsible for a strong variability in drug absorption and tolerance [47]. Among these polymorphisms, the rs1128503 is a nucleotide change in exon 12 (C1236T) that does not alter the glycine at position 412, while the triallelic rs2032582 polymorphism in exon 21 (G2677 T/A) leads to an alanine to threonine or serine amino acid substitution (Ala893Thr/Ser). Finally, the rs1045642 synonymous polymorphism in exon 26 (C3435T) does not affect the leucine at position 1145 [48]. To the best of our knowledge, the main products of this gene are two, ABCB1 and ABCB2, but only the first one confers multidrug resistance [49]. This is due to the fact that ABCB2 is commonly expressed by hepatocytes at the canalicular side for the secretion of phosphatidylcholine into the bile fluid [50].

Because of the primary sequence of ABCB1, that contains the characteristic short ATP-binding motifs and in between an additional conserved sequence characteristic of ABC superfamily, ABCB1 has been classified as ABC transporters. ABCB1 is localized in both the luminal and abluminal membranes of brain capillary endothelial cells to carry out its role as "brain sentinel" [51–54]. It is still not clear if the expression of ABCB1 is only restricted to those brain blood vessels that are part of the BBB, or if it is equally expressed in fenestrated capillaries of the circumventricular organs (area postrema in the brainstem, the subfornical organ, the median eminence, the pineal body, the vascular organ of the lamina terminalis, choroid plexus, and neurohypophysis) [55, 56].

Several studies have demonstrated that rat astrocytes and microglial cultures can express multiple membrane transporters including ABCB1, but in lower levels when compared to brain endothelial cells culture [57–59]. Differently, many studies demonstrated the expression of ABCB1 at microglia, astrocytes, and pericytes adjacent to endothelial cells in normal primate brains [60–62]. These authors reported that ABCB1 was distributed along the nuclear envelope, in the caveolae, in cytoplasmic vesicles, and in Golgi complex and rough endoplasmic reticulum. On the other hand, numerous studies did not detect ABCB1 in neuronal or glial cells, suggesting that its expression in these cells may depend by pathological CNS conditions from seizures to tumors [63–67].

Besides its protective function, ABCB1 has also been implicated in resistance to apoptosis. The programmed cell death contributes to tissue remodeling and to elimination of damaged cells, and through the stimulation of this pathway, ABCB1 may affect the regenerative process in lesioned tissue. Several mechanisms that could explain this event have been described. First, ABCB1 might block the caspase-3 activation by inhibiting caspase-8 induction that is normally conducted by Fas. As shown by Ruefli et al. [68], this event needs ATP binding or hydrolysis, because mutations in the ATP-binding regions abolished ABCB1-mediated Fas resistance. Second, ABCB1 may affect the apoptosis induced by ceramide. Indeed, when ceramide is not converted into the nontoxic glucosylceramide, it mediates cell death. ABCB1 promotes the translocation of the nontoxic derivate from the cytosolic to the luminal face of the Golgi and in this way influences directly ceramide metabolism and, indirectly, causes apoptosis resistance [69, 70].

In the beginning, this drug efflux pump was discovered by oncologists as responsible for chemotherapy resistance, but besides this ability, ABCB1 confers resistance to numerous drugs, including immunosuppressive drugs, HIV protease inhibitors, and antibiotics [71–73].

ABCB1 function could be counteracted with competitive inhibitors, such as verapamil and cyclosporine A, or directly by blocking its function, as with elacridar (GF120918) [74–78]. It has been shown that a clinically relevant oral dose of oxytetracycline is able to saturate ABCB1 and, subsequently, to increase the absorption of other drugs [79]. Several side effects may be associated with the inhibition of this transporter activity. A dramatic example is congestive heart failure caused by a combined therapy of verapamil and doxorubicin to inhibit ABCB1 [80]. The use of efflux transporters inhibitors, when applied coincidently for general treatment or to enhance CNS uptake of drugs, are better suited to acute therapies where the aim is to reach short maximal concentrations, as happens in the treatment of brain tumors, than for chronic administration where long-term inhibition will interfere with the normal brain homeostasis. In this context, it is also interesting to consider a recent review by Kalvass et al. [81], in which authors discussed the low probability of modulating transporters at the human BBB by currently marketed drugs. The central conclusion of their work is that, while increased CNS distribution of efflux transport substrates has been commonly observed in animal models and when dosed with nonmarketed inhibitors (e.g., tariquidar) in humans, the overall clinical evidence indicates that drug interactions at the human BBB due to efflux transporter inhibition by marketed drugs are low in magnitude ($\leq$2-fold increase in brain : plasma ratio). Moreover, serious adverse CNS safety events arising from these interactions have not been observed.

Looking at the cerebral proteopathies, a long inhibitory treatment may lead to raised intracerebral concentrations of a large spectrum of neurotoxic substances that enter the brain

or are directly being produced within the brain. These changes of the transport kinetics and the accumulation of neurotoxins species should take in the study of the pathogenesis of neurodegenerative diseases, where dysfunction of ABCB1 has been associated with Parkinson's disease (PD), progressive supranuclear palsy (PSP), multisystem atrophy (MSA), and with depressive disorders [82–85].

Whereas ABCB1 has long dominated the "stage" of transporter-related drug resistance in cancer, other ABC transporters became clinically relevant in the 1990s. In particular, the ABCC family includes 13 related ABC transporters that are able to transport structurally different lipophilic anions. The most intriguing feature of ABCCs is that they provide a transport facility for compounds (drugs, xenobiotics, or physiological substrates) conjugated with glutathione (GSH), glucuronide, or sulfate. For several tissues and cell types, the release of glutathione disulfide (GSSG) has been reported during OS and has been proposed to be an endogenous mechanism of cellular defense [86, 87]. Among ABCC transporter subfamily, ABCC1–4 are known to be responsible for GSH and GSSG transport [88–90]. Indeed, studies in primary cultures of rat astrocytes showed that MK571, a known inhibitor of ABCC, blocks the transport of GSH [91]. Despite the fundamental role of ABCC family in the detoxifying cellular system, in this context, our attention is focalized on the role of ABCB1 and OS in AD.

4. ABCB1 and Oxidative Stress in Alzheimer's Disease

Apart from the fact that ABCB1 remains one of the major cause of chemotherapy resistance in cancer, this transport protein play an important role as export pump of endogenous compounds and exogenous toxic agents in a variety of cells and tissues. Because of its high levels of expression at the BBB, ABCB1 is a powerful gatekeeper to the brain. We can affirm that ABC transporters in general, and ABCB1 in particular, have evolved to counteract OS, indeed toxic compounds generated are eliminated by ABC transporters after they are detoxified by conjugation to GSH, glucuronide, and sulfate. Interestingly, following organ damage or disease, changes in the expression levels of ABC transporters have been observed, probably to compensate the increased load of OS products or to compensate for the loss of efflux pumps in damaged tissues. Alteration of ABCB1 participated in many CNS disorders, such as upregulation in epilepsy [92], neural inflammation [93], and stroke [94]. DeMars et al. [95] demonstrated that middle cerebral artery occlusion increased ABCB1 in the liver in conjunction with increased ABCB1 in the brain. Certainly, understanding mechanisms and signals that modulate ABCB1 expression and activity at the BBB could result in new therapeutic targets for CNS disorders. Many sensors of the cellular and extracellular environment are capable of changing ABC transporter expression at the BBB, in particular oxidative and inflammatory stress, diet, pharmacotherapy, and toxicant exposure [96].

It is known that ROS have been implicated in the regulation of ABCB1. However, there is still substantial controversy about the association between ABCB1 expression and OS. Conditions that generate ROS have been shown to increase ABCB1 expression in the liver [97] and kidney [98]. Conversely, other studies have demonstrated decreased ABCB1 due to increased ROS levels in tumor spheroids [99]. The actual amount of ROS seems to be essential in determining what cellular effects are initiated. Thus, in conditions of OS, ROS may function as an endothelial signal transduction intermediate promoting cell survival, increasing ABCB1 expression. However, ROS may also lead to increased lipid peroxidation, which is implicated in BBB disintegration, with a consequent decrease in ABCB1 expression and activity. OS is a cofactor in nearly every CNS disorders and there is substantial evidence that the accumulation of ROS can acutely disrupt the BBB. Felix and Barrand demonstrated the effect of OS on the expression of ABCB1 at BBB endothelium [100]. They exposed primary cultured rat brain endothelial cells to hydrogen peroxide, causing a concentration-dependent increase in expression and activity of ABCB1. A subsequent study by the same group proposed the involvement of several signaling effectors including ERK1/2, Akt, and JNK, which in turn activated nuclear factor-κB (NF-κB). Consistent with these results, Hong et al. demonstrated that ABCB1 expression was upregulated under conditions of chronic OS-induced GSH depletion in rat brain capillary endothelial cells [101]. These effects were reversed by the ROS scavenger, N-acetylcysteine, suggesting that depletion of GSH leads to elevated ROS, which induces ABCB1 expression. ROS can play a crucial role in signal transduction [102] through various transcriptional factors, such as NF-κB and nuclear factor E2-related factor-2 (Nrf2). In turn, these transcription factors can regulate the expression of ABC transporters. Nrf2 is a transcription factor that regulates the expression of proteins that protect against OS. Nrf2 normally resides in the cytoplasm bound to Kelch-like ECH-associated protein 1 (Keap1). Upon oxidant/electrophile binding on Keap1, Nrf2 is released and translocates to the nucleus where it binds to antioxidant response elements, increasing expression of key players in the antioxidant response, including genes that code for proteins that produce GSH, reduce ROS, and metabolize xenobiotics. Importantly, administering Nrf2 ligands is neuroprotective in animal models of neurological disorders, such as cerebral ischemia, subarachnoid hemorrhage, spinal cord injury, PD, and AD [103–107]. Wang et al. demonstrated that Nrf2 activation with the isothiocyanate sulforaphane (SFN) in vivo or in vitro increases expression and transport activity of ABCB1 at the BBB. Dosing rats with SFN increased ABCB1 expression in the brain capillaries and decreased by 50% brain accumulation of the ABCB1 substrate, verapamil. No such effects were seen in the brain capillaries from Nrf2-null mice, indicating Nrf2 dependence [108]. Oxidative damage is a relative early event in the pathogenesis of AD. Several findings support the hypothesis that Aβ interferes with oxidative phosphorylation, which results in OS and apoptosis in brain cells [109, 110]. According to the amyloid cascade hypothesis, AD results from the accumulation of Aβ in the brain [1]. The neurovascular hypothesis of Zlokovic et al. states that a critical pathological event driving Aβ accumulation in the brain is the reduced

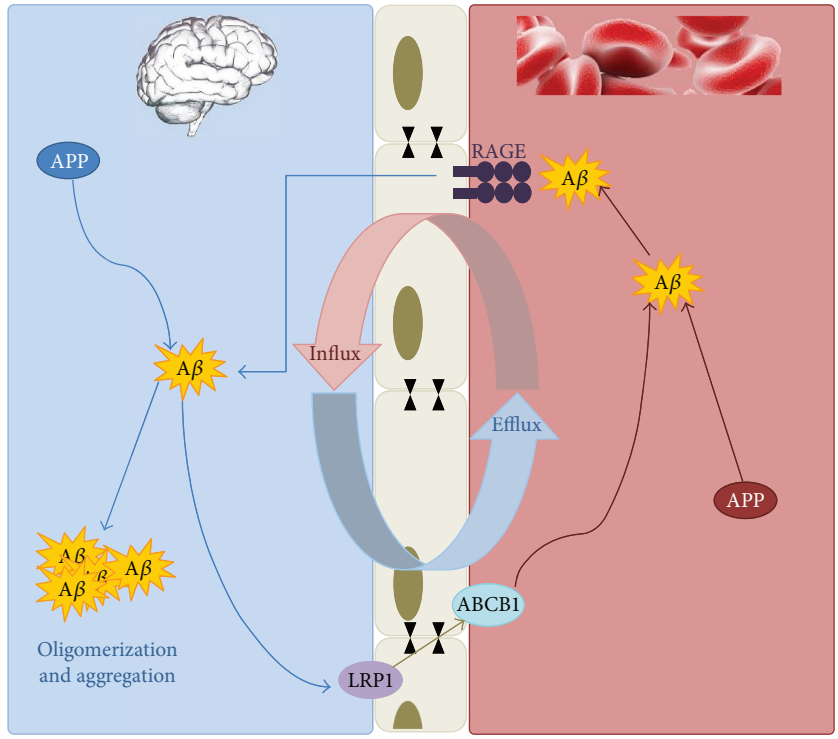

Figure 1: Proposed components of the Aβ efflux and influx across the blood-brain barrier. The two main Aβ efflux transporters include the low-density lipoprotein receptor-related protein 1 (LRP-1) and ABCB1, and the receptor for advanced glycation end-products (RAGE) is the main Aβ influx transporter.

clearance of Aβ from the brain across the BBB [111]. Over the last 10 years, a new AD research field emerged with a focus on ABC transporters at the BBB and in other cells of the CNS. Lam et al. [112] were the first to demonstrate that the potent and efficient efflux transporter ABCB1 is able to transport Aβ. Pharmacological blockade of ABCB1 rapidly decreased extracellular levels of Aβ secretion. They were able to directly measure transport of Aβ peptides across the plasma membranes of ABCB1-enriched vesicles and showed that this phenomenon was both ATP- and ABCB1-dependent. The transport of Aβ at the BBB is bidirectional. As shown in Figure 1, the two main Aβ efflux transporters include the low-density lipoprotein receptor-related protein 1 (LRP-1) and ABCB1, and the receptor for advanced glycation end-products (RAGE) is the main Aβ influx transporter [14, 113].

As demonstrated by Hartz et al. [114], extracellular Aβ first comes into contact with LRP-1 on the abluminal side of the brain endothelial cell. This is followed by transport of Aβ into the vascular lumen by ABCB1, or by a ABCB1-independent pathway. Entry of circulating Aβ into the brain is mediated by RAGE, but can also be restricted by ABCB1 [115]. The mode of interaction between Aβ and ABCB1 is not clear yet. Previous studies raised two possibilities: (i) ABCB1 could mediate Aβ transport directly or (ii) ABCB1 could interact with Aβ but does not transport it. In this case, ABCB1 may anchor Aβ on the plasma membrane and inhibit uptake into the endothelial cells [116]. In pathological conditions, as AD, it is possible to observe a reduction of ABCB1 at the BBB, which is probably associated with the accumulation of Aβ in the brain. As previously demonstrated by Loo and Clarke [117], the ubiquitin-proteasome pathway is responsible, at least in part, for the regulation of ABCB1 trafficking, localization, stability, and functions. In a recent work, Hartz et al. [118] focused on the critical mechanistic steps involved in the reduction of ABCB1 in AD. They investigated in the brain capillaries if $A\beta_{40}$ triggers ABCB1 ubiquitination, internalization, and proteasome-dependent degradation leading to reduction of ABCB1 expression and activity. Indeed, experiments with microtubule and proteasomal inhibitors confirmed that ABCB1 was internalized and degraded by the proteasome. As the authors expected, $A\beta_{40}$ activates the ubiquitin–proteasome system at the BBB, resulting in ABCB1 degradation and in a reduction of its expression and activity levels.

Moreover, it is possible to hypothesize that diminished ABCB1 expression due to increasing age, genetic, or environmental factors may lead to impaired Aβ clearance, followed by the accelerated accumulation of intracerebral Aβ and eventually the development of AD.

Cirrito et al. [14] provided the first evidence of a direct link between BBB, ABCB1, and Aβ brain deposition. Using *Mdrla*/$b^{-/-}$ double-knockout mice, the authors demonstrated that brain clearance of Aβ was significantly lower compared to that in control animals after intracerebroventricular injection of Aβ. Then, the authors dosed transgenic hAPP-overexpressing mice, a well-established AD model, with a selective ABCB1 inhibitor and measured Aβ brain concentrations by microdialysis. As expected, Aβ levels in the brain interstitial fluid were significantly increased compared to

untreated hAPP control mice. Moreover, isolated brain capillaries from transgenic Tg2576 mice showed a 70% decrease in ABCB1 transport activity and a 60% decrease in ABCB1 protein expression compared to age-matched wild-type mice [114]. Consistent with these in vivo studies, a significant negative correlation exists between the densities of senile plaque and ABCB1 levels in the brain capillaries of patients with AD [119]. Interestingly, using ^{11}C-verapamil and positron emission tomography imaging, a clinical study showed significant reduction in ABCB1 activity in AD patients compared to cognitively normal subjects [120]. Another study detected 25% lower ABCB1 protein expression levels in hippocampal blood vessels in postmortem brain samples from AD patients than in samples from age-matched nondemented patients [121]. Despite all these data supporting the involvement of decreased ABCB1 activity in Aβ accumulation in AD, little is known about the mechanisms that could initiate or sustain these transport deficiencies in disease progression. Some studies pointed at Aβ accumulation itself as a causative factor [122, 123]. Park et al. proved that Aβ mediated ABCB1 downregulation in murine brain endothelial cells by RAGE activation. These authors suggested that activation of RAGE by Aβ would enhance NF-κB activity that decreases ABCB1 expression [124]. It is possible that in the early stages of the disease, accumulation of Aβ levels in the brain capillary plasma membrane could directly impair ABCB1 function, lead to Aβ accumulation, and reduce ABCB1 expression. Hartz et al. [125] suggested that Aβ contributes to the loss of BBB integrity that could be responsible for BBB dysfunction and cognitive decline and to the compromised integrity of both specific membrane transporters and proteins of the tight junction complex. Altered BBB homeostasis not only causes neuronal damage but also compromises Aβ clearance at the NVU, resulting in a vicious cycle between Aβ accumulation and BBB dysfunction during AD progression [126]. Although BBB disruption is often detected in AD patients, it is not clear whether it is a specific feature of AD. In this regard, further studies should be conceived to define how BBB function is altered before AD onset and during disease progression. A deeper understanding of how BBB dysfunction is a cause or consequence in AD pathogenesis could allow the development of new therapeutic strategies targeting BBB for this neurodegenerative disease.

In our opinion, also OS and neuroinflammation may play a pivotal role in these transport deficiencies. The AD brain is in a chronic proinflammatory state, and indeed, Aβ causes inflammation in the brain through Toll-like receptor and complement activation [127], and elevated levels of proinflammatory cytokines and acute phase proteins are localized around Aβ plaques [128]. It was reported that ABCB1 downmodulates the function of dendritic cells, which are considered to be crucial regulators of specific inflammatory processes through the secretion of proinflammatory cytokine, resulting in an impaired immune response. This finding suggests a new physiological role for ABCB1 as an immunomodulatory molecule and reveals a possible new target for immunotherapy [129].

Oligomeric Aβ can also generate OS by producing the lipid peroxidation product, 4-hydroxynonenal [130], and through activation of NADPH oxidase, the superoxide-producing enzyme, in microglia [131]. On the other hand, inflammation and/or OS can themselves cause Aβ accumulation in the brain, thus creating a vicious circle. Indeed, OS upregulates proteins involved in Aβ production, such as presenilin 1 [132].

Erickson et al. [133] speculated that downregulation of BBB efflux transporters, as ABCB1, in AD may represent a pathological consequence of prolonged vascular sequestration of Aβ as a result of sustained systemic oxidative and inflammatory state. This possibility is supported by Hartz et al. who showed in a transgenic model of AD that ABCB1 dysfunction at the BBB preceded symptoms of cognitive impairment [114]. It is possible that aging would likely sensitize an organism to inflammation and OS so that the threshold required for Aβ efflux impairment is lowered [134]. Thus, targeting intracellular signals that upregulate ABCB1 in the early stages of AD has the potential to increase Aβ clearance from the brain and reduce its accumulation.

5. What Therapeutic Perspectives?

Taking into account all these considerations, the concept that restoring ABCB1 at the BBB could be a valid therapeutic strategy to lower Aβ brain load, reduce cognitive decline, delay onset, and slow progression of AD has to be critically evaluated. The involvement of ABCB1 in the clearance of Aβ was demonstrated by Brukmann et al. [135]. The authors showed that the absence of ABCB1 results in a significant disturbance of Aβ removal in a transgenic murine model of AD (APP/PS1^{+}/$^{--}$ABCB1), leading to an increased intraparenchymal cerebral amyloid angiopathy. We need to consider that if on the one hand, the BBB and ABCB1 are both neuroprotective; on the other hand, they are substantial obstacles to the delivery of drugs to the CNS. In this view, a recent work suggested that the simultaneous administration of verapamil, a known inhibitor of ABCB1 activity, and berberine, a promising natural anti-inflammatory and antioxidant compound, significantly potentiated their neuroprotective effect on behavioral alterations, OS, mitochondrial dysfunction, neuroinflammation, and histopathological modifications in a streptozocin-induced rat model of sporadic dementia [136].

Thus, increased ABCB1 expression enhances neuroprotection, but at the expense of drug delivery; on the contrary, reduced transporter activity decreases neuroprotection, but provides opportunity to increase drug delivery to the CNS [137]. This dual role of ABCB1 should be carefully considered in AD patients, because elderly patients often have a combination of several chronic diseases and need proper drug delivery.

For less than 10 years, the literature reported studies in AD research to demonstrate the neuroprotective activity of compounds acting on ABCB1. In 2009, Nishida et al. [138] crossed AD transgenic (APPsw) model mice with α-tocopherol transfer protein knock-out (Ttpa$^{-/-}$) mice in which lipid peroxidation in the brain was significantly increased. The resulting double-mutant (Ttpa$^{-/-}$APPsw) mice showed increased Aβ deposits in the brain, which was ameliorated

with α-tocopherol supplementation. Interestingly, the Aβ generation in Ttpa$^{-/-}$APPsw mouse brain was not increased, but the authors considered that accumulated Aβ in Ttpa$^{-/-}$APPsw mouse brain was caused by these impaired Aβ clearance. Aβ aggregation was accelerated in these mice compared with wild-type, while LRP-1 and ABCB1 were upregulated in the small vascular fraction of AD mouse brains, probably to compensate their dysfunctions to transport increased toxic substrates in the brain caused by lipid peroxidation.

