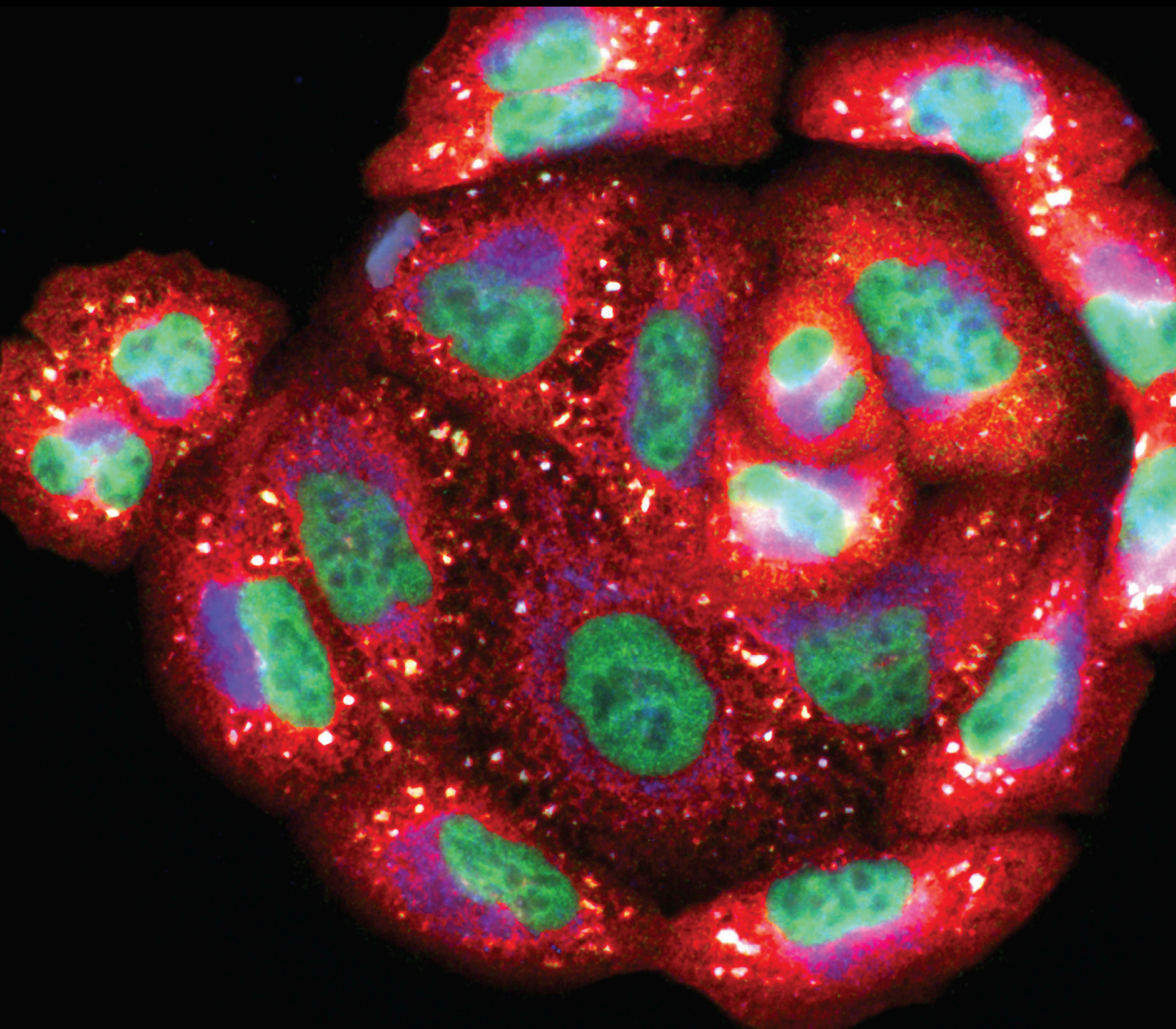
Oxidative Medicine and Cellular Longevity

Lipid Peroxidation Products in Human Health and Disease 2019

Special Issue Editor in Chief: Kota V. Ramana
Guest Editors: Sanjay Srivastava and Sharad S. Singhal



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Editorial

Lipid Peroxidation Products in Human Health and Disease 2019

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Oxidative stress is the major cause of several life-threatening complications including various forms of cancers. Exposure of the body to external pathogens, xenobiotics, allergens, and environmental pollutants leads to increased generation of reactive oxygen species (ROS). The ROS thus generated can interact with important cellular molecules causing the disturbance in the cellular redox balance leading to various pathological consequences. One of the most important molecules directly affected by ROS is polyunsaturated fatty acids. ROS-mediated peroxidation of lipids forms various toxic lipid hydroperoxides and lipid aldehydes which act as secondary signaling intermediates in propagating the oxidative stress signals which contribute to the pathophysiology of human health and disease. Recent studies have demonstrated that the lipid peroxidation-derived lipid aldehydes regulate a number of human pathological complications including cancer, diabetes, cardiovascular, neurological, and various inflammatory diseases. Recent evidence also suggests that lipid peroxidation-derived lipid aldehydes act as biomarkers of various disease processes such as Alzheimer's and Parkinson's. Thus, continuous research work on lipid peroxidation and its generated products is very important in identifying the novel signaling mechanisms involved various human diseases and to explore possible biomarkers of disease and develop better therapeutic approaches. Through a series of special issues, we are continuously encouraging investigators to share their novel research work highlighting the significance of lipid peroxidation products in the pathophysiology of various human diseases.

In the 2019 edition of this issue, the review articles and research articles have discussed how oxidative stress and lipid peroxidation products are involved in various pathological conditions.

An excellent and informative review article by S. Soodaeva et al. described how oxidative and nitrosative stress contributes to the pathophysiology of various respiratory diseases. Specifically, the formation and significance of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the respiratory tract have been exclusively discussed and possible mechanisms through which oxidative and nitrosative stress leads to lung diseases such as asthma and COPD have been suggested. Further, they have also discussed various antioxidant strategies to control the ROS and RNS which could be beneficial in treating lung diseases.

Another review article by H. Sonowal and K. V. Ramana described the significance of lipid peroxidation-derived lipid aldehyde, 4-hydroxynonenal (HNE) in the mediation of various anti- and pro-inflammatory signaling pathways. In this article, the authors have nicely discussed the formation of HNE, its interaction with cellular biomolecules, and involvement in various inflammatory complications. They have also discussed how HNE regulates NRF2-mediated anti-inflammatory signaling leading to the expression of antioxidative defense proteins such as HO1, NQO1, and

GST and also NF-κB-mediated expression of proinflammatory cytokines and chemokines.

The research article by M. A. Ortega et al. investigated the role of oxidative stress and lipid peroxidation in young patients with valvular incompetence leading to chronic venous insufficiency (CVI) disease. In this cohort study involving 110 patients, the authors have analyzed plasma malondialdehyde, iNOS, eNOS, NOX1, and NOX2 in various age groups. Interestingly, they found that patients with a cutoff point of age fifty years showed increased plasma malondialdehyde, a marker for lipid peroxidation, and the expression of iNOS, NOX1, and NOX2 levels. Their results indicate that the increase in oxidative stress and lipid peroxidation reflects the characteristics of the aged CVI patient with valvular incompetence.

Another research article by A. Molfino et al. examined the circulating 19,20-epoxydocosapentaenoic acids (19,20-EDPs) levels in breast cancer patients and healthy control subjects before and after supplementation with docosahexaenoic acid (DHA) for 10 days. The serum levels of 19,20-EDPs were increased in breast cancer as well as in controls after DHA supplementation. Further, they showed that 19,20-EDP levels were lowered in breast cancer patients with BRCA1.2 mutation when compared to breast cancer patients without the mutation. The luminal A-like breast cancer patients showed increased 19,20-EDP after DHA supplementation when compared to nonluminal A breast cancer patients. These results suggest that DHA oral supplementation increases 19,20-EDPs in breast cancer patients independent of breast cancer subsets. However, BRCA1.2 mutated and luminal A-subtype breast cancer patients showed altered ability of DHA epoxidation.

A research study by T. Li et al. examined the protective role of hypoxia-inducible gene domain family member 1A (Higd1a) in high fat-induced lipotoxicity. In this study, the authors have examined the effect of oleic acid and palmitate on Higd1a expression in HepG2 and LO2 cells. Their results indicate that oleic acid and palmitate increased the expression of Higd1a in the cells and knocking down of Higd1a decreased mitochondrial transmembrane potential and caused apoptosis. Further, they found that ROS increase Higd1a expression by increasing the synergistic upregulation of HIF-1 and PGC-1a in the cells. These results suggest a novel protective role of Higd1a in protecting the cells from high fat-induced oxidative stress.

L. Micheli et al. examined the seminal levels of ghrelin, obestatin, malondialdehyde (MDA), glutathione (GSH) and oxidized glutathione (GSSG), IL-6, and TNF-alpha in infertile patients with varicocele or leukocytospemia. In this study, 32 patients with leukocytospemia, 24 with variocele, and 14 control fertile subjects were recruited to examine their semen parameters. The results indicate that when compared to controls, infertile patients with leukocytospemia and variocele showed increased sperm apoptosis, IL-6, and TNF-alpha levels and decreased ghrelin, obestatin, and GSH/GSSG ratio. Similarly, lipid peroxidation aldehyde MDA levels were increased in leukocytospemia and variocele patients when compared to controls. The significance of lipid peroxidation with the etiology of tumorigenesis was reported by Y. Zhang et al. in their research article. In this study, using mouse embryonic fibroblasts, the authors have shown that 15-hydroperoxy-eicosatetraenoic acid (15s-HpETE) causes reversible oxidation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN). They have also observed oxidative dimerization of thioredoxin in the cells. Further, loss of peroxiredoxin III increased 15s-HpETC-induced PTEN oxidation.

A cross-sectional study by S. R. Carneiro et al. has demonstrated the relationship between the lipid peroxidation and physical activity in women and the squamous intraepithelial lesion (SIL) of the cervix. Two groups of 18 SIL patients and 28 control subjects were compared. The lipid peroxidation marker MDA was found higher in SIL patients when compared to controls. At the same time, they also found that the SIL group patients showed a lowered international physical activity questionnaire (IPAQ) score. This study indicates the importance of physical activity in regulation of oxidative stress in patients with SIL.

Another cross-sectional study by S.-S. Wu et al. examined the serum markers of oxidative stress in relation to carotid intima-media thickness (IMT) in patients with metabolic syndrome. In this study, 134 patients with varied metabolic syndromes were examined for their plasma oxidative stress markers. They found that MDA levels and uric acid levels were associated with carotid IMT patients with varied metabolic syndrome scores. These results suggest that determining the MDA levels along with uric acid levels may be promising while monitoring carotid IMT in patients with varied metabolic syndromes.

In conclusion, it is clear from the recent findings that oxidative stress-generated lipid peroxidation products could be potential biomarkers of a broad spectrum of diseases. Therefore, thorough understanding of lipid peroxidation processes and their secondary products is important for early detection of various pathophysiological states and their complications. This could be really important in developing potential therapeutic intervention to combat debilitating diseases such as neurological, aging, cardiovascular, and cancer, where oxidative stress is implicated in the etiology of the disease.

Conflicts of Interest

The editors declare no conflict of interest.

Acknowledgments

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Review Article

4-Hydroxy-Trans-2-Nonenal in the Regulation of Anti-Oxidative and Pro-Inflammatory Signaling Pathways

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Recent studies indicate that 4-hydroxy-trans-2-nonenal (HNE), a major oxidative stress triggered lipid peroxidation-derived aldehyde, plays a critical role in the pathophysiology of various human pathologies including metabolic syndrome, diabetes, cardiovascular, neurological, immunological, and age-related diseases and various types of cancer. HNE is the most abundant and toxic α, β-unsaturated aldehyde formed during the peroxidation of polyunsaturated fatty acids in a series of free radical-mediated reactions. The presence of an aldehyde group at C1, a double bond between C2 and C3 and a hydroxyl group at C4 makes HNE a highly reactive molecule. These strong reactive electrophilic groups favor the formation of HNE adducts with cellular macromolecules such as proteins and nucleic acids leading to the regulation of various cell signaling pathways and processes involved in cell proliferation, differentiation, and apoptosis. Many studies suggest that the cell-specific intracellular concentrations of HNE dictate the anti-oxidative and pro-inflammatory activities of this important molecule. In this review, we focused on how HNE could alter multiple anti-oxidative defense pathways and pro-inflammatory cytotoxic pathways by interacting with various cell-signaling intermediates.

1. Oxidative Stress and Lipid Peroxidation

Free radicals are regularly generated in aerobes because of normal respiration processes and the activity of cellular antioxidant defense machinery maintains a balance of the free radicals utilizing a variety of antioxidant enzymes in the cells. A balanced redox homeostasis is necessary for the maintenance of normal cellular processes in aerobes [1]. Under oxidative stress conditions, disruption of cellular redox homeostasis leads to an imbalance between reactive oxygen species (ROS) generation and their elimination by antioxidant enzymes. The major reasons for the redox imbalance could be the overproduction of free radical species or the inability of the cellular antioxidant defense machinery to eliminate or sequester the free radicals generated in the body. Free radicals such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$), peroxyl radical ($LOO^{\cdot}$) and non-radical oxidants such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), nitrous acid (HNO_2), and singlet oxygen ($^1O_2$), are the most commonly generated ROS in the cells and act as initiators of oxidative damage contributing to pathophysiology of multiple disease complications. Cells upon exposure to external oxidants such as xenobiotics, environmental pollutants, UV radiation, carcinogens and allergens, and internally formed oxidants in the body such as cytokines, growth factors, and chemokines could lead to altered cellular metabolic processes leading to the production of ROS. The activity of enzymes such as NADPH oxidase, xanthine oxidase, and auto-oxidation of glucose can generate ROS under different oxidant stimuli. In addition, mitochondrial oxidative phosphorylation that usually participates in the cellular respiration to generate energy in the cells can also contribute to the generation of free radicals (Figure 1). Apart from inducing damage to cellular macromolecules and dysregulation of cellular homeostasis, the free radicals formed during metabolic processes also act as secondary signaling intermediates and regulate various oxidative and anti-oxidative signaling pathways [2]. Antioxidant peptides such as glutathione (GSH) play an important role in detoxification

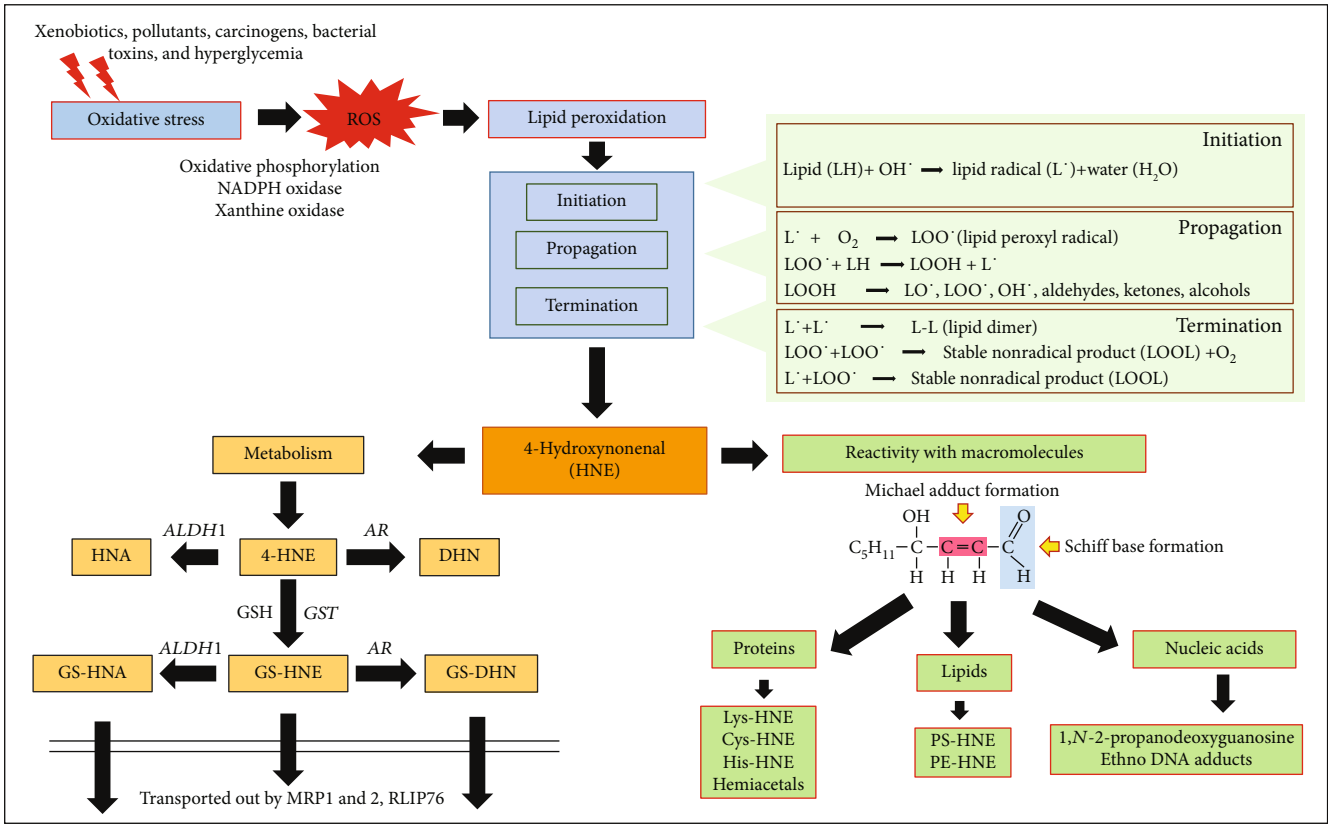

Figure 1: Schematic figure showing the ROS -induced formation of HNE via lipid peroxidation and its metabolism by various antioxidative enzymes.

of ROS. GSH is the most essential regulator of cellular redox homeostasis as it metabolizes or scavenges a number of free radicals or free radical- generated products in the cells. The ratio of GSH (reduced)/GSSG (oxidized) is a key indicator of cellular redox potential. Apart from GSH, enzymes such as glutathione-S-transferases (GSTs), plays a very important role in the maintenance of redox balance in cells. GSTs play a significant role in detoxification of various xenobiotics as well as endogenous toxic products generated in the cells by conjugation with GSH, facilitating further metabolism or detoxification by multiple other antioxidant detoxification and defense pathways [3]. Further, antioxidant enzyme superoxide dismutase (SOD) catalyzes the dismutation of superoxide free radical ($O_2^{\cdot -}$), into hydrogen peroxide and oxygen. The hydrogen peroxide is further catalyzed by catalase into oxygen and water [4]. The activity of these antioxidant enzymes controls the free radical content in the body. However, during pathological complications, alterations in various antioxidant defense pathways along with increased ROS production imbalances cellular homeostasis leading to tissue damage and dysfunction.

Though ROS play an important role in regulating various physiological functions such as the elimination of pathogenic bacteria by immune cells, maintenance of vascular tone, cardiovascular functions, cell proliferation, and differentiation, a disturbance of ROS homeostasis leads to the development of various pathological complications. Several studies have shown that oxidative stress generated by ROS plays a critical role in multiple pathologies including various types of cancers, inflammatory disorders, neurodegenerative diseases, and cardiovascular complications [5, 6]. Apart from the spontaneous direct effect on DNA, RNA, amino acids, proteins and lipids, the secondary products generated by ROS -mediated reactions significantly damage various macromolecules and propagate their deleterious effects in the cells. Most importantly, even though the ROS generated in cells are short-lived, the secondary products generated by ROS are comparatively stable and further acts as important mediators of cellular signaling. The secondary ROS generated products maintain and propagate the effect of ROS long after ROS generation. These secondary toxic products could migrate to distant sites, which are far from their site of origin and can induce tissue damage and organ dysfunction in multiple sites, thus exerting multifactorial side effects. One such effect of ROS is the oxidative damage to the membrane lipids, which is termed as lipid peroxidation. Membrane phospholipids such as poly-unsaturated fatty acids (PUFA) are the major targets of lipid peroxidation induced by ROS. Lipid peroxidation-derived products such as HNE (4-hydroxy-trans-2-nonenal), acrolein and malondialdehyde (MDA) are more stable; have a longer half-life than ROS themselves and have attained considerable attention in the recent past as important mediators of oxidative stress-induced pathological complications [7].

During oxidative stress, lipid peroxidation occurs via three major steps, (a) initiation, (b) propagation and (c) termination.

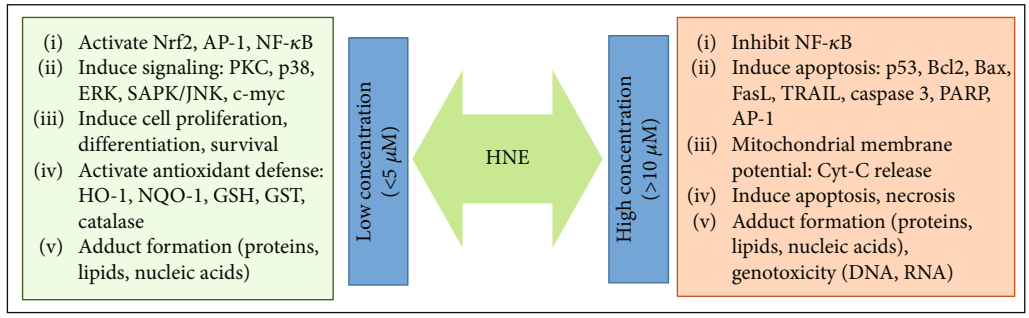

Figure 2: HNE is a pleiotropic signaling molecule: Depending on the concentration and duration of exposure, HNE induces multiple signaling pathways in cells by regulating various signaling intermediates.