Moreover, in a transgenic mouse model of AD (human amyloid precursor protein- (hAPP-) overexpressing mice; Tg2576 strain), brain capillary ABCB1 expression and transport activity were substantially reduced compared with wild-type control mice, suggesting a mechanism by which Aβ accumulates in the brain in AD. Treatment of 12-week-old asymptomatic hAPP mice for 7 days with pregnenolone-16α-carbonitrile to activate the nuclear receptor pregnane X receptor (PXR) restored ABCB1 expression and transport activity in the brain capillaries and significantly reduced the brain Aβ levels compared with untreated control mice [114]. PXR is activated by a number of drugs and dietary constituents, and potent ligands for human PXR include the antibiotic rifampin and the St. John's wort (SJW) constituent hyperforin [139]. In this regard, a clinical trial showed that rifampin dosing lessened cognitive decline in patients with AD over the 12-month treatment period [140]. The mechanistic basis for this observation is not known, but rifampicin activation of PXR leading to induction of ABCB1 in BBB is a likely possibility.

Another compound that modulates ABCB1 activity is oleocanthal, a phenolic component of extravirgin olive oil. The authors provided in vitro and in vivo evidences for the potential of oleocanthal to enhance Aβ clearance from the brain via upregulation of ABCB1 and LRP1 at the BBB. In cultured mice brain endothelial cells, oleocanthal treatment increased ABCB1 and LRP1 expression and activity. Brain efflux index (BEI%) studies of ^{125}I-Aβ_{40} showed that administration of oleocanthal extracted from extravirgin olive oil to C57BL/6 wild-type mice enhanced ^{125}I-Aβ_{40} clearance from the brain and increased the BEI% from $62.0 \pm 3.0\%$ for control mice to $79.9 \pm 1.6\%$ for oleocanthal-treated mice [141]. In a further study, the same authors confirmed its effect in the hippocampal parenchyma and microvessels of TgSwDI mice, a transgenic model of AD [142].

In 2014, Brenn et al. [143] clarified the effect of SJW on the accumulation of Aβ and ABCB1 expression in the brain. The authors showed that long-term administration (60 and 120 days) of SJW extract (final hyperforin concentration 5%) leads to a significant reduction of soluble Aβ_{1-42} (representing mainly small oligomers and monomers) as well as Aβ_{40}- and Aβ_{42}-positive plaque number and size (representing mainly fibrillar and protofibrillar Aβ), while vascular ABCB1 expression was increased in the brains of double transgenic mice.

The expression of ABCB1 at the BBB is also regulated by the vitamin D receptor (VDR). In 2011, Chow et al. [144] showed that mice treated with the physiological ligand of VDR, the 1α,25-dihydroxytitamin D [1,25(OH)$_2$D$_3$] had lower accumulation of digoxin, an ABCB1 substrate. Similarly, one year later Durk et al. [145] demonstrated *in vitro* not only that rat brain endothelial cells (RBE4) and human (hCMEC/D3) cerebral microvessels endothelial cells incubated with 1,25(OH)$_2$D$_3$ showed an increase in ABCB1 expression but also that the treatment counteracted the brain accumulation of Aβ. These findings were confirmed by a second study conducted in 2014 by the same authors [146] with two transgenic mouse models of AD, one at a preplaque formation age (Tg2576) and the other at a plaque formation or already formed age (TgCRND8). In this study, Durk et al. showed that ABCB1 expression via VDR activation decreased soluble Aβ and reduced plaque formation in young TgCRND8 mice, improving also conditioned fear memory. However, the treatment of old TgCRND8 mice (after plaque formation), even if was able to decrease soluble Aβ, it did not reduce the plaque burden. The study underlined not only the role of ABCB1 in AD pathogenesis but also the importance of VDR on its regulation.

Another class of compounds identified as inducer for ABCB1 activity was suggested by Manda et al. [147], which demonstrated in LS-180 cells that fascalpsyn, a marine-derived bis-indole alkaloid, along with its 4,5-difluoro, induced a consistent fold increase in ABCB1 expression. Moreover, these compounds showed an inhibitory activity on acetylcholinestease (AChE), an enzyme strictly involved in the neuronal loss observed in AD patients.

After a study where the activity of rivastigmine to decrease Aβ accumulation was showed, in 2016, Mohamed et al. [148] continued their work by crossing the transgenic AD mouse model APPSWE with mdr1a/b knockout mice to assess rivastigmine activity on three different levels of ABCB1 expression (APP$^+$/mdr1$^{+/+}$, APP$^+$/mdr1$^{+/-}$, and APP$^+$/mdr1$^{-/-}$). The authors showed that the treatment with rivastigmine increased the expression of ABCB1 and LRP1 in isolated brain capillaries of APP$^+$/mdr1$^{+/+}$ and APP$^+$/mdr1$^{+/-}$. Interestingly, ABCB1 deletion caused a significant increase in parenchymal accumulation of Aβ_{40}, but not Aβ_{42}, when compared to APP/mdr1 wild-type mice. This inverse correlation between Aβ_{40} deposition and ABCB1 expression suggests the importance of ABCB1 to maintain Aβ brain homeostasis across the BBB.

The studies we have taken into consideration are summarized in Table 1 and are all very interesting and encouraging, but we believe that they need further investigation. Besides many neuroprotective effects of these compounds, the induction of ABCB1 thus might enhance Aβ clearance from the brain and thereby reduce the risk of developing AD. To test this hypothesis, further studies are warranted to investigate the effects of these compounds for example on animal behavior and memory. However, these studies indicated that the induction of ABCB1 is a promising therapeutic approach to the treatment and/or prevention of neurodegenerative diseases such as AD.

6. Conclusions

In summary, it is now clear that expression/activity of ABCB1 at the BBB is strictly related to Aβ clearance and eventually with AD progression. An understanding of transporter

Table 1: Summary of (a) in vitro and (b) in vivo studies and (c) clinical trials on neuroprotective activity of compounds that targeted Aβ transport across the blood-brain barrier mediated by ABCB1.

(a) *In vitro*

Treatment	Dose	Duration	Cell line	Species	Reference
Sulforaphane (SFN)	0.01–5 μM	0–200 min	Brain capillaries	$Nrf2^{-/-}$ mouse $p53^{-/-}$ mouse	[108]
Oleocanthal (Oleo)	0.5–50 μM	72 h	bEnd3	Mouse	[141]
1α,25-Dihydroxyvitamin D3 ($1,25(OH)_2D3$)	1–100 nM	4 h or 24 h	Brain capillaries	Rat	[145]
		1–3 days	RBE4	Rat	
			hCMEC/D3	Human	
Fascalpsyn	1–100 μM	24–48 h	LS-180 hGF	Human	[147]

(b) *In vivo*

Treatment	Dose	Duration	Model	Species	Reference
SFN	1–10 mg/kg intraperitoneal (i.p.)	2 days	Sprague-Dawley	Rat	[108]
Verapamil	2–5 mg/kg i.p.	21 days	Streptozocin-induced sporadic dementia	Rat	[136]
Berberine	25–100 mg/kg per oral (p.o.)	21 days			
α-Tocopherol	36 mg/kg p.o.	Lifespan	$Ttpa^{-/-}$ APPsw	Mouse	[138]
Pregnenolone-16α-carbonitrile	25 mg/kg i.p.	7 days	APPsw/Tg2576	Mouse	[114]
Oleo	10 mg/kg/12 h i.p.	2 weeks	C57BL/6	Mouse	[141]
Oleo	5 mg/kg/die i.p.	4 weeks	TgSwDI	Mouse	[142]
St. John's wort	1250 mg/kg/die p.o.	60–120 days	$APP/PS^{+/-}$	Mouse	[143]
$1,25(OH)_2D3$	2.5 μg/kg/die i.p.	8 days	$Fxr^{-/-}$	Mouse	[144]
$1,25(OH)_2D3$	2.5 μg/kg/48 h i.p.	8 days	Tg2576 TgCRND8	Mouse	[146]
Rivastigmine	0.3 mg/kg/day alzet pump	8 weeks	APP^{+}/mdr1	Mouse	[148]

(c) Clinical trials

Treatment	Dose	Duration	Diagnosis	Reference
Rifampin Doxycyclin	300 mg/die p.o. 200 mg/die p.o.	12 months	AD	[140]

regulation is critical before we can determine to what extent signaling can be manipulated to improve not only drug delivery to the CNS but also to enhance neuroprotection. Although promising results in animal studies have been achieved, a better understanding into the signaling cascade of this transporter may result in a better understanding of AD etiology and in the development of novel therapeutic strategies.

Conflicts of Interest

The authors declared no conflict of interest.

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References

[1] J. Hardy and D. J. Selkoe, "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics," *Science*, vol. 297, no. 5580, pp. 353–356, 2012.

[2] N. Arce-Varas, G. Abate, C. Prandelli et al., "Comparison of extracellular and intracellular blood compartments highlights redox alterations in Alzheimer's and mild cognitive impairment patients," *Current Alzheimer Research*, vol. 14, no. 1, pp. 112–122, 2017.

[3] F. Morroni, G. Sita, A. Tarozzi, R. Rimondini, and P. Hrelia, "Early effects of $A\beta_{1\text{-}42}$ oligomers injection in mice: involvement of PI3K/Akt/GSK3 and MAPK/ERK1/2 pathways," *Behavioural Brain Research*, vol. 314, pp. 106–115, 2016.

[4] Y. Zhao and B. Zhao, "Oxidative stress and the pathogenesis of Alzheimer's disease," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 316523, 10 pages, 2013.

[5] K. G. Mawuenyega, W. Sigurdson, and V. Ovod, "Decreased clearance of CNS β-amyloid in Alzheimer's disease," *Science*, vol. 330, no. 6012, p. 1774, 2010.

[6] B. Sommer, "Alzheimer's disease and the amyloid cascade hypothesis: ten years on," *Current Opinion in Pharmacology*, vol. 2, no. 1, pp. 87–92, 2002.

[7] J. Dorszewska, M. Prendecki, A. Oczkowska, M. Dezor, and W. Kozubski, "Molecular basis of familial and sporadic Alzheimer's disease," *Current Alzheimer Research*, vol. 13, no. 9, pp. 952–963, 2016.

[8] C. Shepherd, H. McCann, and G. M. Halliday, "Variations in the neuropathology of familial Alzheimer's disease," *Acta Neuropathologica*, vol. 118, no. 1, pp. 37–52, 2009.

[9] H. LeVine, "The amyloid hypothesis and the clearance and degradation of Alzheimer's β-peptide," *Journal of Alzheimer's Disease*, vol. 6, no. 3, pp. 303–314, 2004.

[10] R. Deane, R. D. Bell, A. Sagare, and B. V. Zlokovic, "Clearance of amyloid-β peptide across the blood-brain barrier: implication for therapies in Alzheimer's disease," *CNS & Neurological Disorders - Drug Targets*, vol. 8, no. 1, pp. 16–30, 2009.

[11] D. M. E. van Assema, M. Lubberink, M. Bauer et al., "Blood-brain barrier P-glycoprotein function in Alzheimer's disease," *Brain*, vol. 135, Part 1, pp. 181–189, 2012.

[12] P. M. Jones and A. M. George, "The ABC transporter structure and mechanism: perspectives on recent research," *Cellular and Molecular Life Sciences*, vol. 61, no. 6, pp. 682–699, 2004.

[13] S. Vogelgesang, G. Jedlitschky, A. Brenn, and L. C. Walker, "The role of the ATP-binding cassette transporter P-glycoprotein in the transport of β-amyloid across the blood-brain barrier," *Current Pharmaceutical Design*, vol. 17, no. 26, pp. 2778–2786, 2011.

[14] J. R. Cirrito, R. Deane, A. M. Fagan et al., "P-glycoprotein deficiency at the blood-brain barrier increases amyloid-β deposition in an Alzheimer disease mouse model," *The Journal of Clinical Investigation*, vol. 115, no. 11, pp. 3285–3290, 2005.

[15] D. F. Rolfe and G. C. Brown, "Cellular energy utilization and molecular origin of standard metabolic rate in mammals," *Physiological Reviews*, vol. 77, no. 3, pp. 731–758, 1997.

[16] D. J. Begley, "Delivery of therapeutic agents to the central nervous system: the problems and the possibilities," *Pharmacology & Therapeutics*, vol. 104, no. 1, pp. 29–45, 2004.

[17] P. T. Ronaldson and T. P. Davis, "Blood-brain barrier integrity and glial support: mechanisms that can be targeted for novel therapeutic approaches in stroke," *Current Pharmaceutical Design*, vol. 18, no. 25, pp. 3624–3644, 2012.

[18] P. T. Ronaldson and T. P. Davis, "Targeted drug delivery to treat pain and cerebral hypoxia," *Pharmacological Reviews*, vol. 65, no. 1, pp. 291–314, 2013.

[19] W. A. Banks, "Physiology and pathology of the blood-brain barrier: implications for microbial pathogenesis, drug delivery and neurodegenerative disorders," *Journal of Neurovirology*, vol. 5, no. 6, pp. 538–555, 1999.

[20] E. M. Taylor, "The impact of efflux transporters in the brain on the development of drugs for CNS disorders," *Clinical Pharmacokinetics*, vol. 41, no. 2, pp. 81–92, 2002.

[21] N. J. Abbott and I. A. Romero, "Transporting therapeutics across the blood-brain barrier," *Molecular Medicine Today*, vol. 2, no. 3, pp. 106–113, 1996.

[22] D. J. Begley, C. C. Pontikis, and M. Scarpa, "Lysosomal storage diseases and the blood-brain barrier," *Current Pharmaceutical Design*, vol. 14, no. 16, pp. 1566–1580, 2008.

[23] M. Olah, K. Biber, J. Vinet, and H. W. Boddeke, "Microglia phenotype diversity," *CNS & Neurological Disorders - Drug Targets*, vol. 10, no. 1, pp. 108–118, 2011.

[24] J. H. Heo, S. W. Han, and S. K. Lee, "Free radicals as triggers of brain edema formation after stroke," *Free Radical Biology and Medicine*, vol. 39, no. 1, pp. 51–70, 2005.

[25] P. T. Ronaldson and T. P. Davis, "Targeting transporters: promoting blood-brain barrier repair in response to oxidative stress injury," *Brain Research*, vol. 1623, pp. 39–52, 2015.

[26] G. Lee, L. Schlichter, M. Bendayan, and R. Bendayan, "Functional expression of P-glycoprotein in rat brain microglia," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 299, no. 1, pp. 204–212, 2001.

[27] S. Dallas, L. Schlichter, and R. Bendayan, "Multidrug resistance protein (MRP) 4- and MRP 5-mediated efflux of 9-(2-phosphonylmethoxyethyl)adenine by microglia," *Journal of Pharmacology and Experimental Therapeutics*, vol. 309, no. 3, pp. 1221–1229, 2004.

[28] A. Strasser, D. Stanimirovic, N. Kawai, R. M. McCarron, and M. Spatz, "Hypoxia modulates free radical formation in brain microvascular endothelium," *Acta Neurochirurgica Supplements*, vol. 70, pp. 8–11, 1997.

[29] C. Nito, H. Kamada, H. Endo, K. Niizuma, D. J. Myer, and P. H. Chan, "Role of the p38 mitogen-activated protein kinase/cytosolic phospholipase A_2 signaling pathway in blood-brain barrier disruption after focal cerebral ischemia and reperfusion," *Journal of Cerebral Blood Flow & Metabolism*, vol. 28, no. 10, pp. 1686–1696, 2008.

[30] J. J. Lochhead, G. McCaffrey, L. Sanchez-Covarrubias et al., "Tempol modulates changes in xenobiotic permeability and occludin oligomeric assemblies at the blood-brain barrier during inflammatory pain," *American Journal of Physiology - Heart and Circulatory Physiology*, vol. 302, no. 3, pp. H582–H593, 2012.

[31] P. Pacher, J. S. Beckman, and L. Liaudet, "Nitric oxide and peroxynitrite in health and disease," *Physiological Reviews*, vol. 87, no. 1, pp. 315–424, 2007.

[32] B. J. Thompson and P. T. Ronaldson, "Drug delivery to the ischemic brain," *Advances in Pharmacology*, vol. 71, pp. 165–202, 2014.

[33] G. Schreibelt, G. Kooij, and A. Reijerkerk, "Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase, and PKB signaling," *The FASEB Journal*, vol. 21, no. 13, pp. 3666–3676, 2007.

[34] J. J. Lochhead, G. McCaffrey, C. E. Quigley et al., "Oxidative stress increases blood-brain barrier permeability and induces alterations in occludin during hypoxia-reoxygenation," *Journal of Cerebral Blood Flow & Metabolism*, vol. 30, no. 9, pp. 1625–1636, 2010.

[35] Y. Yang, J. F. Thompson, S. Taheri et al., "Early inhibition of MMP activity in ischemic rat brain promotes expression of tight junction proteins and angiogenesis during recovery," *Journal of Cerebral Blood Flow & Metabolism*, vol. 33, no. 7, pp. 1104–1114, 2013.

[36] K. D. Rochfort, L. E. Collins, R. P. Murphy, and P. M. Cummins, "Downregulation of blood-brain barrier phenotype by proinflammatory cytokines involves NADPH oxidase-dependent ROS generation: consequences for interendothelial adherens and tight junctions," *PLoS One*, vol. 9, no. 7, article e101815, 2014.

[37] A. Elali and S. Rivest, "The role of ABCB1 and ABCA1 in beta-amyloid clearance at the neurovascular unit in Alzheimer's disease," *Frontiers in Physiolology*, vol. 4, p. 45, 2013.

[38] M. I. Borges-Walmsley, K. S. McKeegan, and A. R. Walmsley, "Structure and function of efflux pumps that confer resistance to drugs," *Biochemical Journal*, vol. 376, no. 2, pp. 313–338, 2003.

[39] K. J. Linton, "Structure and function of ABC transporters," *Physiology*, vol. 22, no. 2, pp. 122–130, 2007.

[40] A. H. Schinkel and J. W. Jonker, "Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview," *Advanced Drug Delivery Reviews*, vol. 55, no. 1, pp. 3–29, 2003.

[41] A. G. de Boer, I. C. J. van der Sandt, and P. J. Gaillard, "The role of drug transporters at the blood-brain barrier," *Annual Review of Pharmacology and Toxicology*, vol. 43, no. 1, pp. 629–656, 2003.

[42] W. Löscher and H. Potschka, "Blood-brain barrier active efflux transporters: ATP-binding cassette gene family," *NeuroRx*, vol. 2, no. 1, pp. 86–98, 2005.

[43] E. Bakos and L. Homolya, "Portrait of multifaceted transporter, the multidrug resistance-associated protein 1 (MRP1/ABCC1)," *Pflügers Archiv-European Journal of Physiology*, vol. 453, no. 5, pp. 621–641, 2007.

[44] E. C. M. de Lange, "Potential role of ABC transporters as a detoxification system at the blood–CSF barrier," *Advanced Drug Delivery Reviews*, vol. 56, no. 12, pp. 1793–1809, 2004.

[45] E. M. Leslie, R. G. Deeley, and S. P. C. Cole, "Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense," *Toxicology and Applied Pharmacology*, vol. 204, no. 3, pp. 216–237, 2005.

[46] D. S. Miller, "Regulation of P-glycoprotein and other ABC drug transporters at the blood–brain barrier," *Trends in Pharmacolological Sciences*, vol. 31, no. 6, pp. 246–254, 2010.

[47] A. L. Bartels, "Blood-brain barrier P-glycoprotein function in neurodegenerative disease," *Current Pharmaceutical Design*, vol. 17, no. 26, pp. 2771–2777, 2011.

[48] Á. Fehér, A. Juhász, M. Pákáski, J. Kálmán, and Z. Janka, "ABCB1 C3435T polymorphism influences the risk for Alzheimer's disease," *Journal of Molecular Neuroscience*, vol. 54, no. 4, pp. 826–829, 2014.

[49] J. E. Chin, R. Soffir, K. E. Noonan, K. Choi, and I. B. Roninson, "Structure and expression of the human MDR (P-glycoprotein) gene family," *Molecular and Cellular Biolology*, vol. 9, no. 9, pp. 3808–2380, 1989.

[50] J. J. M. Smit, A. H. Schinkel, R. P. J. Oude Elferink et al., "Homozygous disruption of the murine *MDR2* P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease," *Cell*, vol. 75, no. 3, pp. 451–462, 1993.

[51] R. Bendayan, G. Lee, and M. Bendayan, "Functional expression and localization of P-glycoprotein at the blood brain barrier," *Microscopy Research & Technique*, vol. 57, no. 5, pp. 365–380, 2002.

[52] S. Gazzin, N. Strazielle, C. Schmitt et al., "Differential expression of the multidrug resistance-related proteins ABCb1 and ABCc1 between blood-brain interfaces," *The Journal of Comparative Neurology*, vol. 510, no. 5, pp. 497–507, 2008.

[53] F. Thiebaut, T. Tsuruo, H. Hamada, M. M. Gottesman, I. Pastan, and M. C. Willingham, "Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues," *Proceedings of the National Academy of Sciences of United States of America*, vol. 84, no. 21, pp. 7735–7738, 1987.

[54] A. Wolf, B. Bauer, and A. M. S. Hartz, "ABC transporters and the Alzheimer's disease enigma," *Frontiers in Psychiatry*, vol. 3, pp. 1–14, 2012.

[55] H.-G. G. Bernstein, G. Hölzl, H. Dobrowolny et al., "Vascular and extravascular distribution of the ATP-binding cassette transporters ABCB1 and ABCC1 in aged human brain and pituitary," *Mechanisms of Ageing and Development*, vol. 141-142, pp. 12–21, 2014.

[56] R. F. Keep and D. E. Smith, "Choroid plexus transport: gene deletion studies," *Fluids and Barriers of the CNS*, vol. 8, no. 1, p. 26, 2011.

[57] X. Declèves, A. Regina, J. L. Laplanche et al., "Functional expression of P-glycoprotein and multidrug resistance-associated protein (Mrp1) in primary cultures of rat astrocytes," *Journal of Neuroscience Research*, vol. 60, no. 5, pp. 594–601, 2000.

[58] P. T. Ronaldson, G. Lee, S. Dallas, and R. Bendayan, "Involvement of P-glycoprotein in the transport of saquinavir and indinavir in rat brain microvessel endothelial and microglia cell lines," *Pharmaceutical Research*, vol. 21, no. 5, pp. 811–818, 2004.

[59] P. T. Ronaldson, M. Bendayan, D. Gingras, M. Piquette-Miller, and R. Bendayan, "Cellular localization and functional expression of P-glycoprotein in rat astrocyte cultures," *Journal of Neurochemistry*, vol. 89, no. 3, pp. 788–800, 2004.

[60] P. Ballerini, P. Di Iorio, R. Ciccarelli et al., "Glial cells express multiple ATP binding cassette proteins which are involved in ATP release," *Neuroreport*, vol. 13, no. 14, pp. 1789–1792, 2002.

[61] F. Schlachetzki and W. M. Pardridge, "P-glycoprotein and caveolin-1α in endothelium and astrocytes of primate brain," *Neuroreport*, vol. 14, no. 16, pp. 2041–2046, 2003.

[62] R. Bendayan, P. T. Ronaldson, D. Gingras, and M. Bendayan, "In situ localization of P-glycoprotein (ABCB1) in human and rat brain," *Journal of Histochemistry & Cytochemistry*, vol. 54, no. 10, pp. 1159–1167, 2006.

[63] M. Daood, C. Tsai, M. Ahdab-Barmada, and J. F. Watchko, "ABC transporter (P-gp/ABCB1, MRP1/ABCC1, BCRP/ABCG2) expression in the developing human CNS," *Neuropediatrics*, vol. 39, no. 04, pp. 211–218, 2008.

[64] H. Volk, H. Potschka, and W. Löscher, "Immunohistochemical localization of P-glycoprotein in rat brain and detection of its increased expression by seizures are sensitive to fixation and staining variables," *Journal of Histochemistry & Cytochemistry*, vol. 53, no. 4, pp. 517–531, 2005.

[65] E. Aronica, J. A. Gorter, G. H. Jansen et al., "Expression and cellular distribution of multidrug transporter proteins in two major causes of medically intractable epilepsy: focal cortical dysplasia and glioneuronal tumors," *Neuroscience*, vol. 118, no. 2, pp. 417–429, 2003.

[66] H. Ak, B. Ay, T. Tanriverdi et al., "Expression and cellular distribution of multidrug resistance-related proteins in patients with focal cortical dysplasia," *Seizure*, vol. 16, no. 6, pp. 493–503, 2007.

[67] A. J. Ramos, A. Lazarowski, M. J. Villar, and A. Brusco, "Transient expression of MDR-1/P-glycoprotein in a model of partial cortical devascularization," *Cellular and Molecular Neurobiology*, vol. 24, no. 1, pp. 101–107, 2004.

[68] A. A. Ruefli, K. M. Tainton, P. K. Darcy, M. J. Smyth, and R. W. Johnstone, "P-glycoprotein inhibits caspase-8 activation but not formation of the death inducing signal complex (disc) following Fas ligation," *Cell Death & Differentiation*, vol. 9, no. 11, pp. 1266–1272, 2002.

[69] M. Pallis, J. Turzanski, Y. Higashi, and N. Russell, "P-glycoprotein in acute myeloid leukaemia: therapeutic implications of its association with both a multidrug-resistant and an apoptosis-resistant phenotype," *Leukemia & Lymphoma*, vol. 43, no. 6, pp. 1221–1228, 2002.

[70] J. Turzanski, M. Grundy, S. Shang, N. Russell, and M. Pallis, "P-glycoprotein is implicated in the inhibition of ceramide-induced apoptosis in TF-1 acute myeloid leukemia cells by modulation of the glucosylceramide synthase pathway," *Experimental Hematology*, vol. 33, no. 1, pp. 62–72, 2005.

[71] J. L. Biedler and H. Riehm, "Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies," *Cancer Research*, vol. 30, no. 4, pp. 1174–1184, 1970.

[72] B. Bauer, A. M. S. Hartz, G. Fricker, and D. S. Miller, "Modulation of P-glycoprotein transport function at the blood-brain barrier," *Experimental Biology and Medicine*, vol. 230, no. 2, pp. 118–127, 2005.

[73] B. Sarkadi, L. Homolya, G. Szakács, and A. Váradi, "Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system," *Physiological Reviews*, vol. 86, no. 4, pp. 1179–1236, 2006.

[74] F. Hyafil, C. Vergely, P. Du Vignaud, and T. Grand-Perret, "In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarbonxamide derivative," *Cancer Research*, vol. 53, no. 19, pp. 4595–4602, 1993.

[75] A. H. Dantzig, R. L. Shepard, J. Cao et al., "Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979," *Cancer Research*, vol. 56, no. 18, pp. 4171–4179, 1996.

[76] P. Breedveld, J. H. Beijnen, and J. H. M. Schellens, "Use of P-glycoprotein and BCRP inhibitors to improve oral bioavailability and CNS penetration of anticancer drugs," *Trends in Pharmacological Sciences*, vol. 27, no. 1, pp. 17–24, 2006.

[77] T. B. Ejsing, J. Hasselstrøm, and K. Linnet, "The influence of P-glycoprotein on cerebral and hepatic concentrations of nortriptyline and its metabolites," *Drug Metabolism and Drug Interactions*, vol. 21, no. 3-4, pp. 139–162, 2006.

[78] E. Pussard, M. Merzouk, and H. Barennes, "Increased uptake of quinine into the brain by inhibition of P-glycoprotein," *European Journal of Pharmaceutical Sciences*, vol. 32, no. 2, pp. 123–127, 2007.

[79] J. Schrickx and J. Fink-Gremmels, "P-glycoprotein-mediated transport of oxytetracycline in the Caco-2 cell model," *Journal of Veterinary Pharmacology and Therapeutics*, vol. 30, no. 1, pp. 25–31, 2007.

[80] R. F. Ozols, R. E. Cunnion, R. W. Klecker Jr et al., "Verapamil and adriamycin in the treatment of drug-resistant ovarian cancer patients," *Journal of Clinical Oncology*, vol. 5, no. 4, pp. 641–647, 1987.

[81] J. C. Kalvass, J. W. Polli, D. L. Bourdet et al., "Why clinical modulation of efflux transport at the human blood-brain barrier is unlikely: the ITC evidence-based position," *Clinical Pharmacology & Therapeutics*, vol. 94, no. 1, pp. 80–94, 2013.

[82] A. L. Bartels, A. T. M. Willemsen, R. Kortekaas et al., "Decreased blood-brain barrier P-glycoprotein function in the progression of Parkinson's disease, PSP and MSA," *Journal of Neural Transmission*, vol. 115, no. 7, pp. 1001–1009, 2008.

[83] M. Enokido, A. Suzuki, R. Sadahiro et al., "Implication of P-glycoprotein in formation of depression-prone personality: association study between the C3435T *MDR1* gene polymorphism and interpersonal sensitivity," *Neuropsychobiology*, vol. 69, no. 2, pp. 89–94, 2014.

[84] Y. Li, Y. Li, S. Pang et al., "Novel and functional ABCB1 gene variant in sporadic Parkinson's disease," *Neuroscience Letters*, vol. 566, pp. 61–66, 2014.

[85] M. Santos, S. Carvalho, L. Lima et al., "Common genetic polymorphisms in the *ABCB1* gene are associated with risk of major depressive disorder in male Portuguese individuals," *Genetic Testing and Molecular Biomarkers*, vol. 18, no. 1, pp. 12–19, 2014.

[86] T. P. Akerboom, M. Bilzer, and H. Sies, "Competition between transport of glutathione disulfide (GSSG) and glutathione S-conjugates from perfused rat liver into bile," *FEBS Letters*, vol. 140, no. 1, pp. 73–76, 1982.

[87] T. Akerboom, M. Inoue, H. Sies, R. Kinne, and I. M. Arias, "Biliary transport of glutathione disulfide studied with isolated rat-liver canalicular-membrane vesicles," *European Journal of Biochemistry*, vol. 141, no. 1, pp. 211–215, 1984.

[88] J. Hirrlinger and R. Dringen, "Multidrug resistance protein 1-mediated export of glutathione and glutathione disulfide from brain astrocytes," *Methods in Enzymology*, vol. 400, pp. 395–409, 2005.

[89] P. T. Ronaldson and R. Bendayan, "HIV-1 viral envelope glycoprotein gp120 produces oxidative stress and regulates the functional expression of multidrug resistance protein-1 (Mrp1) in glial cells," *Journal of Neurochemistry*, vol. 106, no. 3, pp. 1298–1313, 2008.

[90] P. Borst, C. de Wolf, and K. van de Wetering, "Multidrug resistance-associated proteins 3, 4, and 5," *Pflugers Archiv – European Journal of Physiology*, vol. 453, no. 5, pp. 661–673, 2007.

[91] J. Hirrlinger, J. König, D. Keppler, J. Lindenau, J. B. Schulz, and R. Dringen, "The multidrug resistance protein MRP1 mediates the release of glutathione disulfide from rat astrocytes during oxidative stress," *Journal of Neurochemistry*, vol. 76, no. 2, pp. 627–636, 2001.

[92] S. M. Dombrowski, S. Y. Desai, M. Marroni et al., "Overexpression of multiple drug resistance genes in endothelial cells from patients with refractory epilepsy," *Epilepsia*, vol. 42, no. 12, pp. 1501–1506, 2001.

[93] A. M. S. Hartz, B. Bauer, M. L. Block, J.-S. Hong, and D. S. Miller, "Diesel exhaust particles induce oxidative stress, proinflammatory signaling, and P-glycoprotein up-regulation at the blood-brain barrier," *FASEB Journal*, vol. 22, no. 8, pp. 2723–2733, 2008.

[94] A. Spudich, E. Kilic, H. Xing et al., "Inhibition of multidrug resistance transporter-1 facilitates neuroprotective therapies

after focal cerebral ischemia," *Nature Neuroscience*, vol. 9, no. 4, pp. 487-488, 2006.

[95] K. M. DeMars, C. Yang, K. E. Hawkins, A. O. McCrea, D. M. Siwarski, and E. Candelario-Jalil, "Spatiotemporal changes in P-glycoprotein levels in brain and peripheral tissues following ischemic stroke in rats," *Journal of Experimental Neuroscience*, vol. 11, article 1179069517701741, 2017.

[96] D. S. Miller and R. E. Cannon, "Signaling pathways that regulate basal ABC transporter activity at the blood- brain barrier," *Current Pharmaceutical Design*, vol. 20, no. 10, pp. 1463–1471, 2014.

[97] K. I. Hirsch-Ernst, T. Kietzmann, C. Ziemann, K. Jungermann, and G. F. Kahl, "Physiological oxygen tensions modulate expression of the mdr1b multidrug-resistance gene in primary rat hepatocyte cultures," *Biochemical Journal*, vol. 350, no. 2, pp. 443–451, 2000.

[98] F. Thévenod, J. M. Friedmann, A. D. Katsen, and I. A. Hauser, "Up-regulation of multidrug resistance P-glycoprotein via nuclear factor-κB activation protects kidney proximal tubule cells from cadmium- and reactive oxygen species-induced apoptosis," *Journal of Biological Chemistry*, vol. 275, no. 3, pp. 1887–1896, 2000.

[99] M. Wartenberg, K. Fischer, J. Hescheler, and H. Sauer, "Redox regulation of P-glycoprotein-mediated multidrug resistance in multicellular prostate tumor spheroids," *International Journal of Cancer*, vol. 85, no. 2, pp. 267–274, 2000.

[100] R. A. Felix and M. A. Barrand, "P-glycoprotein expression in rat brain endothelial cells: evidence for regulation by transient oxidative stress," *Journal of Neurochemistry*, vol. 80, no. 1, pp. 64–72, 2002.

[101] H. Hong, Y. Lu, Z.-N. Ji, and G.-Q. Liu, "Up-regulation of P-glycoprotein expression by glutathione depletion-induced oxidative stress in rat brain microvessel endothelial cells," *Journal of Neurochemistry*, vol. 98, no. 5, pp. 1465–1473, 2006.

[102] L. Behrend, G. Henderson, and R. M. Zwacka, "Reactive oxygen species in oncogenic transformation," *Biochemical Society Transactions*, vol. 31, no. 6, pp. 1441–1444, 2003.

[103] G. Wu, L. Zhu, X. Yuan et al., "Britanin ameliorates cerebral ischemia-reperfusion injury by inducing the Nrf2 protective pathway," *Antioxidants & Redox Signaling*, vol. 27, no. 11, pp. 754–768, 2017.

[104] X. Zhao, L. Wen, M. Dong, and X. Lu, "Sulforaphane activates the cerebral vascular Nrf2-ARE pathway and suppresses inflammation to attenuate cerebral vasospasm in rat with subarachnoid hemorrhage," *Brain Research*, vol. 1653, pp. 1–7, 2016.

[105] M. Zhang, C. An, Y. Gao, R. K. Leak, J. Chen, and F. Zhang, "Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection," *Progress in Neurobiology*, vol. 100, pp. 30–47, 2013.

[106] F. Morroni, G. Sita, A. Tarozzi, G. Cantelli-Forti, and P. Hrelia, "Neuroprotection by 6-(methylsulfinyl)hexyl isothiocyanate in a 6-hydroxydopamine mouse model of Parkinson's disease," *Brain Research*, vol. 1589, pp. 93–104, 2014.

[107] L. Wang, M. Wang, J. Hu et al., "Protective effect of 3H-1, 2-dithiole-3-thione on cellular model of Alzheimer's disease involves Nrf2/ARE signaling pathway," *European Journal of Pharmacology*, vol. 795, pp. 115–123, 2017.

[108] X. Wang, C. R. Campos, J. C. Peart et al., "Nrf2 upregulates ATP binding cassette transporter expression and activity at the blood-brain and blood-spinal cord barriers," *The Journal of Neuroscience*, vol. 34, no. 25, pp. 8585–8593, 2014.

[109] S. W. Pimplikar, R. A. Nixon, N. K. Robakis, J. Shen, and L.-H. Tsai, "Amyloid-independent mechanisms in Alzheimer's disease pathogenesis," *The Journal of Neuroscience*, vol. 30, no. 45, pp. 14946–14954, 2010.

[110] D. E. Barnes and K. Yaffe, "The projected effect of risk factor reduction on Alzheimer's disease prevalence," *The Lancet Neurology*, vol. 10, no. 9, pp. 819–828, 2011.

[111] B. V. Zlokovic, R. Deane, A. P. Sagare, R. D. Bell, and E. A. Winkler, "Low-density lipoprotein receptor-related protein-1: a serial clearance homeostatic mechanism controlling Alzheimer's amyloid β-peptide elimination from the brain," *Journal of Neurochemistry*, vol. 115, no. 5, pp. 1077–1089, 2010.

[112] F. C. Lam, R. Liu, P. Lu et al., "β-amyloid efflux mediated by P-glycoprotein," *Journal of Neurochemistry*, vol. 76, no. 4, pp. 1121–1128, 2001.

[113] R. Deane, S. Du Yan, R. K. Submamaryan et al., "RAGE mediates amyloid-β peptide transport across the blood-brain barrier and accumulation in brain," *Nature Medicine*, vol. 9, no. 7, pp. 907–913, 2003.

[114] A. M. S. Hartz, D. S. Miller, and B. Bauer, "Restoring blood-brain barrier P-glycoprotein reduces brain amyloid-β in a mouse model of Alzheimer's disease," *Molecular Pharmacology*, vol. 77, no. 5, pp. 715–723, 2010.

[115] P. Candela, F. Gosselet, J. Saint-Pol et al., "Apical-to-basolateral transport of amyloid-β peptides through blood-brain barrier cells is mediated by the receptor for advanced glycation end-products and is restricted by P-glycoprotein," *Journal of Alzheimer's Disease*, vol. 22, no. 3, pp. 849–859, 2010.

[116] S. Ohtsuki, S. Ito, and T. Terasaki, "Is P-glycoprotein involved in amyloid-β elimination across the blood–brain barrier in Alzheimer's disease?," *Clinical Pharmacology & Therapeutics*, vol. 88, no. 4, pp. 443–445, 2010.

[117] T. W. Loo and D. M. Clarke, "The transmembrane domains of the human multidrug resistance P-glycoprotein are sufficient to mediate drug binding and trafficking to the cell surface," *Journal of Biological Chemistry*, vol. 274, no. 35, pp. 24759–24765, 1999.

[118] A. M. S. Hartz, Y. Zhong, A. Wolf, H. LeVine, D. S. Miller, and B. Bauer, "Aβ40 reduces P-glycoprotein at the blood-brain barrier through the ubiquitin-proteasome pathway," *The Journal of Neuroscience*, vol. 36, no. 6, pp. 1930–1941, 2016.

[119] S. Vogelgesang, I. Cascorbi, E. Schroeder et al., "Deposition of Alzheimer's β-amyloid is inversely correlated with P-glycoprotein expression in the brains of elderly non-demented humans," *Pharmacogenetics and Genomics*, vol. 12, no. 7, pp. 535–541, 2002.

[120] D. M. van Assema, M. Lubberink, P. Rizzu et al., "Blood–brain barrier P-glycoprotein function in healthy subjects and Alzheimer's disease patients: effect of polymorphisms in the ABCB1 gene," *European Journal of Nuclear Medicine and Molecular Imaging Research*, vol. 2, no. 1, p. 57, 2012.

[121] H. C. Wijesuriya, J. Y. Bullock, R. L. Faull, S. B. Hladky, and M. A. Barrand, "ABC efflux transporters in brain vasculature of Alzheimer's subjects," *Brain Research*, vol. 1358, pp. 228–238, 2010.

[122] A. Carrano, J. J. M. Hoozemans, S. M. van der Vies, A. J. M. Rozemuller, J. van Horssen, and H. E. de Vries, "Amyloid

beta induces oxidative stress-mediated blood-brain barrier changes in capillary amyloid angiopathy," *Antioxidants & Redox Signaling*, vol. 15, no. 5, pp. 1167–1178, 2011.

[123] K. D. Kania, H. C. Wijesuriya, S. B. Hladky, and M. A. Barrand, "Beta amyloid effects on expression of multidrug efflux transporters in brain endothelial cells," *Brain Research*, vol. 1418, pp. 1–11, 2011.

[124] R. Park, S.-Y. Kook, J.-C. Park, and I. Mook-Jung, "A$\beta_{1\text{-}42}$ reduces P-glycoprotein in the blood–brain barrier through RAGE–NF-κB signaling," *Cell Death & Disease*, vol. 5, no. 6, article e1299, 2014.

[125] A. M. S. Hartz, B. Bauer, E. L. B. Soldner et al., "Amyloid-β contributes to blood-brain barrier leakage in transgenic human amyloid precursor protein mice and in humans with cerebral amyloid angiopathy," *Stroke*, vol. 43, no. 2, pp. 514–523, 2012.

[126] Y. Yamazaki and T. Kanekiyo, "Blood-brain barrier dysfunction and the pathogenesis of Alzheimer's disease," *International Journal of Molecular Science*, vol. 18, no. 9, p. 1965, 2017.

[127] S. Liu, Y. Liu, W. Hao et al., "TLR2 is a primary receptor for Alzheimer's amyloid β peptide to trigger neuroinflammatory activation," *The Journal of Immunology*, vol. 188, no. 3, pp. 1098–1107, 2012.

[128] H. Akiyama, S. Barger, S. Barnum et al., "Inflammation and Alzheimer's disease," *Neurobiology of Aging*, vol. 21, no. 3, pp. 383–421, 2000.

[129] G. Kooij, R. Backer, J. J. Koning et al., "P-glycoprotein acts as an immunomodulator during neuroinflammation," *PLoS One*, vol. 4, no. 12, p. e8212, 2009.

[130] D. A. Butterfield, T. Reed, S. F. Newman, and R. Sultana, "Roles of amyloid β-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment," *Free Radical Biology and Medicine*, vol. 43, no. 5, pp. 658–677, 2007.

[131] V. D. Bianca, S. Dusi, E. Bianchini, I. Dal Prà, and F. Rossi, "β-Amyloid activates the O_2^- forming NADPH oxidase in microglia, monocytes, and neutrophils. A possible inflammatory mechanism of neuronal damage in Alzheimer's disease," *The Journal of Biological Chemistry*, vol. 274, no. 22, pp. 15493–15499, 1999.

[132] A. Oda, A. Tamaoka, and W. Araki, "Oxidative stress up-regulates presenilin 1 in lipid rafts in neuronal cells," *Journal of Neuroscience Research*, vol. 88, no. 5, pp. 1137–1145, 2010.

[133] M. A. Erickson, P. E. Hartvigson, Y. Morofuji, J. B. Owen, D. A. Butterfield, and W. A. Banks, "Lipopolysaccharide impairs amyloid beta efflux from brain: altered vascular sequestration, cerebrospinal fluid reabsorption, peripheral clearance and transporter function at the blood-brain barrier," *Journal of Neuroinflammation*, vol. 9, p. 150, 2012.

[134] L. Rink, I. Cakman, and H. Kirchner, "Altered cytokine production in the elderly," *Mechanisms of Ageing and Development*, vol. 102, no. 2-3, pp. 199–209, 1998.

[135] S. Bruckmann, A. Brenn, M. Grube et al., "Lack of P-glycoprotein results in impairment of removal of beta-amyloid and increased intraparenchymal cerebral amyloid angiopathy after active immunization in a transgenic mouse model of Alzheimer's disease," *Current Pharmaceutical Design*, vol. 14, no. 6, pp. 656–667, 2017.

[136] A. Kumar, M. J. Ekavali, K. Chopra, and D. K. Dhull, "Possible role of P-glycoprotein in the neuroprotective mechanism of berberine in intracerebroventricular streptozotocin-induced cognitive dysfunction," *Psychopharmacology*, vol. 233, no. 1, pp. 137–152, 2016.

[137] D. S. Miller, "Regulation of ABC transporters blood-brain barrier: the good, the bad, and the ugly," *Advances in Cancer Research*, vol. 125, pp. 43–70, 2015.