In the initial initiation step, the free radicals (e.g. $OH^{\cdot}$) attack PUFA and generates lipid radicals ($L^{\cdot}$). The removal of hydrogen atoms from the lipids leads to the reduction of ROS into water and generation of lipid radicals. During propagation step, the unstable lipid radicals react with oxygen leading to the generation of lipid peroxyl radicals ($LOO^{\cdot}$) and additional lipid radicals ($L^{\cdot}$), which further reacts in a chain reaction and new lipid radicals and lipid peroxides are generated. The presence of metal ions such as iron (Fe^{2+}) and copper (Cu^{2+}) is shown to accelerate the propagation reaction. In the final termination step, hydroperoxides are generated by the reaction of peroxyl radicals with vitamin E (α-tocopherol). The lipid peroxides and lipid radicals react with each other to generate more stable non-radical products during the termination of the lipid peroxidation process (Figure 1). The β-oxidation of lipid peroxides leads to the generation of various toxic lipid aldehydes such as alkanals, alkenals, hydroxyalkenals, and alkadienes [8, 9]. The formation of lipid aldehydes such as MDA and HNE is often considered as the most common toxic end products of lipid peroxidation and generally used as indicators of oxidative damage in the cells and tissues. Further, the α,β-unsaturated hydroxyalkenal, HNE has been shown to be the most abundant and toxic lipid peroxidation end product generated during lipid peroxidation [8, 9]. HNE with its highly reactive electrophilic groups can interact with cellular proteins, GSH, nucleic acids and can cause cytotoxicity or genotoxicity (Figure 1). Recent studies have postulated that HNE is a biomarker of oxidative stress-induced pathological complications and correlated its damaging effects to various human diseases such as cancer, neurodegenerative, inflammatory and autoimmune diseases, various metabolic diseases, and mitochondrial dysfunction [8, 10–15]. Besides its reactivity with macromolecules, HNE can also alter the membrane potential, electron transport and ion imbalance in the cells leading to neurological disorders [16, 17]. Further, HNE also plays an important role in cell survival and death by signaling through different pathways mediated by caspase3, Bax, Bcl2, death receptors, multiple kinases and transcription factors such as Nrf2, AP-1 and NF-κB (Figure 2) [18–21]. The effect of HNE on the cellular macromolecules is dependent on the type of tissue and concentration of HNE, with specificities for proteins containing cysteine, histidine and lysine residues [8, 9, 22]. The detoxification and metabolism of HNE is also differential in different tissues [23]. In the succeeding section, we have discussed the reactivity of HNE with different macromolecules and its metabolism.

2. Reactivity and Metabolism of HNE

HNE has three functional groups 1) carbonyl group on C1 (C=O), 2) a double bond between C2 and C3 (C=C) and 3) hydroxyl group on C4 (which facilitates C=C polarization and cyclization reactions), which makes HNE a highly reactive aldehyde product of lipid peroxidation process (Figure 1). HNE can react with different macromolecules such as proteins, phospholipids, nucleic acids, and glutathione (GSH). Membrane-bound entities and proteins containing an abundance of cysteine, lysine and histidine residues and phospholipids such as phosphatidyl-ethanolamine are preferred targets for adduct formation by HNE. Electrophilic sites of HNE leads to Schiff base formation between an amino group and the carbonyl group at C1 and Michael addition of thiol or amino compounds at C3 [9, 24, 25]. The reactivity of HNE towards amino acids has been shown to be in the order of cysteine>histidine>lysine [26–28]. First, the amino acids undergo Michael addition at the C=C double bond of HNE. Michael addition to the C=C bond confers rotational freedom at the C2-C3 bond, which facilitates secondary reactions of primary amines with the carbonyl group of HNE to form Schiff bases [29]. Although cysteine is the most preferred amino acid for reactivity with HNE, HNE-histidine adducts have been shown to be more stable as compared to cysteine- and lysine-HNE adducts [28, 30]. Further, HNE-induced covalent modification of nucleophilic residues of amino acids regulate protein activation/inactivation, which could alter cellular signaling pathways. Specifically, important cellular signaling pathways controlling apoptosis, cell cycle, oxidative and nitrosative stress-associated pathways are reported to be significantly affected by HNE leading to cellular toxicities [31–33]. HNE also inhibits the proteasomes such as 20S proteasome and hence impairs the cellular proteasomal degradation of damaged or modified protein subunits generated subsequent to oxidative stress [34]. Chaperone activities specifically mediated by HSP 72 and HSP 90 have been shown to be modified by HNE adduct formation [35, 36]. HSP 90 function has been reported to be modified by the modification of Cys-572 residue by HNE, which has important pathological implications in alcoholic liver diseases

(ALD) [36, 37]. HNE can directly interact with guanosine bases in DNA to form 1,N-2-propano-deoxyguanosine with an ability to form 1.2 ± 0.5 adducts/10^7 nucleotides [38]. Etheno-DNA (ε-DNA) adducts have been shown to be generated by the reaction of HNE with nucleotides in DNA that could lead to mutational changes in DNA, increasing the susceptibility to cancerous transformation in cells [39]. Formation of HNE-DNA adducts indicate the genotoxic and mutagenic effects induced by lipid peroxidation [40]. HNE-DNA adduct formation hampers DNA repair mechanism in the cells. Studies have shown that HNE inhibits nucleotide excision repair in DNA damage-induced either by UV radiation or carcinogens such as benzo[a]pyrene diol epoxide (BDPE); a major environmental pollutant and component of cigarette smoke [41, 42]. Thus, these studies provide evidence that HNE is a multifactorial effector of signaling and cellular functions in the body.

HNE is short-lived in the cells with a half-life of less than 2 min and is immediately conjugated with other macromolecules or metabolized by various antioxidant enzymes [43]. Based on cellular antioxidant defense capacity and cell types, multiple studies have demonstrated varying concentrations and half-life of HNE in vitro and in vivo [44]. Major cellular metabolic pathways which play an important role in the detoxification of HNE are: (a) alcohol dehydrogenase (ADH) or aldose reductase (AR, AKR1B1), which reduces HNE to 1,4-dihydroxy-2-nonene (DHN), (b) aldehyde dehydrogenase (ALDH), which oxidizes HNE to 4-hydroxy-2-nonanoic acid (HNA) and (c) glutathione-S-transferases (GSTs), which catalyze the conjugation of HNE to GSH forming GS-HNE, which is then transported out of the cell in an ATP- dependent manner by various drug transporters such as MRP1, MRP2, and RLIP76 (Figure 1) [8, 45–47]. HNE has the ability to induce modifications in enzymes involved in cellular detoxification such as GSTs [48], glutathione reductase (GR) [49], either by Michael adduct formation or by covalent modifications of amino acid residues in the proteins. Interestingly, the cellular GSH levels control the concentration of HNE in cells and on the other hand, HNE has been shown to regulate the expression of enzymes responsible for GSH synthesis. Exposure of cells to HNE has been shown to increase γ-glutamate cysteine ligase (GCL) activity. GCL catalyzes the rate-limiting step in GSH biosynthesis and hence is important for the maintenance of cellular GSH levels. Further, HNE has also been shown to induce the transcriptional activation of γ-glutamyl cysteine synthase, thus contributing to enhanced GSH biosynthesis [50]. HNE can conjugate with GSH spontaneously or by the conjugation reactions catalyzed by the specific GST isozymes [51, 52]. GSTA4-4 and GST5.8 have been shown to specifically participate in the GSH-conjugation with HNE [53, 54]. Studies have shown that overexpression of HNE metabolizing GST (mGSTA4-4, hGSTA4-4, or hGST5.8) confers protection to cells against oxidative injury [55]. Exposure of hepatoma cells to exogenous HNE leads to the formation of major metabolites of HNE: glutathione-HNE (GS-HNE) indicating the importance of HNE-GSH conjugation as a major metabolic route for its detoxification [47]. Apart from GST, studies have also demonstrated that aldose reductase (AR; AKR1B1) further metabolizes GS-HNE to GS-DHN and aldehyde dehydrogenase (ALDH) isozymes metabolize GS-HNE to GS-HNA [54, 56]. Several studies have demonstrated that aldo-keto reductases are an important class of enzymes upregulated by oxidative stress or exposure to HNE [57]. Up-regulation of AR has been shown to play an important role in HNE detoxification in HepG2 cells. Burczynski et al., have demonstrated that HNE generated in the cells induces its own metabolism and detoxification by up-regulating the aldo-keto reductase isozyme AKR1C1 [58]. Both HNE and its GSH metabolite, GS-HNE, have been shown to be reduced by AR to 1,4-dihydroxy-2-nonene (DHN) and GS-DHN, respectively [59–62]. Further, AR reduces GSH conjugates of aldehydes (e.g. GS-HNE and GS-acrolein) more effectively as compared to their parent aldehydes (e.g. HNE and acrolein) indicating that AR has a specific binding site for glutathionylated aldehydes [63, 64]. Indeed, Singh et al., have crystallized AR bound GS-analogue and found that AR has specific GS-aldehyde binding domain [65]. These studies demonstrate that catalytic activity mediated by AR is a major step in the detoxification of lipid peroxidation-derived aldehydes such as HNE and their glutathione conjugates. Indeed multiple studies modulating AR activity have shown to exert therapeutic effects in various oxidative stress-induced inflammatory pathologies including cancer [56, 66–68].

HNE-mediated regulation of signaling pathways is pleiotropic and the signaling pathways activated or inhibited by HNE depends on the concentration of HNE and the type of cells used in the study. In cell culture studies, HNE concentration greater than 10 μM has been reported to induce apoptosis whereas sub-lethal dose $\leq 5\,\mu$M induces cell proliferation. Concentration-dependent activation of various pro-inflammatory and anti-oxidative pathways by HNE is shown to regulate multiple kinases and transcription factors important for disease pathology (Figure 2). In certain pathologies, a very high concentration of HNE has been observed. Elevated plasma levels of HNE (~100 μM) have been reported in children with systemic lupus erythematosus [69] and in the liver tissues of mouse models of experimental alcoholic liver disease [70, 71]. However, it is still unclear how the cells cope with such high levels of HNE in vivo and the deleterious effect exerted by such high concentrations of HNE in various human diseases. With the increasing evidence of the importance of ROS and lipid peroxidation-derived aldehydes in different human pathologies, and advancement in analytical techniques to detect and identify the specific role of free radical intermediates in various diseases [72], studies on the roles of lipid peroxidation-derived aldehydes in modulating cellular signaling pathways has attained a considerable attention in the recent years. In the following section, we have briefed some of the important findings on the role of HNE in mediating anti - and pro-inflammatory signaling pathways.

3. Regulation of Anti-oxidative Pathways by HNE

NFE2-related nuclear factor 2 (Nrf2) is a master transcription factor regulating the expression of genes involved in the anti-oxidative and anti-inflammatory pathways [73].

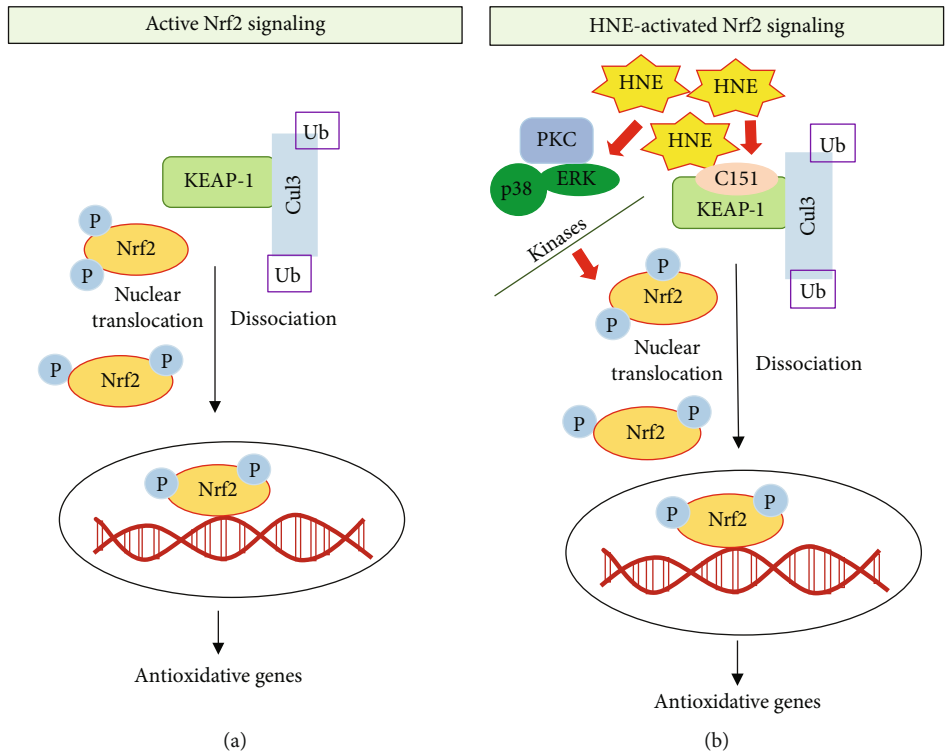

FIGURE 3: Role of HNE on Nrf2-mediated anti-oxidative signaling: Nrf2 is a master regulator of various anti-oxidative and anti-inflammatory pathways in the cells. Under basal conditions, Nrf2 is bound to KEAP-1 and remains inactive. KEAP-1 is an adaptor protein and association of KEAP-1 with Cul3, promotes proteasomal degradation of the Nrf2-KEAP1 complex. However, when activated by an external stimulus, (a) phosphorylation and dissociation of Nrf2 occurs from the Nrf2-Keap1 complex leading to nuclear translocation of Nrf2 and transcription of target genes. (b) HNE could promote Nrf2 activation either by modifying the C151 residues in KEAP-1 or by promoting Nrf2 phosphorylation directly. Apart from directly affecting Nrf2 and KEAP-1, various upstream kinases such as p38MAPK, PKC and ERK can also be modulated by HNE, which further initiates the activation of Nrf2.

Various exogenous stimuli such as xenobiotics, flavonoids, and antioxidants activate Nrf2 nuclear translocation, which then binds to its consensus antioxidant response elements (ARE) and induces the transcription of anti-oxidative genes. Under the basal conditions, Nrf2 is sequestered in the cytoplasm by its association with KEAP1, which keeps the complex inactive. The Nrf2-KEAP1 complex is ubiquitylated and subsequently, the association of Nrf2-KEAP1 complex with Cul3 induces proteasomal degradation. However, under conditions of oxidative stress or antioxidant stimuli, phosphorylation of Nrf2 triggers its dissociation from KEAP1. The active Nrf2 is then translocated to the nucleus and binds to its consensus ARE to activate respective antioxidant defense genes [74, 75]. (Figure 3).

Activation of the KEAP1-Nrf2 signaling pathway has been shown to be a major approach for HNE-induced cellular antioxidant defense. HNE is electrophilic in nature and hence activates the EpRE/ARE response elements. The highly reactive cysteine residues in KEAP1 makes it a preferred target for electrophilic attack by HNE [76]. Specifically, HNE has been shown to modify the cysteine amino acid residue at C151 in the BTB domain of KEAP1 leading to the dissociation of KEAP1 from the Nrf2-KEAP1 complex [76]. Three distinct cysteine amino acid-based sensors in KEAP1 have been reported to be recognized by carbonyl groups of HNE [77]. Apart from directly modulating KEAP1-Nrf2 interaction, HNE can also modulate various upstream protein kinases that are required for the phosphorylation Nrf2. Among these, PKC [78], PI3K [79] p38-MAPK, and ERK [80] have been shown to be important cellular protein kinases whose activities are specifically regulated by HNE. Although the precise mechanism of how these kinases modulate HNE-induced Nrf2 activation is not known, it is hypothesized that the adduct formation of protein kinases with HNE could alter the physiological functions of these protein kinases and favor phosphorylation of Nrf2 at specific sites (Figure 3).

Activation of Nrf2 either directly or indirectly acts as an important transcriptional regulator of various Phase -II detoxifying enzymes conferring protection to cells against oxidative damage [81]. Some important Phase- II detoxification enzymes activated by HNE include aldo-keto reductases (AKRs), γ-glutamylcysteine ligase (GCL), glutathione peroxidase (GPX), glutathione-S-transferase (GST), NADPH quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), thioredoxin (Trx), thioredoxin reductase (TrxR), and drug transporter proteins such as MRPs, Pgp1 and RLIP76. All the Phase-II detoxification enzymes have been shown to have specific ARE consensus sequences for Nrf2 binding in response to oxidative stress [82]. It is important that under oxidative stress, the major cellular detoxification pathways are activated in a concerted mechanism as a

defense response to maintain the cellular redox balance homeostasis to confer protection against oxidative injury.

Several studies have demonstrated that the activation of Nrf2 plays an important role in the detoxification of xenobiotics, including lipid peroxidation products such as HNE [83, 84]. Miller et al., have shown that the intraperitoneal injection of Nrf2-ARE activators such as sulforaphane or carnosic acid protects cerebral cortical mitochondria from HNE-induced toxicity. Further, treatment with sulforaphane or carnosic acid up-regulated the mRNA levels of antioxidant enzymes such as HO-1 and prevented HNE-induced disruption of mitochondrial respiration [85]. HNE has also been shown to induce the expression of antioxidant enzymes AKR1C1, GSTA4, and HO-1 in HeLa cells, which play an important role in the protection against oxidative stress-induced toxicity [86]. Using siRNA-mediated silencing of Nrf2, this study has provided strong evidence that Nrf2 plays an important role in exerting protection against HNE-induced toxicity in HeLa cells [86]. Exposure to low concentrations of HNE (<5 μM) is likely to induce an elevated antioxidant response, which prepares the cells to cope with oxidative insults. Incubation of macrophages and vascular smooth muscle cells with HNE has been shown to activate Nrf2 and protect cells from oxidative injury during vascular complications. Abundant formation of lipid peroxidation products such as HNE has been reported in atherosclerotic plaques and hence identifying the signaling pathways regulated by HNE in vascular cells is of immense importance in atherosclerosis [87]. Gargiulo et al., have shown that the exposure of monocytes to HNE induces various inflammatory cytokines such as IL-1β, IL-8, and TNF-α which regulate atherosclerotic plaque stability [88, 89]. Incubation of HNE with murine peritoneal macrophages isolated from Nrf2 knockout mouse models did not show increased expression of HO-1, Prx1, and A170 antioxidant proteins, indicating the significance of Nrf2 in HNE-induced protective effects [88]. HNE-induced upregulation of CD36, a major scavenger receptor, has been shown to exert protective functions during atherosclerosis and oxLDL-induced oxidative damage in an Nrf2 dependent manner [90]. Studies have demonstrated that multiple antioxidant response elements and oxLDL uptake receptors are HNE targets, which play an important role in atherosclerosis progression, the anti-oxidant response in endothelial and vascular cells and atheroprotective functions [91, 92]. In 661 W retinal ganglion cells, apart from upregulating the nuclear translocation and Nrf2-ARE transcription activity, treatment with 5 μM HNE induced the expression of antioxidant genes such as Trx, TrxR, and HO-1, exerting protective functions. Similarly, the up-regulation of antioxidant genes by HNE conferred protection against H_2O_2-induced apoptosis in 661 W cells [93]. This study also demonstrated that siRNA-mediated silencing of Nrf2 failed to confer protection induced by HNE against H_2O_2-induced cell damage [93]. However, the signaling mechanisms activated by HNE are elusive. As we have already discussed in the preceding section that the concentration of HNE used in a study could act as an important factor to properly decipher the signaling mechanisms activated or inhibited by HNE (Figure 2). In studies using primary cultures of human optic nerve head astrocytes, it is observed that HNE concentrations greater than 50 μM induce apoptosis and significantly decrease the cellular GSH levels [94]. Treatment with a lesser concentration of HNE (< 25 μM) also leads to a significant decrease in cellular GSH levels after 1 h and 3 h of treatment. However, when the cells were allowed to recover for 24 h without HNE after 1 h or 3 h treatment with 25 μM HNE, significant restoration of GSH levels have been observed [94]. In this study, real-time PCR gene expression analysis of mRNA also showed a significant increase in the levels of AKR1C1, GSTA4 and GCLC mRNA along-with increase in transcription factor Nrf2 and c-Fos. Thus, the concentration and time of exposure to HNE are important in mediating HNE-induced antioxidant responses, which prepares the cells for protection against oxidant-induced damage [94]. It is already clear that strategies such as using natural or synthesized antioxidants to activate or augment Nrf2 mediated cellular antioxidant defense pathways exert protective functions against oxidative stress injury by multiple oxidants including lipid peroxidation products [86, 95]. Thus, HNE-mediated activation of Nrf2 may be an important step in regulating the HNE-induced antioxidant response in cells and tissues.