[138] Y. Nishida, S. Ito, S. Ohtsuki et al., "Depletion of vitamin E increases amyloid β accumulation by decreasing its clearances from brain and blood in a mouse model of Alzheimer disease," *Journal of Biological Chemistry*, vol. 284, no. 48, pp. 33400–33408, 2009.

[139] R. E. Watkins, G. B. Wisely, L. B. Moore et al., "The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity," *Science*, vol. 292, no. 5525, pp. 2329–2333, 2001.

[140] M. B. Loeb, D. W. Molloy, M. Smieja et al., "A randomized, controlled trial of doxycycline and rifampin for patients with Alzheimer's disease," *Journal of the American Geriatrics Society*, vol. 52, no. 3, pp. 381–387, 2004.

[141] A. H. Abuznait, H. Qosa, B. A. Busnena, K. A. El Sayed, and A. Kaddoumi, "Olive-oil-derived oleocanthal enhances β-amyloid clearance as a potential neuroprotective mechanism against Alzheimer's disease: in vitro and in vivo studies," *ACS Chemical Neuroscience*, vol. 4, no. 6, pp. 973–982, 2013.

[142] H. Qosa, Y. S. Batarseh, M. M. Mohyeldin, K. A. El Sayed, J. N. Keller, and A. Kaddoumi, "Oleocanthal enhances amyloid-β clearance from the brains of TgSwDI mice and in vitro across a human blood-brain barrier model," *ACS Chemical Neuroscience*, vol. 6, no. 11, pp. 1849–1859, 2015.

[143] A. Brenn, M. Grube, G. Jedlitschky et al., "St. John's wort reduces beta-amyloid accumulation in a double transgenic Alzheimer's disease mouse model-role of P-glycoprotein," *Brain Pathology*, vol. 24, no. 1, pp. 18–24, 2014.

[144] E. C. Y. Chow, M. R. Durk, C. L. Cummins, and K. S. Pang, "1α,25-dihydroxyvitamin D_3 up-regulates P-glycoprotein via the vitamin D receptor and not farnesoid X receptor in both *fxr*(−/−) and *fxr*(+/+) mice and increased renal and brain efflux of digoxin in mice in vivo," *Journal of Pharmacology and Experimental Therapeutics*, vol. 337, no. 3, pp. 846–859, 2011.

[145] M. R. Durk, G. N. Y. Chan, C. R. Campos et al., "1α,25-Dihydroxyvitamin D_3-liganded vitamin D receptor increases expression and transport activity of P-glycoprotein in isolated rat brain capillaries and human and rat brain microvessel endothelial cells," *Journal of Neurochemistry*, vol. 123, no. 6, pp. 944–953, 2012.

[146] M. R. Durk, K. Han, E. C. Y. Chow et al., "1α,25-Dihydroxyvitamin D_3 reduces cerebral amyloid-β accumulation and improves cognition in mouse models of Alzheimer's disease," *Journal of Neuroscience*, vol. 34, no. 21, pp. 7091–7101, 2014.

[147] S. Manda, S. Sharma, A. Wani et al., "Discovery of a marine-derived bis-indole alkaloid fascaplysin, as a new class of potent P-glycoprotein inducer and establishment of its structure-activity relationship," *European Journal of Medical Chemistry*, vol. 107, pp. 1–11, 2016.

[148] L. A. Mohamed, J. N. Keller, and A. Kaddoumi, "Role of P-glycoprotein in mediating rivastigmine effect on amyloid-β brain load and related pathology in Alzheimer's disease mouse model," *Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease*, vol. 1862, no. 4, pp. 778–787, 2016.

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Review Article

The Significance of Hypothiocyanite Production via the Pendrin/DUOX/Peroxidase Pathway in the Pathogenesis of Asthma

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Inhaled corticosteroids (ICSs) are used as first-line drugs for asthma, and various novel antiasthma drugs targeting type 2 immune mediators are now under development. However, molecularly targeted drugs are expensive, creating an economic burden on patients. We and others previously found pendrin/SLC26A4 as a downstream molecule of IL-13, a signature type 2 cytokine critical for asthma, and showed its significance in the pathogenesis of asthma using model mice. However, the molecular mechanism of how pendrin causes airway inflammation remained elusive. We have recently demonstrated that hypothiocyanite ($OSCN^-$) produced by the pendrin/DUOX/peroxidase pathway has the potential to cause airway inflammation. Pendrin transports thiocyanate (SCN^-) into pulmonary lumens at the apical side. Peroxidases catalyze SCN^- and H_2O_2 generated by DUOX into $OSCN^-$. Low doses of $OSCN^-$ activate NF-κB in airway epithelial cells, whereas $OSCN^-$ in high doses causes necrosis of the cells, inducing the release of IL-33 and accelerating inflammation. $OSCN^-$ production is augmented in asthma model mice and possibly in some asthma patients. Heme peroxidase inhibitors, widely used as antithyroid agents, diminish asthma-like phenotypes in mice, indicating the significance of this pathway. These findings suggest the possibility of repositioning antithyroid agents as antiasthma drugs.

1. Introduction

Asthma is a common and chronic respiratory disease characterized by variable symptoms and features—wheezing, shortness of breath, cough, and expiratory airflow limitation [1]. Asthma is estimated to affect at least 300 million people worldwide, making it a significant medical and social problem. Inhaled corticosteroids (ICSs) are used as first-line drugs for asthma. Although ICSs are very effective, 5–10% of asthma patients are estimated as having severe asthma characterized by difficulty to achieve disease control despite high-dose ICSs plus long-acting β2-agonists or oral corticosteroids, which accounts for about 50% of the total costs for treating asthma [2, 3]. It is known that type 2 inflammation is dominant in the pathogenesis of asthma [4]. Based on this immunological background, various novel antiasthma drugs targeting type 2 immune mediators—interleukin- (IL-) 4, IL-5, IL-13, TSLP, IL-33, and CRTH2—are now under development [5]. However, molecularly targeted drugs, mostly biologics, are expensive, creating an economic burden on patients. Therefore, it is of great importance to elucidate the pathogenesis of severe asthma to help identify therapeutic strategies that will be more affordable for these patients.

It has been sporadically reported that IL-4 and/or IL-13, signature type 2 cytokines, influence anion transport in airway tissues [6]. However, the underlying mechanism of how anion transport in airway tissues leads to inflammation has not been sufficiently explained. We and others previously found that pendrin/SLC26A4, an anion transporter located

at the apical side of airway epithelial cells, is a downstream molecule of the IL-4/IL-13 signals that plays an important role in the pathogenesis of asthma [7, 8]. We then investigated how pendrin causes airway inflammation, pinpointing the significance of the hypothiocyanite ($OSCN^-$) production via the pendrin/DUOX/peroxidase pathway [9, 10]. These results have revealed for the first time the involvement of anion or its derivative in the pathogenesis of asthma. Moreover, these findings suggest to us that we can apply antithyroid agents, pan-heme peroxidase inhibitors, to drug repositioning for antiasthma drugs.

In this article, we describe how we started our research and how we have arrived at these findings.

2. Discovery of Pendrin as a Downstream Molecule of the IL-4/IL-13 Signals

IL-4 and IL-13 are signature cytokines of type 2 inflammation produced by T_H2 cells, follicular helper T cells, group 2 innate lymphoid cells (ILC2), eosinophils, mast cells, and basophils [11–14]. A number of analyses using asthma model mice have established the significance of IL-4 and/or IL-13, particularly the latter, in the pathogenesis of asthma [11, 15–17]. Based on these findings, several IL-4 or IL-13 signaling antagonists such as tralokinumab and dupilumab are now under clinical development as antiasthma drugs [18, 19]. To identify a novel mediator involved in asthma pathogenesis downstream of the IL-13 signals, we and others previously used DNA microarray to search for IL-13-induced molecules in human airway epithelial cells, finding that the *SLC26A4* gene encoding pendrin is a downstream molecule of IL-13 [7, 20, 21]. Moreover, Pedemonte et al. found that IL-4 increases thiocyanate (SCN^-) transport in human airway epithelial cells independently of the cystic fibrosis transmembrane conductance regulator (CFTR) [22]. They found that among the investigated transporters, the *SLC26A4* gene was significantly induced by IL-4 and that pendrin is responsible for the SCN^-/Cl^- exchange. Thus, pendrin appears to be an IL-4- or IL-13-inducible molecule.

In agreement with the *in vitro* experiments, we and others have demonstrated that pendrin is highly expressed in the lungs of asthma model mice such as ovalbumin-inhaled, IL-13-inhaled, and IL-13 transgenic mice [7, 20–23]. We showed that pendrin is expressed in the apical side of airway epithelial cells in ovalbumin-inhaled mice [7]. Nonciliated airway epithelial cells are likely the main pendrin-expressing cells when stimulated by IL-4/IL-13, because pendrin expression is upregulated in the IL-13-overexpressing mice, in which STAT6 is expressed only in nonciliated airway epithelial cells [21]. Moreover, pendrin expression was enhanced in model mice of both acute and chronic asthma [23].

Since the *SLC26A4* gene is an IL-4/IL-13-inducible molecule, it was reasonable to think that STAT6, a transcriptional factor critical for the IL-4/IL-13 signals, regulates the expression of the *SLC26A4* gene. Nofziger et al. found that there exist two consensus binding sites for STAT6 ($TTC(N_4)GAA$) at −3472 to −3463 (motif 1) and −1812 to −1803 (motif 2) of the 5′-flanking region of the *SLC26A4* gene [24, 25]. Vanoni et al. showed that although both consensus sequences can bind STAT6 following IL-4 exposure, IL-4- or IL-13-inducible pendrin expression requires only motif 2 [25]. These results suggest that IL-4 or IL-13 induces expression of the *SLC26A4* gene in a *cis*-regulating manner.

It has been thereafter demonstrated that in addition to IL-4 and IL-13, pendrin expression in lung tissues or airway epithelial cells can have other causes. These include various cytokines, such as IL-1β [22, 26] and IL-17A [27–29]. Also possible are various environmental stimuli such as silica [30], welding fumes [31], C_{60} fullerene [32], and single-wall carbon nanotubes [33]. In addition, pathogenic microbes or microbe-derived molecules—pertussis toxin [27, 34] and a combination of interferon-γ (IFN-γ) and rhinovirus [8]—can be a cause. These findings expand the potential of pathophysiological roles of pendrin. It is of note that the combination of IL-13 and IL-17A enhances pendrin expression in airway epithelial cells [29]. Since expression of IL-17A is a hallmark of severe asthma correlated with infiltration of neutrophils [35], pendrin may be maximally expressed in severe asthma patients.

3. The Pathological Roles of Pendrin in Asthma

Using model mice, we and Nakagami et al. have previously demonstrated the significance of pendrin in the pathogenesis of airway allergic inflammation [7, 8]. Overexpression of pendrin in bronchial tissues leads to mucus hyperproduction, enhanced airway hyperreactivity (AHR), and upregulation of chemokine expression followed by infiltration of neutrophils [7]. Reciprocally, ovalbumin challenge for pendrin-deficient mice decreases airway reactivity and infiltration of inflammatory cells, including eosinophils in bronchoalveolar lavage fluid (BALF), although systemic IgE production, mucus production, and the production of type 2 cytokines do not change [8]. Accordingly, it has been reported that pendrin expression was enhanced in asthma patients compared to control subjects [36], although there is a contradictory report [21]. Moreover, it has been shown that pendrin is highly expressed in the nasal mucosa of patients with chronic rhinosinusitis with nasal polyps (CRSwNP) and allergic rhinitis (AR) [29, 37]. It is known that the existence of eosinophilic CRS in asthma patients is a risk factor for worsening asthma and that the presence of concomitant AR can affect the severity of asthma [38]. These results support the pathogenic significance of pendrin in both upper and lower respiratory allergic inflammation.

The involvement of pendrin in the pathogenesis of airway inflammation has been expanded into COPD and pertussis disease. We found that pendrin expression was enhanced in the lung tissues of esterase-inhaled mice mimicking COPD [7]. In COPD model mice, pendrin is expressed at the apical side of epithelial cells, as it is in asthma model mice, followed by expression of Muc5ac and Muc5b. Since it is known that expression of IL-17A and IL-13 is enhanced in COPD patients [39–41], the combination of these cytokines may induce pendrin expression in COPD. Scanlon et al., moreover, have demonstrated that

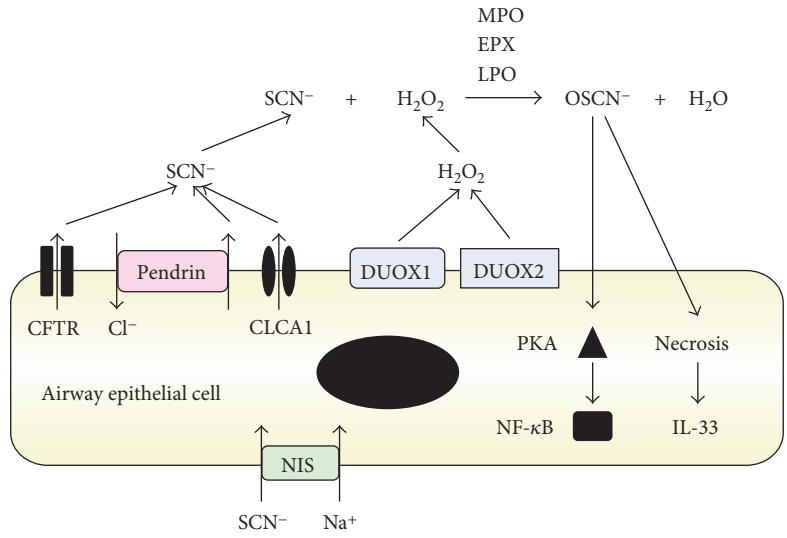

Figure 1: Schematic model of $OSCN^-$ production via the pendrin/DUOX/peroxidase pathway in airway epithelial cells (modified from [9]). In airway epithelial cells, SCN^- is actively transported into pulmonary lumens via NIS/SLC5A5 at the basal side and via several anion transporters including CFTR and pendrin/SLC26A4 at the apical side. SCN^- together with H_2O_2 generated by Duox1 and Duox2 is catalyzed by peroxidases into $OSCN^-$. Three peroxidases including MPO, EPX, and LPO are involved in this reaction. A low dose of $OSCN^-$ activates NF-κB via PKA, whereas a high dose of $OSCN^-$ causes necrosis followed by release of IL-33 in airway epithelial cells. It is of note that if peroxidases are inhibited, it would protect airway epithelial cells against inflammation.

Bordetella pertussis induces pendrin expression in an IL-17A-dependent manner and that pendrin deficiency improves *Bordetella pertussis*-induced inflammation but does not affect bacteria colonization [27].

4. The Role of Pendrin in Airway Surface Liquid (ASL)

Airway surfaces are covered by a thin layer of fluid, ASL, whose composition and volume are critical to ensure proper mucociliary clearance and maintain innate defense systems [6]. The amount and composition of ASL are regulated by the balance between fluid secretion and absorption coordinated by several ion channels and transporters, including pendrin. Nakagami et al. and Lee et al. showed that ASL is thickened in pendrin-deficient tracheal cells stimulated by IL-13 and deaf patients carrying pendrin mutations, probably because of dysregulated anion transport [8, 42]. This suggests the possibility that thickened ASL can enhance mucus clearance and improve airway function, which may at least partially explain how periostin deficiency improves asthma-like phenotypes. Increased pendrin expression following allergen challenges may lead to ASL dehydration and then to airway inflammation and obstruction, thereby exacerbating asthma.

5. The Pathological Role of the Pendrin/DUOX/Peroxidase Pathway in Asthma

The finding that pendrin is important for the onset of airway inflammation suggested to us that anions transported by pendrin, or their derivatives, could play an important role in asthma.

Among various anions, we focused on SCN^-, because pendrin can transport SCN^- into the apical side of airway epithelial cells [22] and $OSCN^-$ derived from SCN^- plays a critical role in the innate defense of mucosal surfaces [43–45]. SCN^- is incorporated from the basal side into airway epithelial cells by the Na^+I^- symporter (NIS)/SLC5A5 and then is actively transported into pulmonary lumens at the apical side by CFTR and pendrin (Figure 1). In contrast, DUOX1 and/or DUOX2, members of the NOX/DUOX family, generate hydrogen peroxide (H_2O_2) in pulmonary lumens. SCN^- and H_2O_2 are catalyzed into $OSCN^-$ by three peroxidases—myeloperoxidase (MPO), eosinophil peroxidase (EPX), and lactoperoxidase (LPO)—expressed in neutrophils, eosinophils, and epithelial cells, respectively, in the lung tissue. $OSCN^-$ has potent antimicrobial properties against bacteria, viruses, and fungi, as seen in cystic fibrosis patients, whose susceptibility to chronic respiratory infections increases in proportion to impaired CFTR function [46].

We examined whether the production of $OSCN^-$ leads to inflammation in airway epithelial cells (H292 cells) using an *in vitro* $OSCN^-$ production system [9]. In this system, when we added only β-D-glucose and glucose oxidase (GOX) in the reaction mixture, H_2O_2 was generated by GOX using β-D-glucose and oxygen (Figure 2(a)). When SCN^- and LPO were furthermore added in the mixture, $OSCN^-$ was generated by LPO via the oxidation of SCN^- with H_2O_2 (Figure 2(b)). Using this system, we compared the ability of H_2O_2 and $OSCN^-$ to activate NF-κB, finding that $OSCN^-$, but not H_2O_2, activated NF-κB (Figure 1). Activation of NF-κB in airway epithelial cells is important for production of chemokines and inflammatory cytokines as well as for expression of adhesion molecules accelerating type 2 immunity [47]. $OSCN^-$ was sensed by protein kinase A (PKA) followed by the dimerization of PKA.

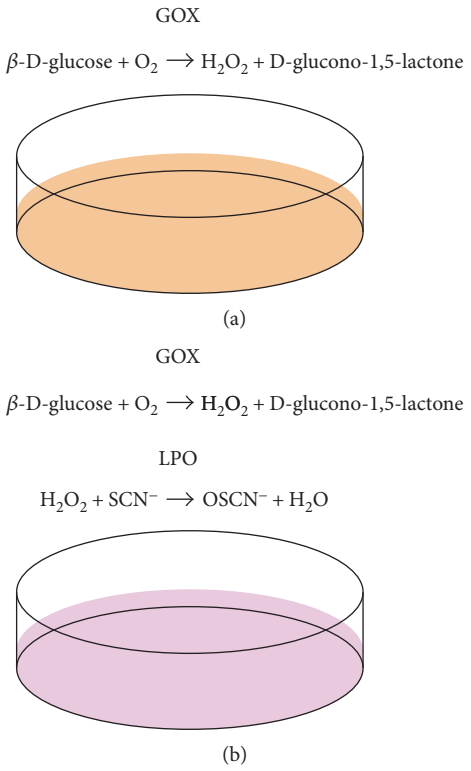

Figure 2: *In vitro* $OSCN^-$ production system (modified from [9]). When only β-D-glucose and glucose oxidase (GOX) are added into the system using airway epithelial cells (H292 cells), H_2O_2 is generated (a). When more SCN^- and LPO are added, H_2O_2 is catalyzed into $OSCN^-$ (b). Thus, in this system, the oxidative activities of H_2O_2 and $OSCN^-$ can be estimated with or without addition of SCN^- and LPO.

The regulatory subunit of type I PKA is dimerized through a disulfide bond followed by increased affinity for the substrates, demonstrating that oxidative stress is changed to intracellular signaling through PKA, independently of cAMP [48]. The stronger oxidative ability of $OSCN^-$ compared to H_2O_2 may be due to the existence of a detoxifying system for H_2O_2, mainly by catalase in airway epithelial cells. Furthermore, $OSCN^-$ in high doses caused necrosis of the cells, inducing release of IL-33, which acts on several immune cells—ILC2, mast cells, basophils, eosinophils, and T_H2 cells accelerating type 2 inflammation [49]. To our knowledge, $OSCN^-$ is the first anion to activate NF-κB in epithelial cells. Thus, we have shown that $OSCN^-$ produced by the pendrin/DUOX/peroxidase pathway potentially plays an important role in the pathogenesis of asthma.

6. Enhancement of the $OSCN^-$ Production System in Asthma

We next examined whether $OSCN^-$ production via the pendrin/DUOX/peroxidase pathway is enhanced in asthma model mice (Table 1) [10]. Expression of pendrin was significantly enhanced, as shown in a previous study [7], whereas expression of CFTR did not change. Expression of all three heme peroxidases (*Mpo*, *Epx*, and *Lpo*) together with peroxidase activities in BALFs was enhanced in allergen-challenged mice. Moreover, the expression of DUOX1, but not DUOX2, was also significantly enhanced in allergen-challenged mice, consistent with a previous report showing that IL-4 can induce DUOX1 [50]. These results suggest that the $OSCN^-$ production machinery is enhanced in asthma model mice. We then investigated whether expression of the heme peroxidases was enhanced in the bronchial tissues of mild to moderate asthma patients well controlled with inhaled corticosteroids [50]. The peroxidase activities and the expression levels of *LPO* were not statistically enhanced. However, some patients showed distinctly high peroxidase activities and *LPO* expression. The clinical severity of their asthma, modified treatment for these patients, and/or heterogeneity among asthma patients may affect airway peroxidase expression, although the precise factor causing the difference is unclear at this moment. Expression of neither *EPO* nor *MPO* was detected. These results confirm that $OSCN^-$ production via pendrin/DUOX/peroxidase is augmented in asthma model mice and possibly in some asthma patients.

Table 1: Change of the machineries of the $OSCN^-$ production system in asthma model mice and asthma patients.

Molecule	Asthma model mouse	Asthma patient
Pendrin	↑	↑/→*
CFTR	→	
Heme peroxidase		
Myeloperoxidase	↑	ND
Eosinophil peroxidase	↑	ND
Lactoperoxidase	↑	↑/→
DUOX1	↑	→
DUOX2	→	↑/→

Expression changes of pendrin, CFTR, MPO, EPX, LPO, DUOX1, and DUOX2 in asthma model mice and asthma patients are depicted. ND: not detected. *Referred from [9, 10].