Studies to decipher the molecular mechanisms of HNE-mediated antioxidant defense response has provided evidence that apart from Nrf2-mediated regulation of antioxidant response, HNE has the ability to directly regulate the transcriptional activity of other antioxidant enzymes responsible for GSH biosynthesis. HNE regulates GSH levels by regulating the rate-limiting step of GSH biosynthesis by modifying Cys553 of the catalytic subunit (~73 kDa GCLC) and Cys35 of the modulatory subunit (~31 kDa, GCLM) of glutamate-cysteine ligase (GCL) [96]. GCL is a heterodimeric holoenzyme complex consisting of a 73 kDa catalytic subunit (GCLC) and a 31-kDa modifier subunit, which catalyzes the first and rate-limiting step of GSH biosynthesis [97]. Further, HNE induces the promoter activity of γ-glutamyl transpeptidase (GGT) by interacting with an electrophile response element (EpRe) in the proximal region of GGT promoter region 5 (GP5). GGT plays an important role in glutathione homeostasis and metabolism of GSH and hence increasing the transcriptional activity of GGT confers protection against oxidative injury. Moreover, HNE has been shown to be involved in Nrf2 binding to the GP5 EpRE complex [98]. Apart from directly inducing GGT promoter activity, HNE could also activate various upstream MAPK kinases such as ERK and p38-MAPK, which are involved in the activation of GGT. The transcriptional regulation of EpRE by p38-MAPK and ERK has been shown to induce GGT mRNA transcription, which regulates antioxidant response and cellular homeostasis [99]. MAPKs could act as important components in the regulation of Nrf2 activity induced by HNE as they have been reported to directly induce phosphorylation of Nrf2 leading to its activation [80]. Further, the transcription factor AP1 has been shown to be involved in the HNE-induced increase in the glutamate cysteine ligase (GCL) expression by regulating antioxidant response [100, 101]. These studies have provided evidence that multiple signaling pathways co-operate to induce antioxidant defense

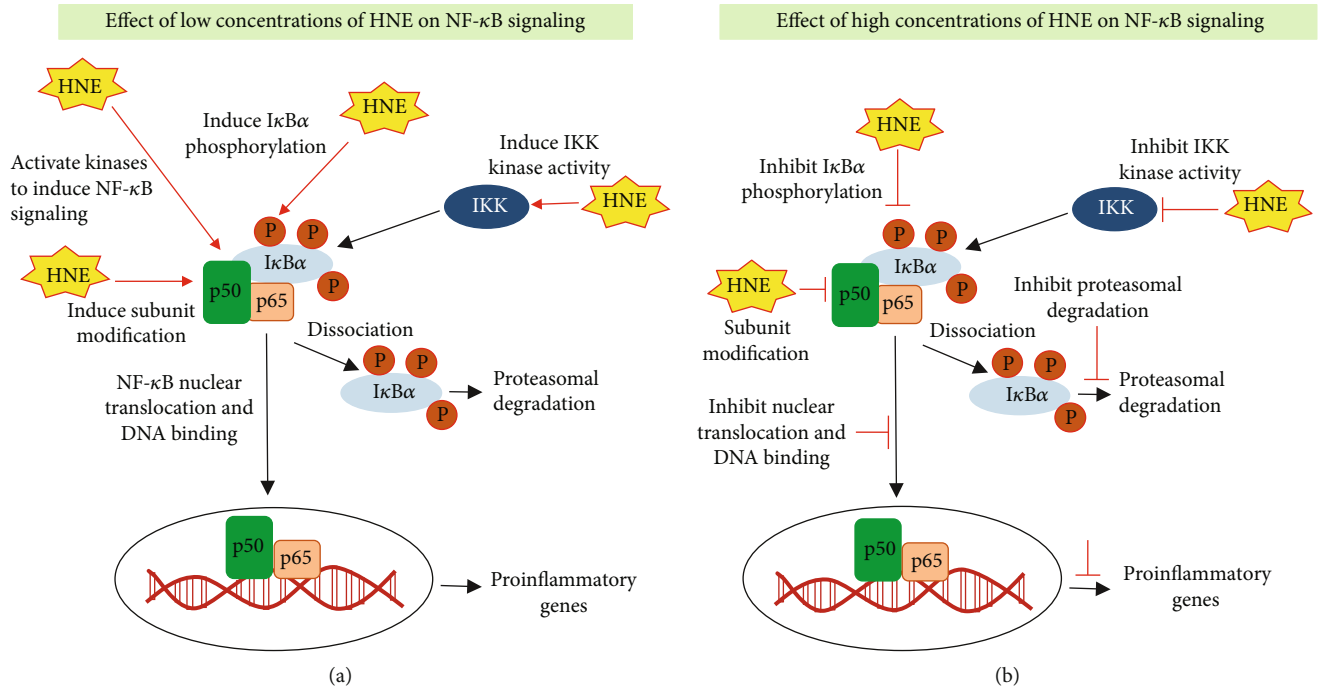

Figure 4: Role of HNE on NF-κB-mediated pro-inflammatory signaling: During oxidative stress conditions ROS activate a series of protein kinases such as IKK which phosphorylates IkBα leading to dissociation of the inactive complex of NF-κB-IκBα. The active NF-κB is translocated to the nucleus and binds to its consensus sequences to induce the transcription of target genes. HNE exerts pleiotropic effects on NF-κB; (a) the low concentrations of HNE (<5 μM) could activate NF-κB whereas (b) high concentrations (>5 μM) of HNE could inhibit NF-κB. HNE could modulate NF-κB activity by interacting with upstream protein kinases and can directly modify NF-κB subunits.

response induced by HNE. Low concentrations of HNE has been shown to induce the expression of cytoprotective genes such as thioredoxin reductase 1 (TrxR1) in a Nrf2/ERK/Akt-dependent pathway in PC12 cells [102]. Further, the expression of TrxR1 has been shown to induce an adaptive response and enhances cellular tolerance to oxidative insult. The HNE-induced ERK and AKT signaling pathways may directly or indirectly affect Nrf2 expression in PC12 cells [102]. HNE-mediated activation of ERK also plays an important role in the regulation of HO-1 expression induced by HNE. In rat pulmonary epithelial cells, HO-1 expression is reported to be translationally regulated by HNE-mediated phosphorylation of ERK [103].

ROS are important for HNE production; ROS-induced lipid peroxidation is a major source of HNE generation in cells [7, 9]. Furthermore, studies have provided evidence that HNE itself could induce ROS generation in cells; the HNE-induced ROS further propagates the effects of HNE-mediated antioxidant defense mechanisms in cells [104]. On the other hand, a few studies showed that ROS could play a bystander effect on HNE-induced cell signaling. Studies on PC12 cell lines provide evidence that HNE-induced cell damage was a result of HNE-induced modification of cellular GSH or modification in cysteine and histidine residues in proteins rather than the direct effect of ROS alone. HNE-induced apoptosis, intercellular Ca^{+2} accumulation, caspase activation and cellular quiescence were not ameliorated by scavenging the ROS generated in cells [105]. Thus, protein modifications, rather than ROS generation in HNE exposed cells seem to be a major regulator of HNE-mediated cell signaling [105]. Several other pathways such as the unfolded protein response- (UPR) and ER stress-associated signaling pathways have been shown to be affected by HNE [105]. In the same study, they have demonstrated that HNE induces ER stress by regulating ATF4 expression which regulates the unfolded protein response (UPR). A significant increase in UPR target genes such as GRP78 and CHOP has been observed in PC12 cells treated with HNE [105]. Thus, HNE by interacting with various protein kinases such as MAPK and AKT, transcription factors and AREs regulates the Nrf2-mediated antioxidant pathways in the cells. Furthermore, exposure of cells to low concentrations of HNE prepares the cells to withstand further oxidative injury, whereas a high concentration activates the apoptotic pathways leading to cellular toxicity.

4. Regulation of Pro-Inflammatory Pathways by HNE

Besides the regulation of Nrf2-mediated anti-oxidant pathways, HNE has been shown to regulate various pro-inflammatory pathways mediated by NF-κB and AP1. The activation of these pathways leads to the expression of multiple cytokines, chemokines and growth factors responsible for inflammatory response and various disease pathologies. NF-κB; a major redox-sensitive transcription factor activated during oxidative stress conditions, is a key regulator of cell viability and death [106]. Various studies have provided evidence of the myriad and elusive role of HNE as a critical regulator of NF-κB-mediated inflammatory signaling

pathways in cells. NF-κB is a heterodimeric transcription factor with five subunits consisting of p50, p52, p65, c-Rel, and RelB. In the inactive state, the NF-κB complex is localized in the cytoplasm due to its association with IκBα. Upon stimulation by exogenous stimuli, NF-κB is phosphorylated and translocated to the nucleus where it binds to its consensus DNA sequence element and induces transcription of target genes. The IκB kinases (IKK) play an important role in the activation of NF-κB by inducing phosphorylation on serine residues of IκBα, which leads to dissociation of IκBα from NF-κB, leading to NF-κB activation and nuclear translocation. After dissociation, IκBα is polyubiquitinylated and degraded by the proteasome [106].

HNE can induce either activation or inhibition of NF-κB depending on the cell type and concentration (Figure 4). Although the mechanisms are not clearly understood, it has been shown in many studies that exposure to a high concentration of HNE inhibits the activation of NF-κB [107]. In fact, oxidative stress-induced HNE and NF-κB activation are positively correlated with the increased inflammatory response in many studies [108–113].

It has been demonstrated in THP1 cells that HNE prevents phosphorylation of IκBα, which is necessary for NF-κB activation. Specifically, pre-treatment of THP1 cells with 25 μM HNE resulted in the reduction of LPS-induced phosphorylation in Ser-32 of IκBα, thus preventing the activation of NF-κB by HNE [114]. Besides LPS, HNE also inhibits phosphorylation of IκBα induced by IL-1β and Phorbol 12-myristate 13-acetate (PMA) in monocytes [114]. Studies have further demonstrated that HNE could directly regulate the activity of IKK Kinase by forming covalent adducts with the IKK complex. IKK-induced phosphorylation IκBα is necessary for NF-κB activation [115]. Studies using H1299 and Jurkat T-cells have shown that pretreatment with HNE blocks TPA- or ionomycin-induced activation of NF-κB [115]. HNE could also regulate NF-κB by directly forming adducts with important signaling intermediates. In a model of long-term hepatic injury following alcohol exposure to rodents, HNE-IκBα adduct formation has been demonstrated to inhibit NF-κB, which is independent of IKK phosphorylation [116]. Inhibition of NF-κB by HNE exerts anti-inflammatory effects. HNE-induced inhibition of NF-κB prevents LPS-induced production of IL-6 in rat kupffer cells [117]. HNE-IKK adduct formation and suppression of IκBα phosphorylation could lead to the inhibition of NF-κB. HNE has been shown to prevent IL-1β-induced nuclear translocation of p65 and NF-κB-DNA binding [118]. Similarly, in rat cortical neuronal cells, exposure to HNE prevented the NF-κB-DNA binding activity [119].

On the other hand, many studies have provided substantial evidence that HNE activates NF-κB (Figure 4). Exposure of cells to low concentrations of HNE (1 μM) induces the activation of NF-κB in vascular smooth muscle cells [120]. The same concentration of HNE has been shown to induce phosphorylation and DNA-binding of NF-κB [120]. Similarly, another study demonstrates that exposure of vascular smooth muscle cells to HNE increases ROS production and activation of NF-κB/AKT signaling pathway leading to cell proliferation [121]. 5-Lipoxygenase (5-LOX) plays an important role in inflammation and atherosclerotic plaque formation. HNE has been shown to induce 5-LOX mRNA expression in murine macrophages by signaling through NF-κB, p38MAPK and ERK [122]. Similarly, exposure of murine macrophages to LPS increased ROS production and subsequently increased lipid peroxidation products [123]. Results from this study also demonstrate that HNE is an important mediator of LPS-induced inflammation by increasing the release of inflammatory cytokines such as TNF-α, IL-1β, IL-6, and MCP-1 [123]. HNE-mediated increase in the NF-κB activity has been shown to be involved in inflammatory signaling in rheumatoid arthritis. Treatment of synovial cells with 5 μM HNE resulted in a time-dependent increase in IL-1β, IL-6, and TNFα [124]. Exposure to a higher concentration of HNE (50 μM), led to a transient increase in IL-1β, IL-6, and TNF-α, with the maximum increase observed after 1 h. However, the levels of inflammatory cytokines gradually decreased after 6 and 12 h. Increase in Cox2 expression, an important inflammatory mediator was also observed in the same study [124]. Further, HNE has been reported to induce endothelial dysfunction, by increasing the production of NF-κB-mediated expression of IL-8, ICAM, and impairment of endothelial barrier function [125].

Although the literature provides conflicting data on the role of HNE in inhibiting or activating NF-κB based on its concentration, it is clear that exposure of cells to HNE induces a state of oxidative stress imbalance in the cells. Exposure to HNE leads to depletion of cellular GSH [126] and studies have provided evidence that depletion of GSH leads to activation of NF-κB [127]. HNE has also been reported to induce p47phox-mediated NADPH oxidase activity in murine macrophages contributing to oxidative stress [128]. Although the involvement of HNE-induced ROS in HNE-mediated signaling is controversial, studies have shown that HNE-induced mitochondrial damage in cells is primarily due to ROS-generation, which is also a major contributor of vascular damage induced by HNE [129]. HNE-induced oxidative stress has been shown to be an inducer of endothelial dysfunction. Several studies have demonstrated that treatment with antioxidants such as NAC and mercaptopropionyl glycine, which inhibits oxidative stress, attenuated the HNE-induced loss of endothelial cell function [130, 131]. These studies highlight that HNE-induced oxidative stress as a major player in HNE-induced endothelial cell damage and dysfunction.

Apart from directly regulating NF-κB, multiple lines of evidence suggest that HNE induces NF-κB -mediated pro-inflammatory signaling by regulating the activation of major protein kinases such as PKC, p38-MAPK, and JNK [132]. PKC is an important kinase in mediating HNE signaling. Treatment of RAW 264.7 macrophages with HNE decreases phorbol 12-myristate 13-acetate (PMA)-induced ROS production in a dose- and time-dependent manner by interacting with PKC [133]. Further, MAPK kinases are also reported to play an important role in HNE-induced endothelial dysfunction. In a study using human macrophages, induction of Cox2 expression was observed upon exposure to HNE. In this study, the authors did not observe any change

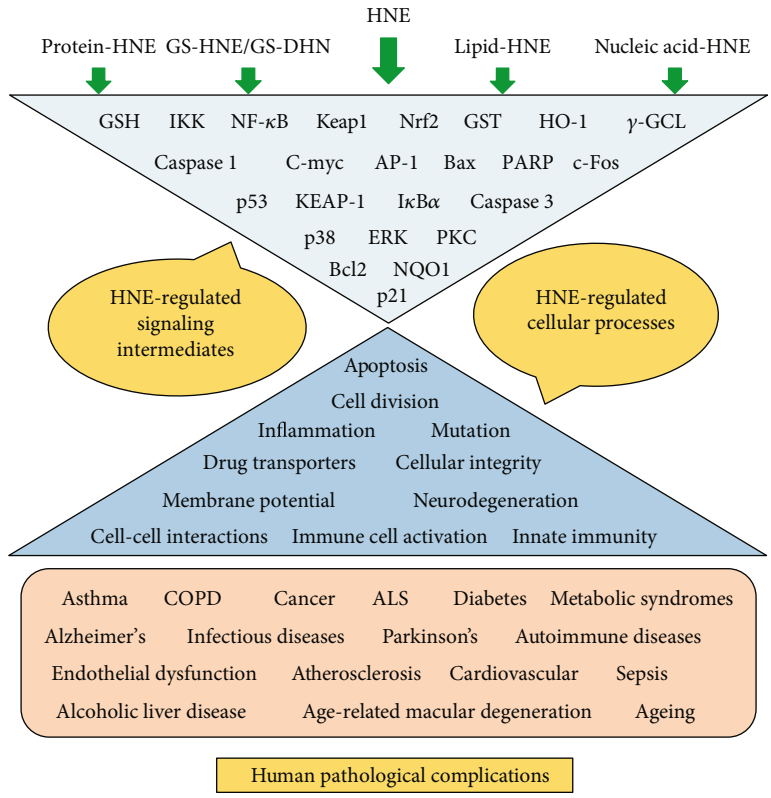

Figure 5: Central role of HNE and its metabolites in the regulation of various signaling pathways leading to various human disease pathologies. A summary of important signaling intermediates and cellular processes affect by HNE and its metabolites which can contribute to the pathophysiology of various human disorders are listed.

in the expression of iNOS or NF-κB but p38-MAPK was found to be an important regulator of HNE-induced inflammatory signaling in macrophages [87]. HNE also alters the balance of calcium ions in cells, which is important for signal transduction mediated by PKC and PI3K isozymes. In human chondrocytes, treatment with HNE induced the HNE-IKK adduct formation leading to inhibition of nuclear translocation of p65 and DNA-binding. Surprisingly, data from the same study show that HNE induces Cox2 and PGE2 in chondrocytes independent of NF-κB [118].

Activator protein 1 (AP-1) transcription factor is an important regulator of cellular signaling pathways controlling differentiation, proliferation, and apoptosis. HNE is well known to alter AP-1 transcriptional activity and affects various cellular processes. In rat cortical neurons, HNE-induced AP-1 nuclear translocation and DNA-binding plays an important role in neuronal calcium homeostasis and mitochondrial dysfunction [119]. Furthermore, the regulation of AP-1 activity by HNE has been shown to induce vascular smooth muscle cell proliferation leading to vascular complications [134].

HNE has been shown to be involved in the regulation of both intrinsic and extrinsic apoptotic signaling pathways in cells [19]. Exposure of SK-N-BE neuroblastoma cell lines to HNE induces the expression of p53, p73, p63, p21 and Bax leading to apoptotic cell death [135]. HNE has also been reported to induce p53-mediated apoptosis in the retinal pigment epithelial cells. Using knockout mouse models of GSTA4-4, Sharma et al., have demonstrated that HNE induces p53-mediated apoptotic signals via p21, Bax, and caspase 3. Inhibiting the cells ability to detoxify HNE leads to HNE-induced phosphorylation and nuclear translocation of p53 and induction of apoptosis [136]. In addition to HNE-induced p53 expression, HNE-mediated cytochrome C release has been shown to be necessary for HNE-induced apoptosis in macrophages [137].

HNE also induces the activation and phosphorylation of Src kinases. Mass spectrophotometric analysis of HNE-Src interaction showed that HNE interacts with His236, Cys241 and Cys248 residues of Src. Activation of Src by HNE leads to the expression of inflammatory mediator Cox2 and transcription factor AP-1, via activation of p38MAPK, JNK and ERK1/2 [138]. The HNE-Src adduct formation plays an important role in pro-inflammatory signaling in aged kidneys [139]. HNE also plays an important role in age-related oxidative stress. Higher plasma levels of HNE has been reported in obese individuals [140]. HNE-induced TNF-α expression in adipocytes was regulated by HNE mediated transcriptional regulation of ETS1 transcription factors [140]. HNE induced inflammation and Cox-2 production in many studies has been reported to be mediated by p38-MAPK mediated signaling and activation of ATF-2/CREB-1, JNK, c-JUN and AP-1 [20]. Many other transcription factors have been reported to play an important role in HNE-induced vascular endothelial cell dysfunction [141]. Transcription factors such as ATF3 and ATF4, have been

reported to play an important role in HNE- induced ER stress in endothelial cells. The siRNA mediated ATF4 deletion prevented HNE-induced monocyte adhesion and IL-8 production and exerted protective functions against HNE-induced toxicity in endothelial cells [126].