To define the pathological roles of peroxidase in bronchial asthma, we applied heme peroxidase inhibitors to an asthma mouse model [10]. We examined the effects of 2-mercapto-1-methylimidazole (methimazole) and 6-propyl-2-thiouracil (PTU), which are agents that inhibit all peroxidases and are widely used as antithyroid agents targeting thyroid peroxidase. The long administration of methimazole (orally every day from the start of sensitization (day 0)) completely inhibited airway inflammation—enhanced AHR, infiltration of inflammatory cells in BALF, and histological changes (Table 2). Short administration (from two days before the start of the allergen airway challenge (day 20)) inhibited inflammation less so, yet significantly. Another peroxidase-inhibiting antithyroid agent, PTU, showed effects similar to but weaker than those of methimazole. These results strongly suggest that heme peroxidase activities are critical for the onset of allergic airway inflammation in these model mice. Our results appear consistent with the findings of several reports showing that accidental administration of antithyroid agents provided beneficial effects to asthma patients [51, 52], although there

Table 2: Effects of peroxidase inhibitor and genetic deficiency of each peroxidase on asthma model mice (referred from [10]).

Phenotype	Met-L	Met-S	*Lpo*$^{-}$	*Epx*$^{-}$	*Mpo*$^{-}$
AHR	↓↓↓	↓↓	↓	↓	→
BALF					
Eosinophil	↓↓↓	↓↓	↓↓	→	→
T cell	↓↓↓	↓	↓↓	↓	→
Neutrophil	↓↓↓	↓↓	↓	→	→
Macrophage	→	→	↓	→	→

Effects of the long (Met-L) or short (Met-S) administration of methimazole or genetic deficiency of *Lpo* (*Lpo*$^{-}$), *Epx* (*Epx*$^{-}$), and *Mpo* (*Mpo*$^{-}$) on enhanced AHR and the numbers of eosinophils, T cells, neutrophils, and macrophages in BALF of asthma model mice are depicted.

is one conflicting report [53]. It is of note that in most patients, bronchial asthma was exacerbated by discontinuing or tapering off antithyroid agents [51, 52].

Next, we examined which peroxidase dominantly contributes to the onset of allergic airway inflammation using mice deficient in each of the three peroxidases (*Mpo*, *Epx*, and *Lpo*) [10]. *Epx*- and *Lpo*-deficient mice showed a nominal but not statistically significant decrease of AHR compared to their control littermates, whereas the *Mpo*-deficient mice showed no change of AHR (Table 2). Furthermore, infiltration of eosinophils and T cells was decreased in the BALF of the *Lpo*-deficient mice, whereas there was no change in infiltration in the *Mpo*- or *Epx*-deficient mice. These results suggest that the contributions of the three peroxidases are redundant in the onset of allergic airway inflammation. However, *Lpo* appears to be dominant among the peroxidases.

Taking these results together, we assume that whereas the $OSCN^{-}$ production system may be an innate host defense mechanism in the lung, this misplaced production of $OSCN^{-}$ is likely to contribute to pulmonary inflammation, causing deleterious effects in response to airway allergen provocation.

7. Clinical Application of the Pathological Significance of the Pendrin/DUOX/Peroxidase Pathway to Asthma

The findings showing the importance of the $OSCN^{-}$ production via the pendrin/DUOX/peroxidase pathway in asthma indicate that all of the machineries of the $OSCN^{-}$ production system can be viewed as potential novel therapeutic targets for asthma. It is of note that we have confirmed that heme peroxidase inhibitors widely used as antithyroid agents are efficacious for inhibiting allergic airway inflammation in mice. This suggests that we can apply antithyroid agents to drug repositioning for antiasthma drugs. Drug repositioning, which is the process of finding new therapeutic indications for existing drugs, is now seen as a less expensive alternative to drug discovery and development [54, 55]. The use of antithyroid agents could be the first example of drug repositioning for asthma. Various antiasthma drugs targeting type 2 immune mediators, such as IL-4, IL-5, IL-13, TSLP, IL-33, and CRTH2, are now under development [5]. However, to develop novel drugs, particularly biologics, huge investments of time and money are required, and safety risks are involved. Moreover, most molecularly targeted drugs for asthma under development are biologics, which are relatively expensive. Clearly, drug repositioning in asthma can potentially decrease the economic burden on asthma patients.

Moreover, the importance of the $OSCN^{-}$ by the pendrin/DUOX/peroxidase pathway in asthma can be applied to airway inflammation in smokers. Plasma SCN^{-} levels in smokers are almost three times higher than in nonsmokers (130–140 μM versus 40–50 μM) [56, 57]. It is well known that smoking is associated with poor control, decrease of lung function, and enhanced corticosteroid resistance in asthma [58, 59]. However, no deleterious effects of SCN^{-} or $OSCN^{-}$ derived from tobacco on the lungs have yet been reported. The findings suggest the possible involvement of the $OSCN^{-}$ production system in how smoking affects asthma or other smoking-related pulmonary diseases, giving us clues on how best to treat asthma patients who smoke.

8. Conclusion

After showing that pendrin/SLC26A4 is a downstream molecule of IL-13 and that it is actively involved in the pathogenesis of airway allergic inflammation, we investigated the underlying molecular mechanism of how this occurs. As a result, we have demonstrated the significance of $OSCN^{-}$ production via the pendrin/DUOX/peroxidase pathway in allergic airway inflammation. The most important clinical point of this finding is that it suggests the possibility of using antithyroid agents, pan-heme peroxidase inhibitors, as repositioned antiasthma drugs. If we can apply this strategy to asthma patients, it should greatly reduce the cost of treating asthma.

Conflicts of Interest

The authors declare that there is no competing interest associated with the present study.

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References

[1] Global Initiative for Asthma, "2017 GINA Report, global strategy for asthma management and prevention," http://ginasthma.org/2017-gina-report-global-strategy-for-asthma-management-and-prevention.

[2] I. M. Adcock and S. J. Lane, "Corticosteroid-insensitive asthma: molecular mechanisms," *Journal of Endocrinolology*, vol. 178, no. 3, pp. 347–355, 2003.

[3] P. P. Hekking, R. R. Wener, M. Amelink, A. H. Zwinderman, M. L. Bouvy, and E. H. Bel, "The prevalence of severe refractory asthma," *The Journal of Allergy and Clinical Immunology*, vol. 135, no. 4, pp. 896–902, 2015.

[4] M. C. Peters, Z. K. Mekonnen, S. Yuan, N. R. Bhakta, P. G. Woodruff, and J. V. Fahy, "Measures of gene expression in sputum cells can identify T_H2-high and T_H2-low subtypes of asthma," *Journal of Allergy and Clinical Immunology*, vol. 133, no. 2, pp. 388–394.e5, 2014.

[5] K. Izuhara, H. Matsumoto, S. Ohta, J. Ono, K. Arima, and M. Ogawa, "Recent developments regarding periostin in bronchial asthma," *Allergology International*, vol. 64, pp. S3–S10, 2015.

[6] K. Izuhara, S. Suzuki, C. Nofziger et al., "The role of pendrin in the airways: links with asthma and COPD," in *The Role of Pendrin in Health and Disease*, pp. 141–154, Springer, Cham, 2017.

[7] I. Nakao, S. Kanaji, S. Ohta et al., "Identification of pendrin as a common mediator for mucus production in bronchial asthma and chronic obstructive pulmonary disease," *Journal of Immunology*, vol. 180, no. 9, pp. 6262–6269, 2008.

[8] Y. Nakagami, S. Favoreto Jr., G. Zhen et al., "The epithelial anion transporter pendrin is induced by allergy and rhinovirus infection, regulates airway surface liquid, and increases airway reactivity and inflammation in an asthma model," *Journal of Immunology*, vol. 181, no. 3, pp. 2203–2210, 2008.

[9] S. Suzuki, M. Ogawa, S. Ohta et al., "Induction of airway allergic inflammation by hypothiocyanite via epithelial cells," *Journal of Biological Chemistry*, vol. 291, no. 53, pp. 27219–27227, 2016.

[10] S. Suzuki, M. Ogawa, S. Ohta et al., "The potential for repositioning antithyroid agents as antiasthma drugs," *Journal of Allergy and Clinical Immunology*, vol. 138, no. 5, pp. 1458–1461.e8, 2016.

[11] K. Izuhara, K. Arima, S. Kanaji, S. Ohta, and T. Kanaji, "IL-13: a promising therapeutic target for bronchial asthma," *Current Medicinal Chemistry*, vol. 13, no. 19, pp. 2291–2298, 2006.

[12] M. Kubo, "T follicular helper and T_H2 cells in allergic responses," *Allergology International*, vol. 66, no. 3, pp. 377–381, 2017.

[13] H. Kabata, K. Moro, S. Koyasu, and K. Asano, "Group 2 innate lymphoid cells and asthma," *Allergology International*, vol. 64, no. 3, pp. 227–234, 2015.

[14] K. Miyake and H. Karasuyama, "Emerging roles of basophils in allergic inflammation," *Allergology International*, vol. 66, no. 3, pp. 382–391, 2017.

[15] M. Wills-Karp, J. Luyimbazi, X. Xu et al., "Interleukin-13: central mediator of allergic asthma," *Science*, vol. 282, no. 5397, pp. 2258–2261, 1998.

[16] G. Grünig, M. Warnock, A. E. Wakil et al., "Requirement for IL-13 independently of IL-4 in experimental asthma," *Science*, vol. 282, no. 5397, pp. 2261–2263, 1998.

[17] Z. Zhu, R. J. Homer, Z. Wang et al., "Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production," *The Journal of Clinical Investigation*, vol. 103, no. 6, pp. 779–788, 1999.

[18] C. E. Brightling, P. Chanez, R. Leigh et al., "Efficacy and safety of tralokinumab in patients with severe uncontrolled asthma: a randomised, double-blind, placebo-controlled, phase 2b trial," *Lancet Respiratory Medicine*, vol. 3, no. 9, pp. 692–701, 2015.

[19] S. Wenzel, M. Castro, J. Corren et al., "Dupilumab efficacy and safety in adults with uncontrolled persistent asthma despite use of medium-to-high-dose inhaled corticosteroids plus a long-acting β2 agonist: a randomised double-blind placebo-controlled pivotal phase 2b dose-ranging trial," *Lancet*, vol. 388, no. 10039, pp. 31–44, 2016.

[20] G. Zhen, S. W. Park, L. T. Nguyenvu et al., "IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production," *American Journal of Respiratory Cell and Molecular Biology*, vol. 36, no. 2, pp. 244–253, 2007.

[21] D. A. Kuperman, C. C. Lewis, P. G. Woodruff et al., "Dissecting asthma using focused transgenic modeling and functional genomics," *Journal of Allergy and Clinical Immunology*, vol. 116, no. 2, pp. 305–311, 2005.

[22] N. Pedemonte, E. Caci, E. Sondo et al., "Thiocyanate transport in resting and IL-4-stimulated human bronchial epithelial cells: role of pendrin and anion channels," *Journal of Immunology*, vol. 178, no. 8, pp. 5144–5153, 2007.

[23] E. Di Valentin, C. Crahay, N. Garbacki et al., "New asthma biomarkers: lessons from murine models of acute and chronic asthma," *The American Journal of Physiology - Lung Cellular and Molecular Physiology*, vol. 296, no. 2, pp. L185–L197, 2009.

[24] C. Nofziger, V. Vezzoli, S. Dossena et al., "STAT6 links IL-4/IL-13 stimulation with pendrin expression in asthma and chronic obstructive pulmonary disease," *Clinical Pharmacology & Therapeutics*, vol. 90, no. 3, pp. 399–405, 2011.

[25] S. Vanoni, C. Nofziger, S. Dossena et al., "The human pendrin promoter contains two N_4 GAS motifs with different functional relevance," *Cellular Physiology and Biochemistry*, vol. 32, no. 7, pp. 238–248, 2013.

[26] A. Hogmalm, M. Bry, B. Strandvik, and K. Bry, "IL-1β expression in the distal lung epithelium disrupts lung morphogenesis and epithelial cell differentiation in fetal mice," *The American Journal of Physiology - Lung Cellular and Molecular Physiology*, vol. 306, no. 1, pp. L23–L34, 2014.

[27] K. M. Scanlon, Y. Gau, J. Zhu et al., "Epithelial anion transporter pendrin contributes to inflammatory lung pathology in mouse models of *Bordetella pertussis* infection," *Infection and Immunity*, vol. 82, no. 10, pp. 4212–4221, 2014.

[28] K. M. Adams, V. Abraham, D. Spielman et al., "IL-17A induces pendrin expression and chloride-bicarbonate exchange in human bronchial epithelial cells," *PLoS One*, vol. 9, no. 8, article e103263, 2014.

[29] S. Seshadri, X. Lu, M. R. Purkey et al., "Increased expression of the epithelial anion transporter pendrin/SLC26A4 in nasal polyps of patients with chronic rhinosinusitis," *Journal of Allergy and Clinical Immunology*, vol. 136, no. 6, pp. 1548–1558.e7, 2015.

[30] R. Sellamuthu, C. Umbright, J. R. Roberts et al., "Molecular insights into the progression of crystalline silica-induced

pulmonary toxicity in rats," *Journal of Applied Toxicology*, vol. 33, no. 4, pp. 301–312, 2013.

[31] J. H. Oh, M. J. Yang, J. D. Heo et al., "Inflammatory response in rat lungs with recurrent exposure to welding fumes: a transcriptomic approach," *Toxicology and Industrial Health*, vol. 28, no. 3, pp. 203–215, 2012.

[32] K. Fujita, Y. Morimoto, S. Endoh et al., "Identification of potential biomarkers from gene expression profiles in rat lungs intratracheally instilled with C_{60} fullerenes," *Toxicology*, vol. 274, no. 1-3, pp. 34–41, 2010.

[33] K. Fujita, M. Fukuda, H. Fukui et al., "Intratracheal instillation of single-wall carbon nanotubes in the rat lung induces time-dependent changes in gene expression," *Nanotoxicology*, vol. 9, no. 3, pp. 290–301, 2015.

[34] C. E. Connelly, Y. Sun, and N. H. Carbonetti, "Pertussis toxin exacerbates and prolongs airway inflammatory responses during *Bordetella pertussis* infection," *Infection and Immunity*, vol. 80, no. 12, pp. 4317–4332, 2012.

[35] A. Linden and B. Dahlen, "Interleukin-17 cytokine signalling in patients with asthma," *The European Respiratory Journal*, vol. 44, no. 5, pp. 1319–1331, 2014.

[36] C. Y. Yick, A. H. Zwinderman, P. W. Kunst et al., "Transcriptome sequencing (RNA-Seq) of human endobronchial biopsies: asthma versus controls," *The European Respiratory Journal*, vol. 42, no. 3, pp. 662–670, 2013.

[37] A. Ishida, N. Ohta, Y. Suzuki et al., "Expression of pendrin and periostin in allergic rhinitis and chronic rhinosinusitis," *Allergology International*, vol. 61, no. 4, pp. 589–595, 2012.

[38] M. Okano, S. Kariya, N. Ohta, Y. Imoto, S. Fujieda, and K. Nishizaki, "Association and management of eosinophilic inflammation in upper and lower airways," *Allergology International*, vol. 64, no. 2, pp. 131–138, 2015.

[39] G. Caramori, I. M. Adcock, A. Di Stefano, and K. F. Chung, "Cytokine inhibition in the treatment of COPD," *International Journal of Chronic Obstructive Pulmonary Disease*, vol. 9, no. 9, pp. 397–412, 2014.

[40] R. Halwani, S. Al-Muhsen, and Q. Hamid, "T helper 17 cells in airway diseases: from laboratory bench to bedside," *Chest*, vol. 143, no. 2, pp. 494–501, 2013.

[41] E. H. Bel and A. Ten Brinke, "New anti-eosinophil drugs for asthma and COPD: targeting the trait!," *Chest*, 2017.

[42] H. J. Lee, J. E. Yoo, W. Namkung et al., "Thick airway surface liquid volume and weak mucin expression in pendrin-deficient human airway epithelia," *Physiological Reports*, vol. 3, no. 8, article e12480, 2015.

[43] A. J. Ratner and A. Prince, "Lactoperoxidase. New recognition of an "old" enzyme in airway defenses," *American Journal of Respiratory Cell and Molecular Biology*, vol. 22, no. 6, pp. 642–644, 2000.

[44] C. L. Hawkins, "The role of hypothiocyanous acid (HOSCN) in biological systems," *Free Radical Research*, vol. 43, no. 12, pp. 1147–1158, 2009.

[45] T. J. Barrett and C. L. Hawkins, "Hypothiocyanous acid: benign or deadly?," *Chemical Research in Toxicology*, vol. 25, no. 2, pp. 263–273, 2012.

[46] P. Moskwa, D. Lorentzen, K. J. Excoffon et al., "A novel host defense system of airways is defective in cystic fibrosis," *American Journal of Respiratory and Critical Care Medicine*, vol. 175, no. 2, pp. 174–183, 2007.

[47] M. Schuliga, "NF-κB signaling in chronic inflammatory airway disease," *Biomolecules*, vol. 5, no. 3, pp. 1266–1283, 2015.

[48] J. P. Brennan, S. C. Bardswell, J. R. Burgoyne et al., "Oxidant-induced activation of type I protein kinase A is mediated by RI subunit interprotein disulfide bond formation," *Journal of Biological Chemistry*, vol. 281, no. 31, pp. 21827–21836, 2006.

[49] C. Cayrol and J. P. Girard, "IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy," *Current Opinion in Immunology*, vol. 31, pp. 31–37, 2014.

[50] R. W. Harper, C. Xu, J. P. Eiserich et al., "Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium," *FEBS Letters*, vol. 579, no. 21, pp. 4911–4917, 2005.

[51] G. A. Settipane, E. Schoenfeld, and M. W. Hamolsky, "Asthma and hyperthyroidism," *Journal of Allergy and Clinical Immunology*, vol. 49, no. 6, pp. 348–355, 1972.

[52] T. Nakazawa and S. Kobayashi, "Influence of antithyroidal therapy on asthma symptoms in the patients with both bronchial asthma and hyperthyroidism," *Journal of Asthma*, vol. 28, no. 2, pp. 109–116, 1991.

[53] R. D. Grembiale, S. Naty, C. Iorio, N. Crispino, G. Pelaia, and C. M. Tranfa, "Bronchial asthma induced by an antithyroid drug," *Chest*, vol. 119, no. 5, pp. 1598-1599, 2001.

[54] E. L. Tobinick, "The value of drug repositioning in the current pharmaceutical market," *Drug News and Perspectives*, vol. 22, no. 2, pp. 119–125, 2009.

[55] Y. Y. Li and S. J. Jones, "Drug repositioning for personalized medicine," *Genome Medicine*, vol. 4, no. 3, p. 27, 2012.

[56] K. Husgafvel-Pursiainen, M. Sorsa, K. Engstrom, and P. Einisto, "Passive smoking at work: biochemical and biological measures of exposure to environmental tobacco smoke," *International Archives of Occupational and Environmental Health*, vol. 59, no. 4, pp. 337–345, 1987.

[57] Y. Saloojee, C. J. Vesey, P. V. Cole, and M. A. Russell, "Carboxyhaemoglobin and plasma thiocyanate: complementary indicators of smoking behaviour?," *Thorax*, vol. 37, no. 7, pp. 521–525, 1982.

[58] A. Tamimi, D. Serdarevic, and N. A. Hanania, "The effects of cigarette smoke on airway inflammation in asthma and COPD: therapeutic implications," *Respiratory Medicine*, vol. 106, no. 3, pp. 319–328, 2012.

[59] R. Polosa and N. C. Thomson, "Smoking and asthma: dangerous liaisons," *The European Respiratory Journal*, vol. 41, no. 3, pp. 716–726, 2013.

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Research Article

Glycosides from *Stevia rebaudiana* Bertoni Possess Insulin-Mimetic and Antioxidant Activities in Rat Cardiac Fibroblasts

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Stevia rebaudiana Bertoni is a shrub having a high content of sweet diterpenoid glycosides in its leaves, mainly stevioside and rebaudioside A, which are used as noncaloric, natural sweeteners. The aim of this study was to deepen the knowledge about the insulin-mimetic effect exerted by four different mixtures of steviol glycosides, rich in stevioside and rebaudioside A, in neonatal rat cardiac fibroblasts. The potential antioxidant activity of these steviol glycosides was also assessed, as oxidative stress is associated with diabetes. Likewise the insulin effect, steviol glycosides caused an increase in glucose uptake into rat fibroblasts by activating the PI3K/Akt pathway, thus inducing Glut4 translocation to the plasma membrane. The presence of S961, an insulin antagonist, completely abolished these effects, allowing to hypothesize that steviol glycosides could act as ligands of the same receptor engaged by insulin. Moreover, steviol glycosides counteracted oxidative stress by increasing reduced glutathione intracellular levels and upregulating expression and activity of the two antioxidant enzymes superoxide dismutase and catalase. The present work unravels the insulin-mimetic effect and the antioxidant property exerted by steviol glycosides, suggesting their potential beneficial role in the cotreatment of diabetes and in health maintenance.

1. Introduction

Stevia rebaudiana Bertoni is a shrub belonging to the Asteraceae family, native to Paraguay and Brazil, and has been now cultivated in many parts of the world [1–3]. Due to the high content of sweet diterpenoid glycosides in its leaves, the "sweet herb of Paraguay" has been used for many years in South America for sweetening food products and in traditional medicine, particularly as a natural control for diabetes [4]. *Stevia rebaudiana* Bertoni has attracted scientific interest for its potential use as noncaloric [5, 6] and noncariogenic [7, 8] sweetener and also for its multifaceted benefits on human health [9] and therapeutic properties [10, 11]. Several studies indeed suggest that *Stevia* has antihyperglycaemic, antihypertensive, antitumour, antidiarrheal, diuretic, anti-inflammatory, and immune-modulatory effects [12]. Owing to these characteristics, leaf extracts rich in steviol glycosides, such as stevioside and rebaudioside A, have been authorized as commercial sweeteners and food additives [13], as the safety of high-purity steviol glycosides has been extensively reviewed in the published literature and by national and international food safety agencies [14].