HNE also plays an important role in cancer-associated inflammation and cancerous progression as evidenced by recent reports highlighting the importance of HNE-mediated pro-inflammatory signaling in cancer [142]. HNE is an important second messenger molecule modulating cellular signaling pathways in cancer and mediates cancer cell proliferation, apoptosis, and antioxidant defense [44]. HNE either by extrinsic or intrinsic mechanisms has been reported to facilitate cancerous progression [9, 13, 41, 44]. Co-administration of HNE accelerated DSS-induced colitis in mice. Along with the increase in DSS-induced colitis, a significant increase in the expression of pro-inflammatory genes such as IL-6, TLR4, Cox2 along-with infiltration of $CD45^+$ and $CD45^+F4/80^+$ immune cells were observed in HNE + DSS treated mice compared to DSS-alone. Treatment with HNE also impairs intestinal barrier function by reducing the colonic expression of occludin protein. Loss of barrier function leads to an increase in the bacterial LPS in the circulation. Further, by using TLR4 null mouse models they have demonstrated that HNE mediates inflammatory signaling in DSS-induced colitis by signaling through TLR4 [143]. Apart from this, analyzing transcript levels of genes altered by HNE in human colorectal carcinoma cells have provided evidence that exposure to HNE induces an alteration in multiple signaling pathways related to antioxidant response, ER stress, apoptosis and cell cycle regulation in a time- and dose-dependent manner [144]. Apart from cancer progression, studies also provide evidence that HNE plays an important role in inducing apoptosis in colon carcinoma cells. It has been demonstrated that HNE-induced apoptosis in colon carcinoma cells is mediated by signaling through HSF-1. siRNA-mediated inhibition of HSF-1 prevented HNE-induced cleavage of PARP and caspase 3. Overexpression of Bcl-xl attenuated HNE-induced apoptosis in colon carcinoma cells [145]. HNE also plays an central role in the pathogenesis of inflammatory lung pathologies such as COPD. High levels of HNE-modified proteins have been observed in lung tissues from COPD patients with a positive correlation to inflammation and expression of inflammatory mediators [146]. Recent studies have also demonstrated the importance of HNE in neuronal inflammation and maintenance of neuronal cells. Studies using animal models suggest that HNE reduces neuronal intracellular calcium (Ca^{+2}) in CSF and leads to the loss of motor neurons. Calcium levels were also altered in the surviving neurons with no observable morphological alterations. Thus, HNE induces a prominent change in ionic equilibrium in neuronal cells implicating its importance in neurodegenerative diseases [147, 148]. The importance of HNE and lipid peroxidation products in neuronal biology and neuroinflammation is attaining considerable attention in recent years [148, 149]. HNE induces the expression of Cox2, PGE2 and IL-6 expression and inhibition of HNE is a beneficial target to attenuate inflammation during osteoarthritis [150, 151]. Apart from the evidence provided above on the role of HNE in inflammatory pathologies, HNE also plays an important role in the pathogenesis and progression of other inflammatory diseases such as cataract, AMD and COPD [152]. Thus, these studies provide evidence that HNE is an important mediator of various pro-and anti-inflammatory signaling pathways in different cell types (Figure 5) and understanding the cellular signaling pathways regulated by lipid peroxidation-derived aldehydes such as HNE will provide insights into the mechanism of disease and open avenues for the development of new therapeutic strategies in the future.

5. Conclusions and Future Perspectives

Recent studies have shown that depending upon the cell type, concentration and adduct formation with macromolecules, HNE could dictate cells to undergo proliferation or death. Interaction of HNE with cellular GSH has been shown to be a major metabolic route for its detoxification as well as intervening the cellular signaling pathways. Recent studies also suggest that HNE and HNE-protein adducts could act as biomarkers of various disease processes. Importantly, HNE metabolizing enzymes such as AR, ALDH1, and GSTs could regulate various anti-oxidative and pro-inflammatory pathways by generating HNE metabolites such as GS-HNE and GS-DHN which can further act as secondary signaling molecules. These studies opened new dimensions to understand the significance of HNE as well as other lipid aldehydes formed during lipid peroxidation and to understand the significance of aldehyde metabolizing enzymes in the pathophysiology of various diseases. These studies have provided a redox link between lipid aldehyde formation with oxidative stress and immune and inflammatory responses. Most importantly, we have shown that AR that reduces HNE, acrolein and other lipid aldehydes mediate oxidative stress signals initiated by various oxidants such as allergens, bacterial toxins, hyperglycemia, cytokines, and growth factors [56]. In fact, inhibition of AR has been shown to prevent several inflammatory complications including colon cancer, asthma, sepsis, uveitis, and cardiomyopathy [67, 68]. Thus, the specific use of antioxidants or synthetic inhibitors of HNE metabolizing enzymes that control the intracellular levels of HNE and its GSH-metabolites could be developed as potential therapeutic drug targets. It is a challenge for future research to clearly understand the complex interactions of HNE with various cellular proteins and how the protein-HNE adducts regulate various physiological processes. Further, it is still not clear how HNE commands cell signaling pathways based on a specific cell or tissue type. A better understanding of HNE-adduct formation with cellular macromolecules and identification of new HNE-adducts as potential biomarkers for various human pathologies will help to understand the pathophysiology of disease progression. In addition, identification of new immune and inflammatory response pathways regulated by HNE and its metabolites could help to understand the tissue and organ damage and dysfunction. Further, the use of novel transgenic approaches, metabolomics

and next-generation sequencing (NGS) could ease the identification of critical signaling pathways intervened by HNE and its metabolites.

Abbreviations

ADH: Alcohol dehydrogenase
AKR1B1: Aldo-keto reductase family 1 member B1
ALD: Alcoholic liver disease
ALDH: Aldehyde dehydrogenase
AMD: Age-related macular degeneration
AP-1: Activator protein-1
AR: Aldose reductase
ARE: Antioxidant response element
ATF4: Activating transcription factor 4
COPD: Chronic obstructive pulmonary disease
Cox-2: Cyclooxygenase 2
CREB: cAMP response element-binding protein
CSF: Cerebrospinal fluid
Cul3: Cullin3
DHN: 1,4-dihydroxy-2-nonene
DNA: Deoxyribonucleic acid
DSS: Dextran sulfate sodium
EpRE: Electrophile responsive element
ERK: Extracellular signal-regulated kinase
ETS1: ETS proto-oncogene1
GGT: γ-Glutamyl transpeptidase
GPX: Glutathione peroxidase
GRP-78: Glucose regulated protein 78
GSH: Glutathione
GSSG: Glutathione disulfide
GST: Glutathione-S-transferase
H_2O_2: Hydrogen peroxide
HNA: 4-hydroxy-2-nonanoic acid
HNE: 4-hydroxy-trans-2-nonenal
HO-1: Heme oxygenase-1
HSF-1: Heat shock factor-1
ICAM-1: Intercellular adhesion molecule -1
IKK: I-kappaB kinase
IL-6: Interleukin-6
iNOS: Inducible nitric oxide synthase
IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor- alpha
KEAP-1: Kelch like ECH associated protein 1
LPS: Lipopolysaccharide
MCP-1: Monocyte chemoattractant protein-1
MDA: Malondialdehyde
MRP: Multidrug resistance-associated protein
NAC: N-acetylcysteine
NADPH: Nicotinamide adenine dinucleotide phosphate
NGS: Next-generation sequencing
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NQO1: NADPH quinone oxidoreductase 1
Nrf2: NFE2-related nuclear factor 2
OxLDL: Oxidized low-density lipoprotein
p38MAPK: p38 mitogen activated protein kinase
PARP: Poly ADP ribose polymerase
PGE2: Prostaglandin E2
Pgp1: ATP binding cassette subfamily B member 1 (ABCB1)
PKC: Protein kinase C
PMA: Phorbol myristate acetate
Prx1: Peroxiredoxin1
PUFA: Polyunsaturated fatty acid
RLIP76: RalA binding protein 1(RALBP1)
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TLR-4: Toll-like receptor 4
TNF-α: Tumor necrosis factor α
TPA: Tissue plasminogen activator
Trx: Thioredoxin
UPR: Unfolded protein response
γ-GCL: γ-Glutamate cysteine ligase.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Relationships between Ghrelin and Obestatin with MDA, Proinflammatory Cytokines, GSH/GSSG Ratio, Catalase Activity, and Semen Parameters in Infertile Patients with Leukocytospermia and Varicocele

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Ghrelin and obestatin are involved in many biological functions including reproduction. Growing evidences suggest that both peptides could exert protective and antioxidant activities. In this study, the relationships between ghrelin/obestatin, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), malondialdehyde (MDA), reduced glutathione (GSH), oxidized glutathione (GSSG), expressed as the GSH/GSSG ratio, catalase (CAT), and semen parameters in infertile patients with varicocele or leukocytospermia and controls were investigated. Fifty-six infertile patients (32 with leukocytospermia and 24 with varicocele) and 14 controls participated in this study. Semen analysis was performed following the WHO guidelines. Apoptotic and necrotic sperm were scored by annexin V/propidium iodide assay. Seminal plasma samples were used for the following determinations: ghrelin, obestatin, IL-6, and TNF-α were measured by an immunological method, GSH/GSSG by an enzymatic method, and CAT by spectrophotometric determination. With respect to controls, both the leukocytospermia and varicocele groups showed altered sperm parameters, significantly increased sperm apoptosis ($P = 0.009$ and $P = 0.011$, respectively), IL-6 ($P = 0.0001$ and $P = 0.004$, respectively), and TNF-α levels ($P = 0.0001$ and $P = 0.002$, respectively); both groups had significantly decreased levels of ghrelin ($P = 0.0001$), obestatin ($P = 0.0001$ and $P = 0.006$, respectively), and GSH/GSSG ratio ($P = 0.003$ and $P = 0.0001$, respectively). The MDA concentration was significantly increased in the leukocytospermia group *vs.* controls ($P = 0.0001$), in the varicocele group *vs.* controls ($P = 0.011$), and in the leukocytospermia group *vs.* the varicocele group ($P = 0.008$). CAT activity was augmented in both the leukocytospermia and varicocele groups ($P = 0.0001$) *vs.* controls. The results indicate that both ghrelin and obestatin may play a protective role in human semen and this effect is probably due to their antioxidant properties.

1. Introduction

Ghrelin and obestatin peptides are derived from a ghrelin-obestatin preproprotein that is cleaved to yield these two proteins. Although both hormones have the same precursor, their biological activities differ from each other. Various organs produce both hormones; however, most circulating ghrelin derives from A-like or Gr cells situated in the basal part of the glands' gastric oxyntic mucosa. It must be said, however, that ghrelin is more thoroughly investigated than obestatin [1]. Many biological functions such as regulation of food intake, gastrointestinal motility, sleep, cardiovascular functions, and cell proliferation are regulated by ghrelin [2]. It was initially proposed that obestatin exerts opposite effects to those of ghrelin by promoting satiety and thus decreasing the food intake [3]. However, growing evidences suggest that obestatin could be considered a sort of "protective" peptide as it may support cell survival and proliferation and could play a

healing role in different organs [4]. Recently, much attention has been dedicated to the ability of ghrelin to reduce the inflammation processes by promoting a strong inhibition of inflammatory cytokine expression [5] and attenuating the oxidative stress in different organs [6–8]. Ghrelin exerts its antioxidant and anti-inflammatory effects by preventing the peroxidation and enhancing the activity of antioxidant enzymes [9]. Obestatin as well seems to exert antioxidant and antiapoptotic effects in several animal models used to study pathologies that have an inflammatory base [4, 10, 11].