Since the prevalence of diabetes is rapidly rising all over the world, the identification of nontoxic, natural compounds from a plant origin, able to mimic the insulin action, appears to be of great interest and importance, according to the last WHO expert committee recommendations [15]. In this context, the use of *Stevia* extracts as natural sweeteners is notably useful in restricting or controlling caloric intake in the diet or as a substitute for sucrose in the treatment of diabetes. The mechanisms by which *Stevia* leaves and its steviol glycosides exert a marked antidiabetic effect have been intensively studied [16]. Some reports account for an increased insulin secretion by stevioside [17] or rebaudioside A [18]; other demonstrated that stevioside increases insulin sensitivity in rodent models [19], inducing antihyperglycaemic effects on diabetic rats [20] and on diabetic subjects [21, 22]; moreover, insulin-mimetic properties of steviol and stevioside have been reported in L6 and 3T3L1 cell lines [23]. Furthermore, as oxidative stress is among the features correlated with diabetic condition, the antioxidant activity recently attributed to *Stevia* further highlights the potential synergic beneficial effect of this plant [24–27].

We previously demonstrated that steviol glycosides exert a marked insulin-like effect on glucose transport activity in cancer cell lines [28]. The aim of this study, graphically reported in Figure 1, was to investigate the molecular mechanisms underpinning the insulin-mimetic effect showed by steviol glycosides in a nontransformed cell system. Moreover, the potential antioxidant property of steviol glycosides was also examined. Neonatal rat cardiac fibroblasts were chosen, as they are fully involved in high glucose-induced cardiac fibrosis, a pathological consequence of diabetes, leading to cardiac dysfunction [29]. Cardiac fibroblasts, indeed, express the insulin receptor (IR) [30], the insulin growth factor-like 1 and its receptor (IGF-1/IGF-1R) [31], and the insulin-responsive glucose transporter (Glut4) [32].

2. Materials and Methods

2.1. Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), penicillin, streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), insulin, 2-deoxy-glucose (DOG), phloretin, DAPI, RIPA lysis buffer, 10% SDS solution, mammalian protease inhibitor mixture, phosphatase inhibitor cocktail PhosSTOP (Roche™), hydrogen peroxide, Tris-HCl, sodium pyruvate, NADH, monochlorobimane (MCB), SOD assay kit, primary antibody to β-actin, bovine serum albumin (BSA), and all other chemicals were purchased from Sigma-Aldrich. S961 insulin receptor antagonist was from Phoenix Peptide. 2-Deoxy-D-[2,6-3H]-glucose and Ultima Gold MV scintillation cocktail were from PerkinElmer. Anti-Glut4 (sc-1606) antibody was obtained from Santa Cruz Biotechnology. Rabbit anti-goat IgG (H+L) secondary antibody Alexa Fluor® 488 conjugate (A11078) was purchased from Life Technologies. Primary antibodies against phospho-Akt (Ser473) (#4058), Akt (#9272), phospho-AMPKα (Thr172) (#2535), AMPKα (#4058), and IGF-1 receptor β (IGF-1R) (#3027) and horseradish peroxidase-conjugated secondary antibodies anti-rabbit (#7074) and anti-mouse (#7076) were purchased from Cell Signaling Technologies. Primary antibodies anti-phospho-PI3 kinase p85 pTyr458/p55 pTyr199 (#PA5-17387) and anti-phospho-IGF-1R pTyr1165+pTyr1166 (#PA5-35452) were from Thermo Scientific. Anti-PI3 kinase (#06-195) antibody was purchased from Millipore. DC™ Protein Assay, 4–20% Mini-PROTEAN® TGX™ Precast Gels, Precision Plus Protein™ Unstained Standards, and Clarity™ Western ECL Substrate were from Bio-Rad. Catalase Assay Kit was from Cayman Chemical. RNA-to-cDNA Conversion Kit was from Applied Biosystems. RNA Miniprep Kit was from Agilent Technologies. RT-PCR primers for superoxide dismutase 1 (SOD1), catalase (CAT), β-2-microglobulin (B2M), and actin (ACT) were manufactured from Sigma-Aldrich.

Four different mixtures of steviol glycosides, differing in their relative content, were provided by *Stevia* extraction companies and used in this study.

REB A 97 (R97) was from Pure Circle SDN BHD (Negeri Sembilan, Malaysia) and, according to the certificate of analysis, contains >97% rebaudioside A and <3% other steviol glycosides.

RA60 (R60) was from HYET Sweet B.V. (Breda, the Netherlands) and, according to the certificate of analysis, contains 95.48% total steviol glycosides, of which 63.43% are rebaudioside A, 22.85% are stevioside, 8.21% are rebaudioside C, 0.73% are dulcoside A, and 0.26% are other steviol glycosides.

SG95 (SG) was from Pure Circle SDN BHD (Negeri Sembilan, Malaysia) and, according to the certificate of analysis, contains >95.0% total steviol glycosides, of which >50.0% are rebaudioside A and at least 25% are stevioside.

RA50 (TRU) was from Eridania Italia SpA. (Bologna, Italy) and contains 95.0% total steviol glycosides, of which at least 50% are rebaudioside A and 25% are stevioside and 20% other steviol glycosides are not analytically quantified.

The structures of the main steviol glycosides, stevioside and rebaudioside A, are reported in Figure 2.

2.2. Cell Culture. Neonatal Sprague-Dawley rat cardiac fibroblasts were a kind gift of Dr. Antonello Lorenzini (Department of Biomedical and Neuromotor Sciences, Alma Mater Studiorum—University of Bologna, Italy). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (*v*/*v*) foetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in a humidified atmosphere maintained at 5% CO_2.

2.3. Cell Viability. Cell viability was evaluated by the MTT assay. Neonatal rat cardiac fibroblasts were treated with increasing concentrations of R97, R60, SG, and TRU (0.5–5 mg/mL). After 24 h, cells were stressed or not with 100 μM H_2O_2 for 30 min and then incubated with 0.5 mg/mL MTT for 4 h at 37°C. To dissolve the blue-violet formazan salt crystals formed, a solubilisation solution (10% SDS, 0.01 M HCl) was added and the plates were incubated overnight in a humidified atmosphere (37°C, 5% CO_2) to ensure complete lysis. The absorbance

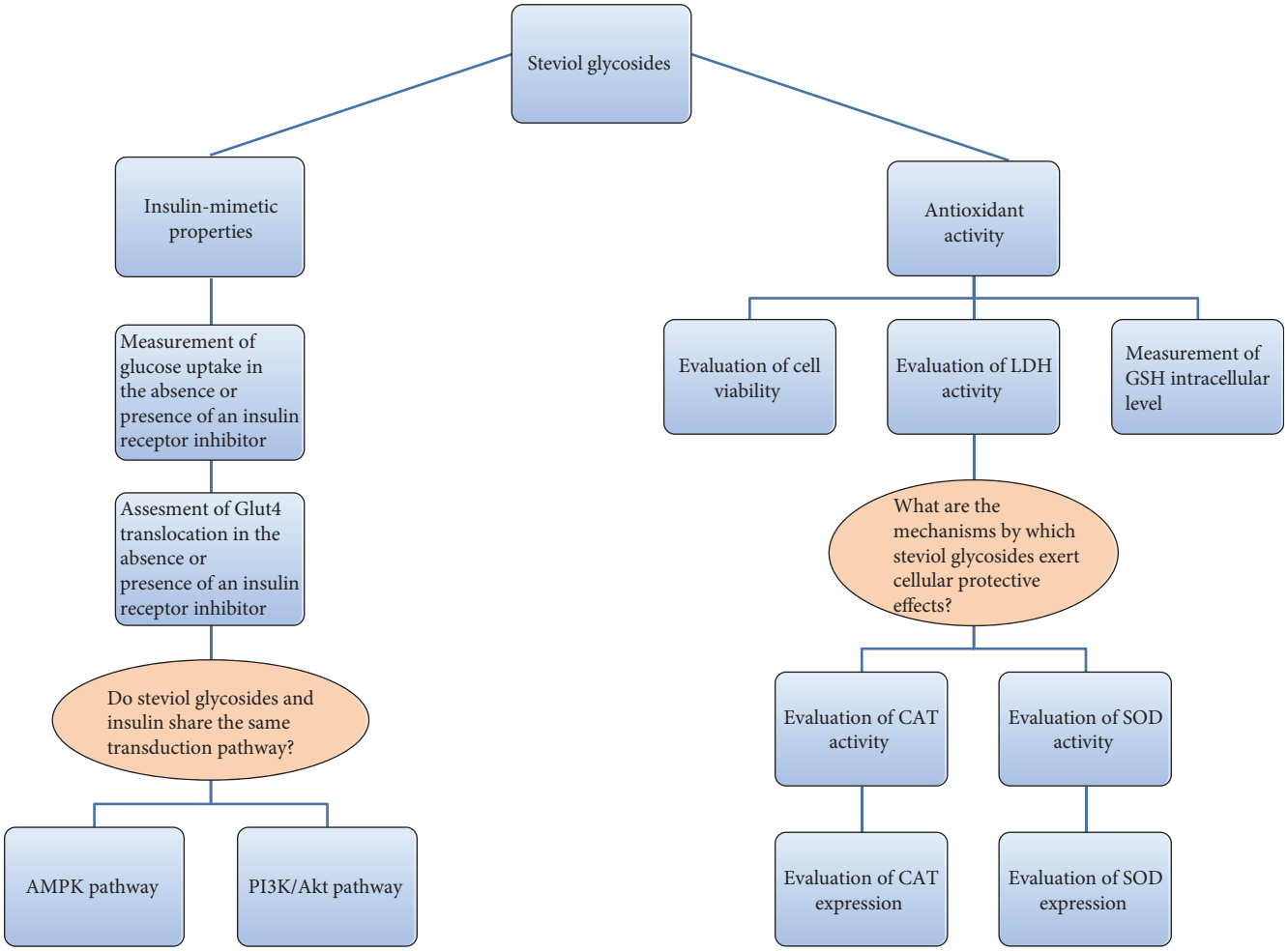

Figure 1: Graphical representation of the experimental design.

at 570 nm was measured using a multiwell plate reader (Wallac Victor2, PerkinElmer).

2.4. Glucose Transport Assay. Glucose transport assay was performed as described in [28, 33]. Cells were incubated or not with R97, R60, SG, or TRU (1 mg/mL) for 1 h or with insulin (100 nM) for 30 minutes, in the presence (or not) of S961 (10 nM) for 90 min. Then, they were washed in PBS and treated for 10 min at 37°C with a mixture of 2-deoxy-D-[2,6-3H] glucose (0.8 μCi/assay) and 1.0 mM unlabeled glucose analogue (DOG), under conditions where the uptake was linear at least for 20 min. The transport was stopped by adding phloretin (final concentration 0.3 mM), a potent inhibitor of glucose transport activity. Cells were washed twice with PBS, detached and resuspended with 1 mL cold PBS, and added to Ultima Gold MV scintillation cocktail (PerkinElmer). Radioactivity was measured by liquid scintillation counting (Tri-Carb® liquid scintillation analyzer, PerkinElmer).

2.5. Immunofluorescence. Neonatal rat cardiac fibroblasts, grown on coverslips, were treated with R97 or R60 (1 mg/mL) for 1 hour or with insulin (100 nM) for 30 min in the presence or absence of 10 nM S961 for 90 min and then fixed in 3% (*w*/*v*) paraformaldehyde for 15 min. Cells were washed twice with PBS, blocked with 1% (*w*/*v*) PBS/BSA for 1 hour, and then incubated for 1 hour with 20 μg/mL of goat anti-Glut4 antibody raised against a peptide within an extracellular domain of the glucose transporter protein. Subsequently, cells were treated for 1 hour with fluorescent FITC-conjugated rabbit anti-goat IgG in the dark, nuclei were stained with DAPI, and coverslips were mounted on slides. Confocal imaging was performed by a Nikon A1 confocal laser scanning microscope (Nikon Instruments, Japan).

2.6. Immunoblotting Analysis. After treatments with R97, R60, SG, or TRU (1 mg/mL) for 1 hour or with insulin (100 nM) for 30 min, rat cardiac fibroblasts were washed with ice-cold PBS and lysed with RIPA buffer containing mammalian protease and phosphatase inhibitor mixtures. Protein concentration was measured by Bio-Rad DC Protein Assay (Bio-Rad Laboratories). Proteins were separated on 4–20% SDS-PAGE Mini-PROTEAN TGX Precast Gels using a Mini-PROTEAN II apparatus (Bio-Rad Laboratories) and electrophoretically transferred to the nitrocellulose membrane (Hybond-C; GE Healthcare). To avoid nonspecific binding, membranes were incubated in blocking buffer containing 5% (*w*/*v*) albumin in Tris-buffered saline

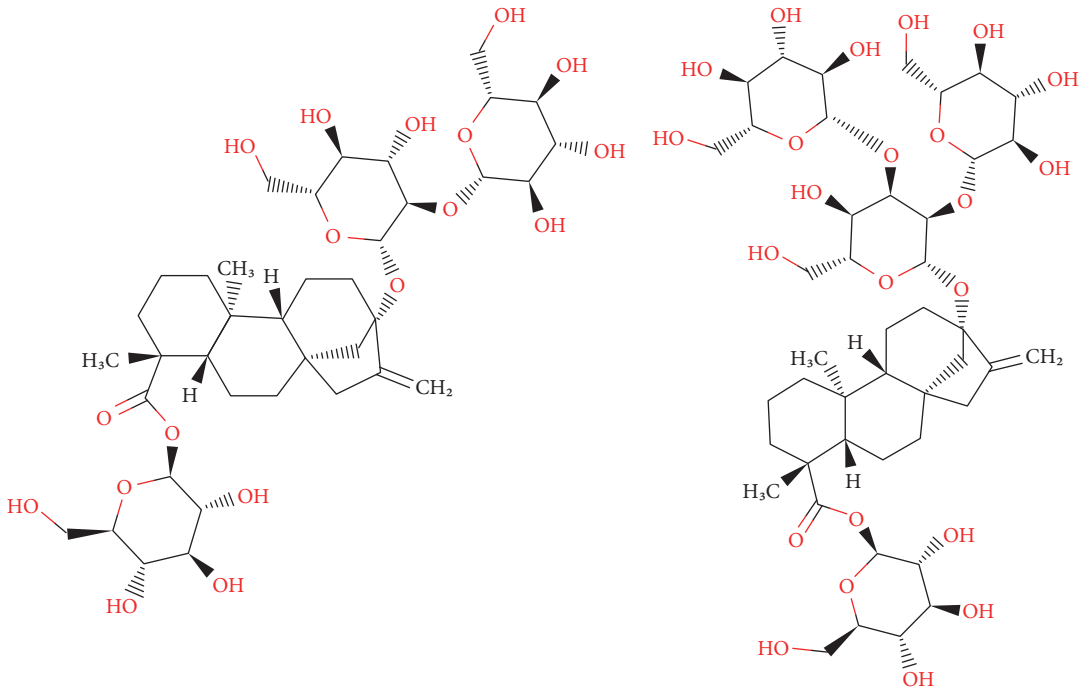

Figure 2: Chemical structures of stevioside (a) and rebaudioside A (b).

(TBS)/Tween and probed overnight at 4°C with primary antibodies (anti-phospho-IGF-1R, anti-IGF-1R, anti-phospho-PI3K, anti-PI3K, anti-phospho-Akt, anti-Akt, anti-phospho-AMPK, anti-AMPK, or anti-β-actin as internal normalizers). Nitrocellulose membranes were then washed with TBS/Tween and incubated with horseradish peroxidase-labelled secondary antibodies in 5% albumin TBS/Tween at room temperature for 1 hour and successively washed with TBS/Tween. Chemiluminescence detection was performed using Clarity Western ECL Substrate (Bio-Rad Laboratories). Bands were acquired with a CCD imager (ChemiDoc™ MP System, Bio-Rad) and analyzed by using Image Lab analysis software (Bio-Rad).

2.7. Lactate Dehydrogenase Assay. Rat fibroblasts were incubated with R97, R60, SG, or TRU (1 mg/mL) for 24 hours and then stressed using 100 μM H_2O_2 for 30 min. Lactate dehydrogenase (LDH) release from cells into the culture medium was detected by monitoring LDH activity through a spectrophotometric assay based on the reduction of pyruvate to lactate coupled with NADH oxidation to NAD^+. The decrease in absorbance at 340 nm resulting from the oxidation of NADH was monitored at 37°C in a Varian Cary 50 Spectrophotometer.

2.8. Determination of Glutathione (GSH) Levels. Reduced GSH levels were determined by the monochlorobimane (MCB) fluorometric assay as previously reported [34, 35]. Briefly, rat cardiac fibroblasts were incubated for 24 hours with R97, R60, SG, or TRU (1 mg/mL) and exposed or not to 100 μM H_2O_2 for 30 min. After treatment, the culture medium was removed and the cells were washed with cold PBS and incubated for 30 min at 37°C with 50 μM MCB in PBS. The strong fluorescence of the GSH-MCB adduct was measured in a multiwell plate reader (Wallac Victor2, PerkinElmer). Excitation wavelength was 355 nm and emission wavelength was 460 nm.

2.9. Superoxide Dismutase Assay. Rat cardiac fibroblasts were incubated for 24 hours with R97, R60, SG, or TRU (1 mg/mL), and then, superoxide dismutase (SOD1) activity was measured using the SOD Assay Kit provided by Sigma-Aldrich and following the manufacturer's instructions. SOD Assay Kit-WST is a colorimetric indirect assay method based on Dojindo's highly water-soluble tetrazolium salt WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that is reduced by superoxide anion to a stable water-soluble formazan with high molar absorptivity. The absorbance at 440 nm is proportional to the amount of superoxide anion generated from xanthine oxidase provided in the kit; SOD activity was quantified as inhibition activity by spectrophotometrically following the decrease in the color development at 440 nm in a multiwell plate reader (Wallac Victor2, PerkinElmer).

2.10. Catalase Assay. Rat cardiac fibroblasts were incubated for 24 hours with R97, R60, SG, or TRU (1 mg/mL), and then, catalase (CAT) activity was quantified by Cayman's Catalase Assay Kit following the manufacturer's protocol. This kit, according to the method of Johansson et al. [34], exploits the peroxidative activity of CAT, and it is based on the oxidation, catalyzed by CAT, of methanol (the electron donor) in the presence of an optimal concentration of H_2O_2. The produced formaldehyde was measured spectrophotometrically

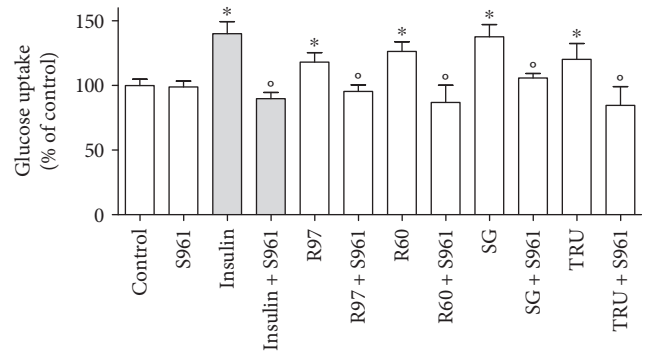

Figure 3: Effect of different steviol glycoside mixtures or insulin on glucose transport activity in rat cardiac fibroblasts in the presence or absence of the antagonist of the insulin receptor S961. Cells were treated with R97, R60, SG, or TRU (1 mg/mL) for 1 hour or with insulin (100 nM) for 30 min, in the presence or absence of 10 nM S961 for 90 min. Glucose uptake was assayed as described in the experimental procedure section. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. Statistical analysis was performed by Bonferroni's multiple comparison test following one-way ANOVA. $^{*}p < 0.05$ significantly different from control cells; $^{\circ}p < 0.05$ significantly different from the corresponding cells not treated with S961.

with the chromogen Purpald® (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole), which reacts with aldehydes producing purple color. The absorbance was monitored at 540 nm in a multiwell plate reader (Wallac Victor2, PerkinElmer). CAT activity was calculated from the amount of formaldehyde produced in the assay.

2.11. Analysis of mRNA Expression by RT-PCR. Total RNA was extracted from fibroblasts using a commercially available kit (Absolutely RNA Miniprep Kit, Agilent Technologies), according to the manufacturer's instructions. The quantification of RNA was performed using a NanoVue Spectrophotometer (GE Healthcare) by analyzing at A260/A280 and A260/A230. mRNA was reverse-transcribed into cDNA starting from 1 μg of total RNA using a high-capacity RNA-to-cDNA Conversion Kit (Applied Biosystems). The subsequent PCR was carried out in a total volume of 20 μL containing 2 μL of cDNA, 10 μL SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad Laboratories), and 1 μL (500 nM) of each primer. The primers used are as follows: CAT (forward) 5′-CAAGTTCCATTACAAGACTGAC-3′ and (reverse) 5′-TAAATGGGAAGGTTTCTGC-3′, SOD (forward) 5′-AATGTGTCCATTGAAGATCG-3′ and (reverse) 5′-CACATAGGGAATGTTTATTGGG-3′, β-actin (forward) 5′-AAGACCTCTATGCCAACAC-3′ and (reverse) 5′-TGATCTTCATGGTGCTAGG-3′, and β2-microglobulin (forward) 5′-ACTGGTCTTTCTACATCCTG-3′ and (reverse) 5′-AGATGATTCAGAGCTCCATAG-3′.

All primers were produced by Sigma-Aldrich. β-Actin and β2-microglobulin were used as reference genes. cDNA amplification was started by activating the polymerase for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. A melt curve was run to ensure quality control and the generation of a single product. Normalized expression levels were calculated relative to control cells according to the $2^{-\Delta\Delta CT}$ method.

2.12. Statistical Analysis. Results are expressed as means ± SD. Differences among the means were determined by Bonferroni's multiple comparison test following one-way ANOVA and were considered significant at $p < 0.05$.

3. Results

3.1. Effect of Steviol Glycosides on Cell Viability. Rat cardiac fibroblasts were treated with increasing concentrations of R97, R60, SG, or TRU (0.5–5 mg/mL) for 24 hours to investigate their direct effect on cell integrity/damage. There was no observed decrease in the ability of cardiac fibroblasts to reduce MTT following exposure to the four different mixtures up to 5 mg/mL, indicating the absence of toxicity in this range of concentrations (data not shown). These results are in agreement with previously reported data obtained in different cell types [28].

3.2. Effect of Different Steviol Glycosides on Glucose Transport Activity and on Glut4 Translocation to the Plasma Membrane. Neonatal rat cardiac fibroblasts were incubated for 1 hour with R97, R60, SG, or TRU (1 mg/mL) or with 100 nM insulin and then assayed for glucose transport activity. Figure 3 shows that all mixtures were able to significantly enhance glucose uptake at a similar extent, and, interestingly, the increase in glucose transport activity was comparable to that induced by insulin exposure. To deeper study the mechanism of glucose transport induced by steviol glycosides, S961, a 43-amino-acid biosynthetic peptide antagonist of insulin receptor [36], was used. Cell preincubation with 10 nM S961 completely abolished the increase in glucose transport due to steviol glycosides or insulin treatment (Figure 3).

It is well known that insulin induces the translocation of Glut4 from cytosolic storage vesicles to the plasma membrane, thereby enhancing glucose transport activity [37]. Therefore, the translocation of Glut4 to the plasma membrane following treatment with steviol glycosides or insulin was assessed by utilizing a Glut4-specific antibody targeting an epitope in the extracellular domain near the N-terminus and visualized through confocal microscopy. Figure 4 shows the effect of two representative mixtures,

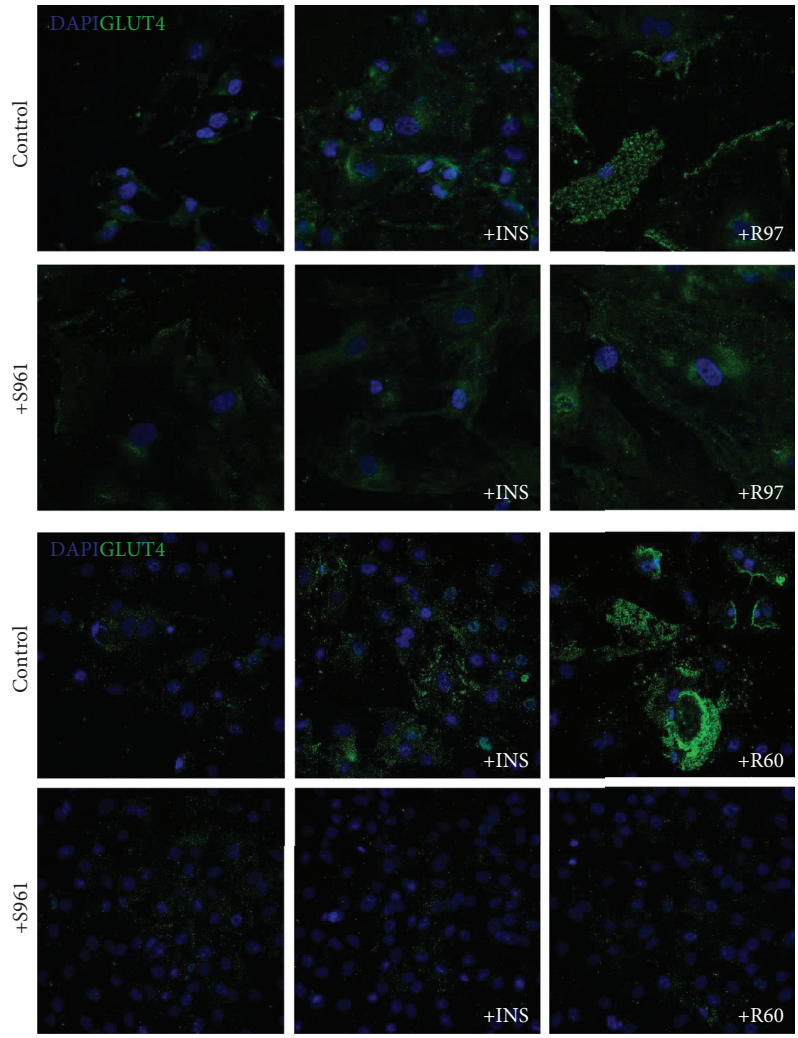

Figure 4: Effect of R97, R60, or insulin on Glut4 translocation to the plasma membrane in rat cardiac fibroblasts in the presence or absence of the antagonist of the insulin receptor S961. Cells were treated with R97 or R60 (1 mg/mL) for 1 hour or with insulin (100 nM) for 30 min in the presence or absence of 10 nM S961 for 90 min. Glut4 translocation to the plasma membrane was detected using immunofluorescence staining with anti-Glut4 antibody and DAPI nuclear staining as a counterstain. Images were acquired by the Nikon A1 confocal laser scanning microscope (Nikon Instruments, Japan). The results are representative of two independent experiments.

R97 and R60, in comparison with that of insulin, in the presence or absence of the antagonist S961. Results indicate that R97 and R60 are able to induce Glut4 translocation to the plasma membrane with comparable efficiency and that their effect is similar to that obtained by insulin stimulation. Moreover, cell pretreatment with S961 counteracts the observed Glut4 translocation both in insulin-stimulated cells and in steviol glycoside-treated cells. Experiments with SG and TRU revealed similar results, indicating that all the samples share the ability to modulate Glut4 translocation with the same efficiency (data not shown). These results are in accordance with those obtained in the evaluation of glucose transport activity.

3.3. Effect of Steviol Glycosides on PI3K/Akt and AMPK Pathways. To assess whether steviol glycosides exert their effect on glucose transport activity through the IR or IGF-1R, the activation of the PI3K/Akt pathway, which mediates the intracellular signaling triggered by the IR family through IR substrates [38], was investigated. The effects of R97, R60, SG, or TRU on activation/phosphorylation of IGF-1R, PI3K, and Akt were examined by immunoblotting, and the results are shown in Figure 5. Cell treatment with 1 mg/mL of the four different mixtures or with 100 nM insulin caused a significant increase in the phosphorylated isoforms of IGF-1R, PI3K, and Akt, indicating a common pathway in insulin and steviol glycoside signaling mechanisms.

The activation of the metabolic pathway regulated by AMP-activated protein kinase (AMPK) is also known to increase Glut4 translocation and glucose uptake in the heart, especially during physical activity [39]. To test the possible contribution of the AMPK-dependent metabolic

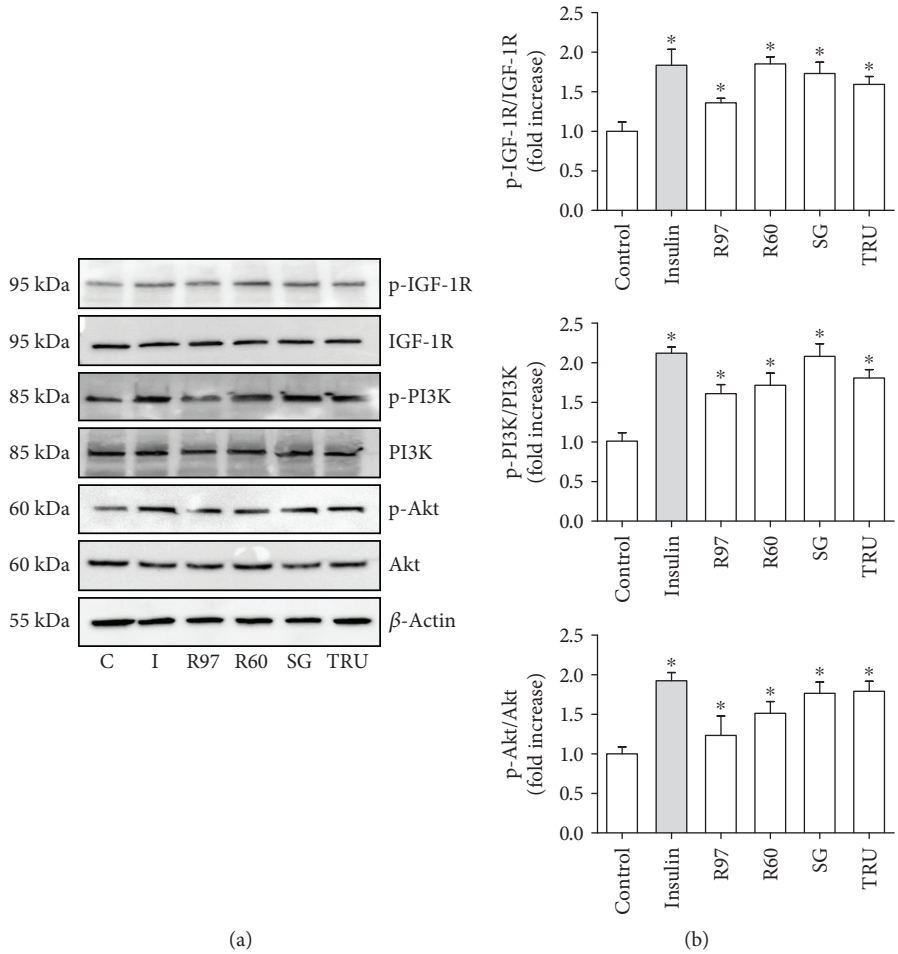

Figure 5: Effect of different steviol glycoside mixtures or insulin on the PI3K/Akt pathway in rat fibroblasts. (a) Rat cardiac fibroblasts treated with R97, R60, SG, or TRU (1 mg/mL) for 1 hour or with insulin (100 nM) for 30 min were lysed with RIPA buffer. Cell lysates were electrophoresed and immunoblotted with the indicated antibodies, as described in the experimental procedure section. Actin detection was used as a load control. Immunoblots are representative of three independent experiments. (b) Densitometric analysis of protein phosphorylation status is expressed as phospho-protein/total protein and reported as folds to control. $^{*}p < 0.05$ significantly different from control cells.

pathway to the glucose transport stimulation induced by steviol glycosides, the phosphorylation level of AMPK was investigated by the immunoblotting technique. Results in Figure 6 indicate that treatment with R97, R60, SG, TRU, or insulin did not alter the phosphorylation level of AMPK in these experimental conditions.

3.4. Evaluation of the Potential Antioxidant Activity of Steviol Glycosides in Rat Cardiac Fibroblasts. In order to evaluate a potential antioxidant/protective effect exerted by the four mixtures under study, fibroblasts were treated with R97, R60, SG, or TRU for 24 hours, stressed (or not) by exogenous addition of H_2O_2, and tested for viability by the MTT assay (Figure 7(a)). The viability of cells pretreated with steviol glycosides was significantly increased compared to that of H_2O_2-treated cells, evidencing their potential protective role against oxidative stress. Moreover, lactate dehydrogenase (LDH) activity in the culture medium was quantified under stressed conditions, as a nonspecific marker of cell damage. As shown in Figure 7(b), fibroblast pretreatment with steviol glycosides was able to significantly counteract the LDH release from the cells.

The effect of steviol glycosides was also evaluated on intracellular glutathione (GSH) level and on the activities of cytosolic superoxide dismutase (SOD1) and catalase (CAT).

Rat cardiac fibroblasts were pretreated with R97, R60, SG, or TRU for 24 hours, and then, GSH intracellular levels were measured. Figure 8(a) shows that basal GSH levels were significantly higher in cells pretreated with steviol glycosides in respect to controls. When fibroblasts were exposed to H_2O_2 (Figure 8(b)), a significant decrease in basal GSH levels occurred, whereas cell pretreatment with steviol glycosides significantly counteracted this GSH depletion, allowing the maintenance of high GSH levels.

SOD and CAT are considered primary antioxidant enzymes, directly involved in the removal of ROS. Figure 9 shows that CAT (Figure 9(a)) and SOD1 (Figure 9(b)) activities significantly increase in rat fibroblasts treated with steviol glycosides for 24 hours compared to controls.

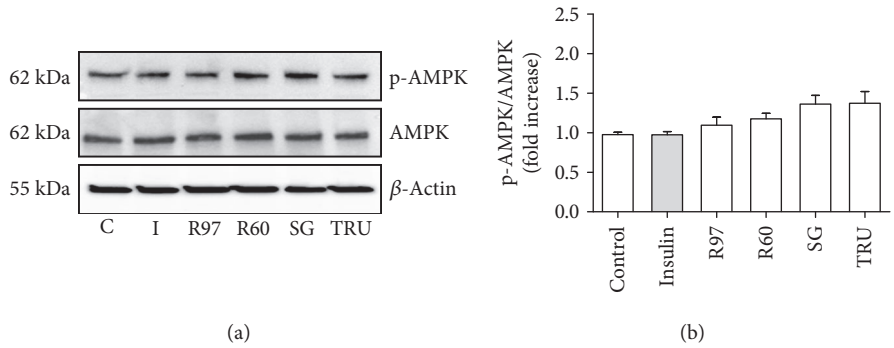

Figure 6: Effects of different steviol glycoside mixtures or insulin on AMPK phosphorylation level in rat cardiac fibroblasts. (a) Rat cardiac fibroblasts treated with R97, R60, SG, or TRU (1 mg/mL) for 1 hour or with insulin (100 nM) for 30 min were lysed with RIPA buffer. Cell lysates were electrophoresed and immunoblotted with anti-AMPK and anti-phospho-AMPK, as described in the experimental procedure section. Actin detection was used as a control. Immunoblots are representative of three independent experiments. (b) Densitometric analysis of AMPK phosphorylation level is expressed as phospho-AMPK/total AMPK and reported as folds to control.

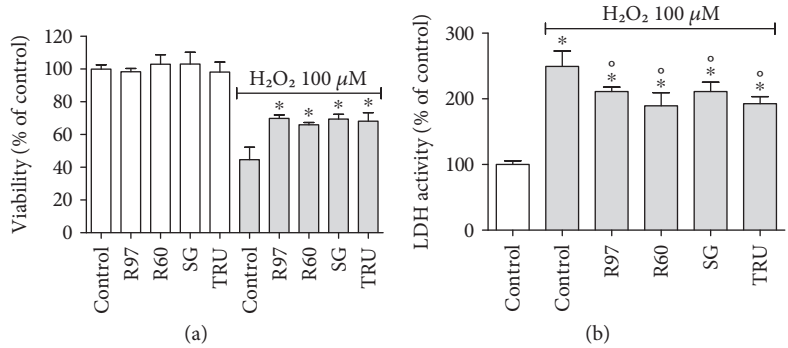

Figure 7: Effect of different steviol glycoside mixtures on rat cardiac fibroblast viability/proliferation and lactate dehydrogenase (LDH) activity. Rat cardiac fibroblasts were treated with R97, R60, SG, or TRU (1 mg/mL) for 24 hours; then, cells were stressed (or not) with 100 μM H_2O_2 for 30 min. Cell viability/proliferation was evaluated by the MTT assay (a) or by the LDH assay (b). Results are expressed as means ± SD of three independent experiments. Statistical analysis was performed by Bonferroni's multiple comparison test following one-way ANOVA. $^*p < 0.05$ with respect to the control; $^\circ p < 0.05$ with respect to control cells treated with H_2O_2.

In order to verify whether steviol glycoside was able to induce an upregulation of these antioxidant enzymes also at the transcriptional level, fibroblasts treated with 1 mg/mL of R97, R60, SG, or TRU for 24 h were lysed and analyzed for SOD1 and CAT cytosolic mRNA content by reverse tran scriptase-polymerase chain reaction (RT-PCR). Figure 10(a) shows that an increase in CAT mRNA amount of about 3-4 folds with respect to that of control is observed upon cell treatment with the steviol glycosides, and also, SOD1 mRNA content (Figure 10(b)) is significantly increased, although at a minor extent.

4. Discussion

Stevia rebaudiana Bertoni and its glycosides are known not only as natural, noncaloric sweeteners but also for their anti-hypertensive, anti-inflammatory, immune-modulatory, and antihyperglycaemic effects [9]. In particular, the hypoglycaemic, antidiabetic, and insulin-like properties of steviol glycosides have been investigated in 3T3-L1 adipocytes [40], in L6 myoblasts [21], and in cancer-derived cell lines [28].

In this paper, we focused on the insulin-mimetic and antioxidant activities of steviol glycosides in neonatal rat cardiac fibroblasts. We demonstrated that both insulin and steviol glycosides are able to increase glucose entry into the cells by activating the PI3K/Akt—but not the AMPK—pathway, thus inducing Glut4 translocation to the plasma membrane. We also showed that steviol glycosides counteract oxidative stress increasing GSH levels and SOD and CAT expressions and activities.

Fibroblasts express the insulin receptor (IR) [41]; furthermore, it has been reported that the IGF-1/IGF-1R system is crucial in the modulation of rat cardiac fibroblast growth [31]. Both IGF-1R and IR receptors belong to the same family, which includes the IR in two isoforms, IR-A and IR-B, forming homo- or heterodimers, and in cells expressing both IR and IGF-1R, also IGF-1R/IR hybrids [42]. Insulin binds with high affinity both to IR and IGF-1R [41]. Indeed, as reported in Figure 4, IGF-

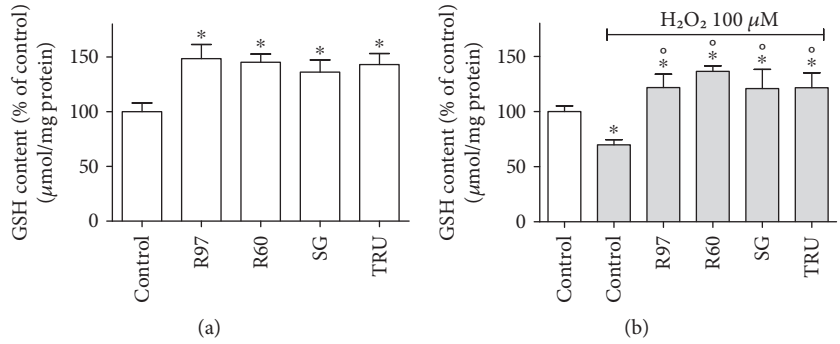

Figure 8: Effect of different steviol glycoside mixtures on intracellular GSH levels in rat cardiac fibroblasts. (a) Rat cardiac fibroblasts were treated with R97, R60, SG, or TRU (1 mg/mL) for 24 hours, and then, intracellular GSH levels were measured using the fluorescence probe MCB as described in the experimental procedure section. (b) Upon pretreatment with steviol glycosides, rat fibroblasts were exposed to 100 μM H_2O_2 for 30 min and then assayed for intracellular GSH levels. Results are expressed as means ± SD of four independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test. $^*p < 0.05$ significantly different from the control; $^\circ p < 0.05$ significantly different from control cells treated with H_2O_2.

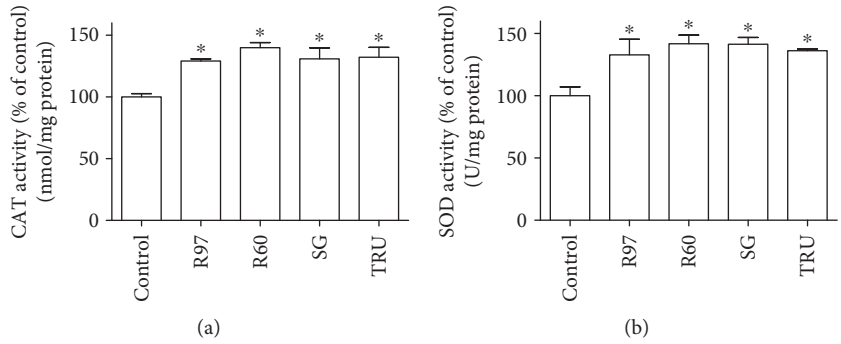

Figure 9: Effect of different steviol glycoside mixtures on catalase (CAT) and superoxide dismutase (SOD1) activities in rat cardiac fibroblasts. Rat cardiac fibroblasts were treated with R97, R60, SG, or TRU (1 mg/mL) for 24 hours and then lysed and assayed for CAT (a) and SOD1 (b), as described in the experimental procedure section. Results are expressed as means ± SD of four independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test. $^*p < 0.05$ significantly different from the control.

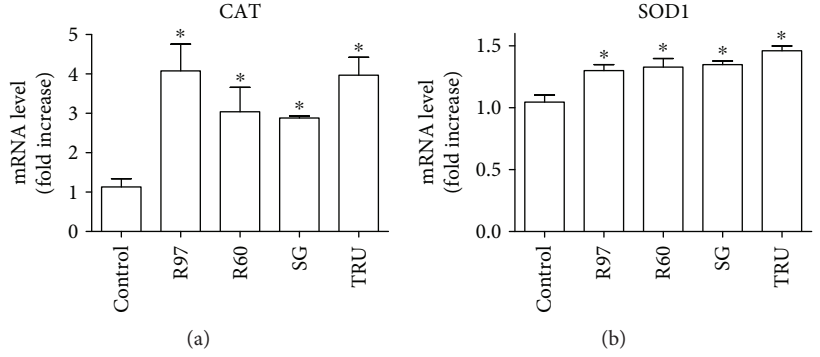

Figure 10: Effect of different steviol glycoside mixtures on catalase (CAT) and superoxide dismutase (SOD1) mRNA levels in rat cardiac fibroblasts. Cells were treated with R97, R60, SG, or TRU (1 mg/mL) for 24 hours, and after RNA extraction, the level of CAT mRNA (a) and SOD1 mRNA (b) was assayed according to the experimental procedure section. Each bar represents the mean ± SD of three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test. $^*p < 0.05$ with respect to the control.

1R is expressed in rat cardiac fibroblasts and it becomes phosphorylated upon cell treatment with insulin. Owing to its physiological role played in cell growth, IGF-1R is also involved in glucose homeostasis through the PI3K/Akt pathway, considered the predominant downstream signaling pathway for the IR family [38].

Given the membership of the two receptors to the same family and the signal proceeding through a common

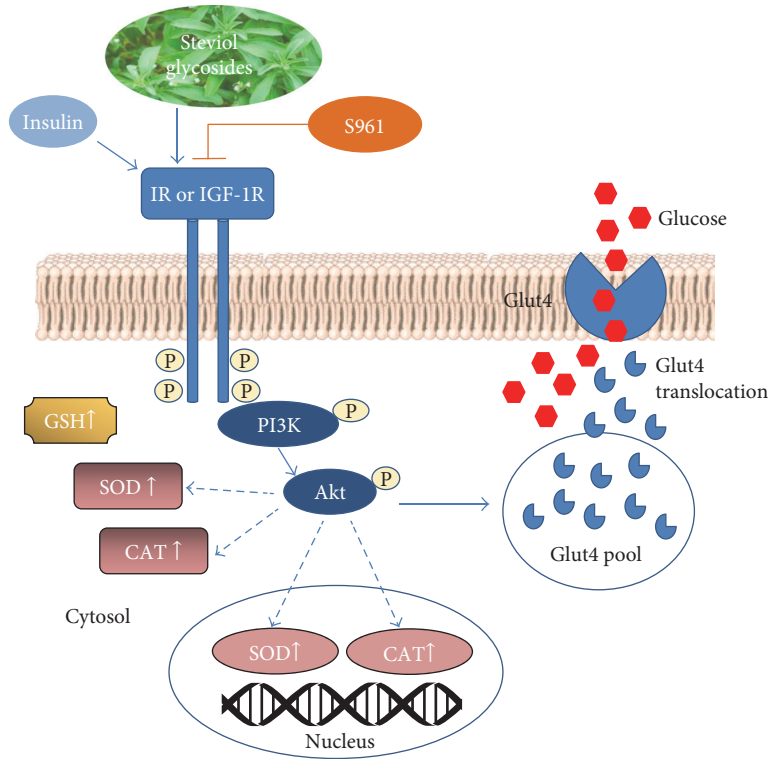

Figure 11: Insulin-mimetic and antioxidant activities of steviol glycoside mixtures. Steviol glycosides are able to act as ligands of the insulin receptor (IR or IGF-1R), triggering the PI3K/Akt pathway. The insulin receptor antagonist, S961, blocks the signal induced by both insulin and steviol glycosides. Upon the receptor activation, steviol glycoside signal leads to Glut4 translocation from intracellular pool to the plasma membrane, allowing glucose entry into the cell and thus mimicking the insulin action. Steviol glycosides are also able to increase the activity and the expression of the antioxidant enzymes SOD and CAT, probably through the activation of the same pathway (dashed blue lines). Moreover, cytosolic GSH levels are significantly increased in rat cardiac fibroblasts treated with steviol glycoside mixtures.

downstream pathway, we did not discriminate between either of receptor engagement upon insulin or steviol glycoside stimulation.

The increased phosphorylation level of the receptor IGF-1R observed upon fibroblast treatment with insulin or the four different mixtures indicates that both insulin and steviol glycosides are able to activate the PI3K/Akt pathways. Through this pathway, they significantly increase the glucose uptake by intensifying the trafficking of Glut4 isoform from intracellular stores to the plasma membrane, thus demonstrating the ability of steviol glycosides to mimic the insulin action.

Similar results were obtained in diabetes-induced myotubes and adipocytes by Bhasker and coworkers [23] who observed an increased level of the Glut4 protein and glucose transport, together with an enhanced transcription of Glut4 mRNA upon steviol or stevioside treatment, although stevioside achieved its efficacy at a higher concentration.

Furthermore, to confirm the possibility that stevioside and rebaudioside A could act as agonists of the same receptor engaged by insulin, we used the insulin antagonist S961. This molecule is a single-chain peptide reported to be a full IR antagonist [36] and stated to be active with a similar potency in cells expressing mostly IR or IGF-1R, probably through a hybrid receptor IR/IGF-1R-mediated response [43]. These authors described also a mixed agonist/antagonist activity for S961, depending on the cell type and concentration, and when using S961 as an IR antagonist *in vitro*, a concentration starting from 10 nM or above is recommended. In rat fibroblasts used in our study, 10 nM S961 behaved as insulin antagonist and completely abolished both the insulin and the steviol glycoside effects on glucose transport activity, confirming the ability of these molecules to trigger the PI3K/Akt signaling pathway through the same insulin receptor.

Insulin and contraction are the two key *stimuli* that acutely regulate Glut4 recruitment to the plasma membrane of the heart, and cardiac fibroblast contractility is one of the characteristics of myocardial remodeling [44]. These two *stimuli* initiate distinct signaling mechanisms, but both lead to increased Glut 4 translocation and glucose uptake [39]. The contraction signaling pathway proceeds through AMPK, and although the downstream targets mediating Glut4 vesicular trafficking have not been completely identified, it is known that AMPK stimulation of glucose transport does not involve downstream activation of the PI3K/Akt pathway [45]. Since crosstalk between these two pathways cannot be excluded [46] and neonatal fibroblasts are isolated from rat heart, the potential involvement of the AMPK pathway in the observed enhanced glucose transport exerted by steviol glycosides was investigated, but the results reported in Figure 6 ruled out this possibility.

These data support the hypothesis that steviol glycosides are able to mimic the insulin action, explaining, at least in part, the antidiabetic effect ascribed to *Stevia rebaudiana* Bertoni.

However, other mechanisms may be responsible for this effect; in fact, increased oxidative stress caused by prolonged hyperglycaemia has also been reported to play a major role in the pathogenesis of this disease [47]. It is well known that the permanent hyperglycaemia characterizing diabetes causes glucose autoxidation and glycation of proteins [48], which thereby depletes the antioxidant defence system thus promoting free radical generation. Moreover, advanced glycation end products and insulin both activate NAD(P)H oxidases, leading to further ROS production [49]. To this regard, many authors have reported a direct antioxidant activity of extracts from leaves of *Stevia rebaudiana* Bertoni, owing to the presence of alkaloids, flavonoids, and polyphenols [9]; nevertheless, biological systems may operate via multiple mechanisms [50]. Indeed, some phytochemicals exert beneficial effects by triggering adaptive stress response signaling pathways, resulting in the increased production of cytoprotective proteins, including antioxidant and phase 2 enzymes, heat shock proteins, growth factors, and protein generally involved in the regulation of cellular redox homeostasis and energy metabolism [51, 52].

Our data demonstrate that steviol glycosides exhibit a significant protective role against H_2O_2-induced damage, supporting fibroblast viability and inhibiting LDH release from the cells. According to Bender and coworker [24], no direct antioxidant activity is expected from purified stevioside or rebaudioside A, since steviol glycosides can hardly be absorbed by cells *in vitro*. In order to clarify the mechanism responsible for this observed protective effect, we investigated whether steviol glycosides, by activating the IR/IGF-1R pathway, can modulate endogenous enzymatic and nonenzymatic systems involved in the antioxidant equipment at protein and/or transcriptional level. It has been reported that the stimulation of IGF-1R upon IGF-1 treatment diminishes the oxidative stress and apoptotic effect of high dose of H_2O_2 on human umbilical vascular endothelial cells, and it was demonstrated that this protective effect is mediated by the PI3K/Akt pathway [53]. Interestingly, a significant increase in the basal GSH intracellular level together with a significant enhancement in SOD and CAT expressions and activities was observed in rat fibroblasts upon steviol glycoside treatment. GSH plays a central role in coordinating the antioxidant defence processes in the body, and the treatment with steviol glycosides maintained its high level also after the stressful action of H_2O_2. Since a decline in the activity of SOD and CAT antioxidant enzymes together with a reduction in GSH levels was described in diabetic animals [26, 27], our results suggest that these antioxidant effects can contribute to the antidiabetic property exhibited by *Stevia rebaudiana* Bertoni.

5. Conclusions

In conclusion, steviol glycosides exert pleiotropic effects on rat cardiac fibroblasts due to their ability to modulate the signaling pathways involved in glucose uptake and upregulation of the endogenous antioxidant defence system. Figure 11 summarizes the obtained results.

These results suggest a potential role of *Stevia rebaudiana* not only as an antihyperglycaemic agent but also as a powerful cardio protective tool in cardiac fibroblasts that have been recently defined as "the renaissance cells" [54] due to their fundamental role in maintaining cardiac function.

Abbreviations

IR: Insulin receptor
IGF-1: Insulin growth factor-like 1
IGF-1R: Insulin growth factor-like 1 receptor
AMPK: AMP-activated protein kinase.

Disclosure

The funders had no role in the study design, data collection and analysis, decision to publish, or paper preparation.

Conflicts of Interest

The authors declare that no conflict of interest and no competing financial interests exist.

Authors' Contributions

Cecilia Prata, Laura Zambonin, Diana Fiorentini, and Silvana Hrelia contributed equally to this work.

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References

[1] R. Lemus-Mondaca, A. Vega-Galvez, L. Zura-Bravo, and K. Ah-Hen, "Stevia rebaudiana Bertoni, source of a high potency natural sweetener: a comprehensive review on the biochemical, nutritional and functional aspects," *Food Chemistry*, vol. 132, no. 3, pp. 1121–1132, 2012.

[2] S. Purkayastha, A. Markosyan, I. Prakash et al., "Steviol glycosides in purified stevia leaf extract sharing the same metabolic fate," *Regulatory Toxicology and Pharmacology*, vol. 77, pp. 125–133, 2016.

[3] U. Wolwer-Rieck, "The leaves of Stevia rebaudiana (Bertoni), their constituents and the analyses thereof: a review," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 4, pp. 886–895, 2012.

[4] D. D. Soejarto, A. D. Kinghorn, and N. R. Farnsworth, "Potential sweetening agents of plant origin. III. Organoleptic evaluation of Stevia leaf herbarium samples for sweetness," *Journal of Natural Products*, vol. 45, no. 5, pp. 590–599, 1982.

[5] G. Brahmachari, L. C. Mandal, R. Roy, S. Mondal, and A. K. Brahmachari, "Stevioside and related compounds - molecules of pharmaceutical promise: a critical overview," *Archiv der Pharmazie (Weinheim, Germany)*, vol. 344, no. 1, pp. 5–19, 2011.

[6] J. M. Geuns, "Stevioside," *Phytochemistry*, vol. 64, no. 5, pp. 913–921, 2003.

[7] G. F. Ferrazzano, T. Cantile, B. Alcidi et al., "Is Stevia rebaudiana Bertoni a non cariogenic sweetener? A review," *Molecules*, vol. 21, no. 1, article E38, 2015.

[8] A. D. Kinghorn, N. Kaneda, N. I. Baek, E. J. Kennelly, and D. D. Soejarto, "Noncariogenic intense natural sweeteners," *Medicinal Research Reviews*, vol. 18, no. 5, pp. 347–360, 1998.

[9] J. C. Ruiz-Ruiz, Y. B. Moguel-Ordonez, and M. R. Segura-Campos, "Biological activity of Stevia rebaudiana Bertoni and their relationship to health," *Critical Reviews in Food Science and Nutrition*, vol. 57, 2015.

[10] V. Chatsudthipong and C. Muanprasat, "Stevioside and related compounds: therapeutic benefits beyond sweetness," *Pharmacology & Therapeutics*, vol. 121, no. 1, pp. 41–54, 2009.

[11] S. K. Goyal, R. Samsher, and K. Goyal, "Stevia (Stevia rebaudiana) a bio-sweetener: a review," *International Journal of Food Sciences and Nutrition*, vol. 61, no. 1, pp. 1–10, 2010.

[12] C. Boonkaewwan, C. Toskulkao, and M. Vongsakul, "Anti-inflammatory and immunomodulatory activities of stevioside and its metabolite steviol on THP-1 cells," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 3, pp. 785–789, 2006.

[13] C. European Food Safety Authority, "Revised exposure assessment for steviol glycosides for the proposed uses as a food additive," *EFSA Journal*, vol. 9, no. 1, p. 1972, 2011.

[14] J. D. Urban, M. C. Carakostas, and S. L. Taylor, "Steviol glycoside safety: are highly purified steviol glycoside sweeteners food allergens?," *Food and Chemical Toxicology*, vol. 75, pp. 71–78, 2015.

[15] WHO, "WHO expert committee on diabetes mellitus," *World Health Organization Technical Report Series*, vol. 916, p. 160, 2003.

[16] N. H. Mohd-Radzman, W. I. W. Ismail, Z. Adam, S. S. Jaapar, and A. Adam, "Potential roles of Stevia rebaudiana Bertoni in abrogating insulin resistance and diabetes: a review," *Evidence-based Complementary and Alternative Medicine*, vol. 2013, Article ID 718049, 10 pages, 2013.

[17] P. B. Jeppesen, S. Gregersen, C. R. Poulsen, and K. Hermansen, "Stevioside acts directly on pancreatic beta cells to secrete insulin: actions independent of cyclic adenosine monophosphate and adenosine triphosphate-sensitive K^+-channel activity," *Metabolism*, vol. 49, no. 2, pp. 208–214, 2000.

[18] R. Abudula, V. V. Matchkov, P. B. Jeppesen, H. Nilsson, C. Aalkjaer, and K. Hermansen, "Rebaudioside A directly stimulates insulin secretion from pancreatic beta cells: a glucose-dependent action via inhibition of ATP-sensitive K-channels," *Diabetes, Obesity & Metabolism*, vol. 10, no. 11, pp. 1074–1085, 2008.

[19] J. C. Chang, M. C. Wu, I. M. Liu, and J. T. Cheng, "Increase of insulin sensitivity by stevioside in fructose-rich chow-fed rats," *Hormone and Metabolic Research*, vol. 37, no. 10, pp. 610–616, 2005.

[20] P. B. Jeppesen, S. Gregersen, K. K. Alstrup, and K. Hermansen, "Stevioside induces antihyperglycaemic, insulinotropic and glucagonostatic effects in vivo: studies in the diabetic Goto-Kakizaki (GK) rats," *Phytomedicine*, vol. 9, no. 1, pp. 9–14, 2002.

[21] S. Gregersen, P. B. Jeppesen, J. J. Holst, and K. Hermansen, "Antihyperglycemic effects of stevioside in type 2 diabetic subjects," *Metabolism*, vol. 53, no. 1, pp. 73–76, 2004.

[22] M. Ritu and J. Nandini, "Nutritional composition of Stevia rebaudiana- a sweet herb and its hypoglycaemic and hypolipidaemic effect on patients with non insulin dependent diabetes mellitus," *Journal of the Science of Food and Agriculture*, vol. 96, 2016.

[23] S. Bhasker, H. Madhav, and M. Chinnamma, "Molecular evidence of insulinomimetic property exhibited by steviol and stevioside in diabetes induced L6 and 3T3L1 cells," *Phytomedicine*, vol. 22, no. 11, pp. 1037–1044, 2015.

[24] C. Bender, S. Graziano, and B. F. Zimmermann, "Study of Stevia rebaudiana Bertoni antioxidant activities and cellular properties," *International Journal of Food Sciences and Nutrition*, vol. 66, no. 5, pp. 553–558, 2015.

[25] S. Ghanta, A. Banerjee, A. Poddar, and S. Chattopadhyay, "Oxidative DNA damage preventive activity and antioxidant potential of Stevia rebaudiana (Bertoni) Bertoni, a natural sweetener," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 26, pp. 10962–10967, 2007.

[26] N. Shivanna, M. Naika, F. Khanum, and V. K. Kaul, "Antioxidant, anti-diabetic and renal protective properties of Stevia rebaudiana," *Journal of Diabetes and its Complications*, vol. 27, no. 2, pp. 103–113, 2013.

[27] S. Singh, V. Garg, and D. Yadav, "Antihyperglycemic and antioxidative ability of Stevia rebaudiana (Bertoni) leaves in diabetes induced mice," *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 5, no. 2, pp. 297–302, 2013.

[28] B. Rizzo, L. Zambonin, C. Angeloni et al., "Steviol glycosides modulate glucose transport in different cell types," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 348169, 11 pages, 2013.

[29] D. Zhang, Y. Cui, B. Li, X. Luo, B. Li, and Y. Tang, "miR-155 regulates high glucose-induced cardiac fibrosis via the TGF-beta signaling pathway," *Molecular BioSystems*, vol. 13, no. 1, pp. 215–224, 2016.

[30] G. Pandini, F. Frasca, R. Mineo, L. Sciacca, R. Vigneri, and A. Belfiore, "Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved," *The Journal of Biological Chemistry*, vol. 277, no. 42, pp. 39684–39695, 2002.

[31] K. Reiss, W. Cheng, J. Kajstura, E. H. Sonnenblick, L. G. Meggs, and P. Anversa, "Fibroblast proliferation during myocardial development in rats is regulated by IGF-1 receptors," *The American Journal of Physiology*, vol. 269, 3, Part 2, pp. H943–H951, 1995.

[32] N. Longo, G. I. Bell, R. C. Shuster, L. D. Griffin, S. D. Langley, and L. J. Elsas, "Human fibroblasts express the insulin-responsive glucose transporter (GLUT4)," *Transactions of the Association of American Physicians*, vol. 103, pp. 202–213, 1990.

[33] S. Hrelia, D. Fiorentini, T. Maraldi et al., "Doxorubicin induces early lipid peroxidation associated with changes in glucose transport in cultured cardiomyocytes," *Biochimica et Biophysica Acta*, vol. 1567, no. 1-2, pp. 150–156, 2002.

[34] L. H. Johansson and L. A. Borg, "A spectrophotometric method for determination of catalase activity in small tissue

samples," *Analytical Biochemistry*, vol. 174, no. 1, pp. 331–336, 1988.

[35] T. Maraldi, C. Prata, D. Fiorentini, L. Zambonin, L. Landi, and G. Hakim, "Induction of apoptosis in a human leukemic cell line via reactive oxygen species modulation by antioxidants," *Free Radical Biology & Medicine*, vol. 46, no. 2, pp. 244–252, 2009.

[36] L. Schaffer, C. L. Brand, B. F. Hansen et al., "A novel high-affinity peptide antagonist to the insulin receptor," *Biochemical and Biophysical Research Communications*, vol. 376, no. 2, pp. 380–383, 2008.

[37] Y. Benomar, N. Naour, A. Aubourg et al., "Insulin and leptin induce Glut4 plasma membrane translocation and glucose uptake in a human neuronal cell line by a phosphatidylinositol 3-kinase- dependent mechanism," *Endocrinology*, vol. 147, no. 5, pp. 2550–2556, 2006.

[38] H. X. Chen and E. Sharon, "IGF-1R as an anti-cancer target—trials and tribulations," *Chinese Journal of Cancer*, vol. 32, no. 5, pp. 242–252, 2013.

[39] D. Chanda, J. J. Luiken, and J. F. Glatz, "Signaling pathways involved in cardiac energy metabolism," *FEBS Letters*, vol. 590, no. 15, pp. 2364–2374, 2016.

[40] N. H. Mohd-Radzman, W. I. W. Ismail, S. S. Jaapar, Z. Adam, and A. Adam, "Stevioside from Stevia rebaudiana Bertoni increases insulin sensitivity in 3T3-L1 adipocytes," *Evidence-based Complementary and Alternative Medicine*, vol. 2013, Article ID 938081, 8 pages, 2013.

[41] P. Singh, J. M. Alex, and F. Bast, "Insulin receptor (IR) and insulin-like growth factor receptor 1 (IGF-1R) signaling systems: novel treatment strategies for cancer," *Medical Oncology*, vol. 31, no. 1, p. 805, 2014.

[42] A. Belfiore, F. Frasca, G. Pandini, L. Sciacca, and R. Vigneri, "Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease," *Endocrine Reviews*, vol. 30, no. 6, pp. 586–623, 2009.

[43] L. Knudsen, B. F. Hansen, P. Jensen et al., "Agonism and antagonism at the insulin receptor," *PLoS One*, vol. 7, no. 12, article e51972, 2013.

[44] A. Chowdhury, L. Hasselbach, F. Echtermeyer, N. Jyotsana, G. Theilmeier, and C. Herzog, "Fibulin-6 regulates pro-fibrotic TGF-beta responses in neonatal mouse ventricular cardiac fibroblasts," *Scientific Reports*, vol. 7, article 42725, 2017.

[45] D. L. Coven, X. Hu, L. Cong et al., "Physiological role of AMP-activated protein kinase in the heart: graded activation during exercise," *American Journal of Physiology Endocrinology and Metabolism*, vol. 285, no. 3, pp. E629–E636, 2003.

[46] J. S. Fisher, "Potential role of the AMP-activated protein kinase in regulation of insulin action," *Cell*, vol. 2, no. 3, pp. 68–81, 2006.

[47] J. W. Baynes and S. R. Thorpe, "Role of oxidative stress in diabetic complications: a new perspective on an old paradigm," *Diabetes*, vol. 48, no. 1, pp. 1–9, 1999.

[48] S. P. Wolff and R. T. Dean, "Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes," *The Biochemical Journal*, vol. 245, no. 1, pp. 243–250, 1987.

[49] J. L. Rains and S. K. Jain, "Oxidative stress, insulin signaling, and diabetes," *Free Radical Biology & Medicine*, vol. 50, no. 5, pp. 567–575, 2011.

[50] K. L. Wolfe and R. H. Liu, "Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 22, pp. 8896–8907, 2007.

[51] C. Angeloni, M. Malaguti, and S. Hrelia, "Antiglycative activity of sulforaphane: a new avenue to counteract neurodegeneration?," *Neural Regeneration Research*, vol. 10, no. 11, pp. 1750-1751, 2015.

[52] L. Vasko, J. Vaskova, A. Fejercakova, G. Mojžišová, and J. Poráčová, "Comparison of some antioxidant properties of plant extracts from Origanum vulgare, Salvia officinalis, Eleutherococcus senticosus and Stevia rebaudiana," *In Vitro Cellular & Developmental Biology - Animal*, vol. 50, no. 7, pp. 614–622, 2014.

[53] C. N. Hao, Y. J. Geng, F. Li et al., "Insulin-like growth factor-1 receptor activation prevents hydrogen peroxide-induced oxidative stress, mitochondrial dysfunction and apoptosis," *Apoptosis*, vol. 16, no. 11, pp. 1118–1127, 2011.

[54] C. A. Souders, S. L. Bowers, and T. A. Baudino, "Cardiac fibroblast: the renaissance cell," *Circulation Research*, vol. 105, no. 12, pp. 1164–1176, 2009.