Ghrelin, in particular, is found in both the male and female reproductive tracts together with its receptor called growth hormone secretagogue receptor-1a (GHSR-1a) [2, 12]. This suggests that ghrelin may have a role in the energy homeostasis control and reproduction [13]. In the male reproductive system, ghrelin is involved in key aspects of testis physiology, such as proliferation of Leydig cells, steroidogenesis, and regulation of hypothalamic-pituitary gonadal axis [14]. On the other hand, very little is known about obestatin, which was observed for the first time in rat Leydig cells [15]. Different animal models of testicular damage have been used to demonstrate the protective effect of ghrelin towards the oxidative stress [16–18]. The results suggested that the presence of such peptide in the reproductive sphere may play a new important role. At present, very few data on the putative activities of ghrelin and obestatin in the human semen is available. Years ago, some of our group demonstrated the presence of both ghrelin and obestatin in human semen samples and male genital tracts. The mean levels of obestatin in human semen were twenty times ca. higher than those measured in the serum of the homologous patients; the mean concentrations of semen ghrelin were twice as much those of serum ghrelin. In addition, we found that semen obestatin concentrations were positively correlated with sperm concentration and percentage of motile sperm, suggesting a possible protective role of this peptide [19]. A subsequent study reported the immunolocalization of both peptides in almost all organs of the human male reproductive system [20]. These observations prompted us to explore the potential protective effect of these peptide hormones in human semen. We determined the levels of ghrelin, obestatin, inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), malondialdehyde (MDA), a marker of oxidative stress, and antioxidant molecules such as reduced glutathione (GSH), oxidized glutathione (GSSG), expressed as GSH/GSSG ratio, and catalase (CAT). These variables were measured in semen of infertile patients grouped as follows: patients with leukocytospermia and patients with varicocele. The control group was composed of fertile males.

2. Materials and Methods

2.1. Patients. For this study, 56 infertile patients (aged 27-38) attending our laboratory for semen analysis were enrolled (from January through October 2018). All infertile patients did not obtain pregnancy after two years of unprotected sexual intercourses; the female factor was excluded.

The infertile patients were categorized into two groups: 32 infertile patients (aged 28-38) with leukocytospermia and 24 (aged 27-38) with varicocele. Leukocytospermia was detected during sperm analysis and defined as reported in the World Health Organization (WHO) guidelines [21]. The varicocele group was composed of patients who underwent both physical examination and scrotal colour Doppler ultrasonography analysis carried out in laboratories different from ours. For this study, we included patients with grade II and grade III varicocele.

All selected patients satisfied the following selection criteria: nonazoospermic men, 46, XY karyotype, and BMI $< 25\,kg/m^2$. Controls and patients had normal concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T). Hormone levels were assayed in serum by commercial kits (Beckman Coulter Access for FSH, LH, and testosterone). A normal range for FSH was 0.7-11.00 mU/mL (sensitivity 0.2 mUI/mL, intra- and interassay coefficient of variation <10%), for LH 0.8-8.0 mU/L (sensitivity 0.2 mUI/mL, intra- and interassay coefficient of variation <10%), and for T 2.7-10.9 mg/mL (sensitivity 0.1 ng/mL, intra- and interassay coefficient of variation <10%). The subjects considered in this study did not have genitourinary infections. Samples were seeded using a calibrated loop on agar plates, which were incubated overnight at 37°C in normal air with 5% CO_2. The microorganisms were identified by gram stain, oxidase, catalase, and other biochemical tests using Bio-Mérieux products (Bio-Mérieux, Florence, Italy). Spermiocultures were considered positive when the number of colonies was $\geq 10^4\,CFU\,mL^{-1}$ in case of gram-positive cocci and $\geq 10^5\,CFU\,mL^{-1}$ in case of gram-negative rods.

The participants had no chronic diseases and did not receive radiotherapy, chemotherapy, and medication. None of the individuals took an oral antioxidant supplement for at least five months before the analysis. Subjects with a history of recreational drug use, alcohol consumption, and heavy smoking habit (>10 cigarette/day) were excluded in this study.

The control group was composed of 14 fertile men (aged 25-35) which fathered at least one child in the last 4 years. The fertile subjects showed a normal karyotype; they were not affected by infections and anatomical problems. Their semen parameters were higher than 25 percentiles as reported in the WHO guidelines [21].

All patients provided an informed written consent before the inclusion of this study. The informed consent describes the aims of the research, and it is approved by the Ethics Committee of Azienda Ospedaliera Universitaria Senese (CEAOUS).

2.2. Semen Analysis. Semen analysis was performed by a standard procedure according to the WHO guidelines [21]. Briefly, specimens were collected by masturbation after 3-5 days of sexual abstinence and analysed after liquefaction for 30 min at 37°C. Conventional semen parameters were determined: volume, pH, sperm concentration, and motility. The sperm morphology was assessed by the Papanicolaou (PAP) test modified for spermatozoa. Peroxidase stain was used to identify the leukocytes in semen samples. A leukocyte concentration ≥ 1 million cell/mL was considered

abnormal [21]. In each sample, sperm apoptosis and necrosis were detected as subsequently described. Seminal samples were centrifuged (200g for 10 min); the supernatant composed of seminal plasma without spermatozoa was distributed in aliquots and stored at -80°C until different analyses were performed.

2.3. Detection of Sperm Apoptosis and Necrosis. The detection of sperm apoptosis and necrosis was performed by Vybrant apoptosis assay (Invitrogen Ltd., Paisley, United Kingdom) based on fluorescein isothiocyanate- (FITC-) annexin V (AnV, green fluorescence) and propidium iodide (PI, red fluorescence). AnV protein has affinity for phosphatidylserine that during the apoptotic process is translocated from the inner to the outer plasma membrane layer; PI stains necrotic cell with a broken membrane. The detailed procedure is described in Moretti et al. [22]; at least 300 sperm for each sample were analysed, and undamaged sperm (AnV negative, PI negative, not stained), apoptotic sperm (AnV positive, PI negative, green stained), and necrotic sperm (AnV negative, PI positive, red stained) were classified. Sperm with both green and red signals were scored as necrotic since their membrane was damaged.

2.4. Ghrelin, Obestatin IL-6, and TNF-α Determinations. Ghrelin, obestatin IL-6, and TNF-α were assayed in an aliquot of all 56 semen samples, after thawing. Ghrelin and obestatin concentrations were measured by radioimmunoassay using a Ghrelin (total) radioimmunoassay (RIA) kit (LINCO Research, St. Charles, Missouri, USA) and an obestatin (Human, Monkey) RIA kit (Phoenix Pharmaceuticals, Inc., Karlsruhe, Germany). The manufacturer's instructions were followed, and the results were expressed in ng/mL.

IL-6 and TNF-α concentrations were assessed by enzyme-linked immunosorbent assay (ELISA, Human IL-6 BMS213/2CE BMS213/2TENCE, and TNF-α BMS223/4CE BMS223/4TENCE; Bender MedSystems GmbH, Vienna, Austria), and the results were expressed in pg/mL. The calculation was done on the calibration curve as reported in the different kit manuals. For each sample, three specimens were determined.

2.5. Oxidized and Reduced Glutathione. After thawing, the seminal plasma (200 μL) was added to an equal volume of 10% metaphosphoric acid. Samples were centrifuged at low speed (2,000g) for 10 min at 0°C. Total glutathione (GSH) and oxidized glutathione (GSSG) were quantified in a supernatant using a microassay procedure [23] using GSSG as the standard to define the calibration curve. Each sample was determined in triplicate, and the results were expressed in nmol of GSH or GSSG per mg of protein.

2.6. Catalase Activity. After thawing, the seminal plasma was centrifuged at 4,000g for 15 min at 4°C. To determine the catalase (CAT) activity, a microassay procedure was used [24].

This method, which employs 20 μL, is based on the reaction of CAT with methanol in the presence of an optimal concentration of hydrogen peroxide. The formaldehyde production was measured spectrophotometrically at 540 nm with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald, Sigma-Aldrich, Milan, Italy) as a chromogen. One unit of catalase activity was defined as the amount of enzyme that caused the formation of 1 nmol of formaldehyde per min at 25°C. Each sample was determined in triplicate, and the results were expressed as nmol/min/mg of protein.

2.7. Protein Assay. Protein concentrations were determined by the method of Lowry et al., and the calibration curves were prepared with dry bovine serum albumin [25].

2.8. Malondialdehyde Assessment. After thawing, the levels of lipid peroxidation in the seminal plasma were estimated using the evaluation of MDA levels. 500 μL of samples were mixed with 500 μL of a solution containing 0.04 M Tris (hydroxymethyl)methylamine (pH 7.4) and 0.01% butyl hydroxytoluene in acetonitrile were added (1 : 1, v/v) to prevent the artificial oxidation of polyunsaturated free fatty acids during the assay. The samples were centrifuged at 3,000g for 15 min. The supernatant was used for MDA high-performance liquid chromatography (HPLC) analysis with UV detection according to Shara et al. [26]. Each sample was determined in triplicate, and the results were expressed in nmol of MDA per mL of seminal plasma. The MDA samples were determined with respect to its own calibration curve.

2.9. Statistical Analysis. Statistical analysis was performed with the SPSS version 19 software package (SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov test was used to verify the normality of distribution of the variables investigated. Some variables (sperm progressive motility, obestatin, ghrelin, sperm morphology, necrosis, apoptosis, and GSH/GSSG ratio) were normally distributed, and the others (sperm concentration, IL-6, TNF-α, catalase, and MDA) were not normally distributed. These variables were transformed into a logarithmic form, obtaining values with a normal distribution. The assumption of homoscedasticity of the variance of groups was assessed using Levene's test. One-way analysis of variance (ANOVA) was used to identify the significant difference between the groups. Tukey's post hoc multiple comparison test was used under homoscedasticity instead, under heteroscedasticity, and the Games–Howell post hoc test was used. Since we included some subjects that smoke ≤ 10 cigarette/day, the data were adjusted for smoking. Spearman's rank correlation coefficient (rho) was used to assess significant correlation (positive or negative) between the studied variables. A P value less than 0.05 is considered significant.

3. Results

The 70 study selected participants were categorized as patients with leukocytospermia (#32), patients with varicocele (#24), and fertile controls (#14). The considered variables were compared between the three groups in order to explore if relationships between ghrelin, obestatin, cytokines, oxidative stress, and antioxidant ability of human semen could be related to infertility (Tables 1 and 2). The data were adjusted for "smoking" that did not result into a confounding variable.

Sperm concentration, motility, and morphology were significantly increased in the group of fertile men compared

Table 1: Means and standard errors of semen parameters, apoptosis, necrosis, IL-6, and TNF-α assayed in semen samples of 70 men divided into 3 groups according to their clinical diagnoses and controls. Statistical methods are indicated, and the exact values of P are reported.

Variables	Diagnosis			Statistics	
	Leukocytospermia (L no. 32)	Varicocele (V no. 24)	Fertile men (F no. 14)	HDS Tukey's post hoc test (P value)	Games-Howell post hoc test (P value)
Sperm ($mL \times 10^6$)	57.12 ± 5.55	70.89 ± 5.76	171.68 ± 23.36	—	F *vs.* L ($P = 0.001$); F *vs.* V ($P = 0.002$)
Progressive motility (%)	30.47 ± 1.79	29.21 ± 2.39	51.29 ± 1.18	F *vs.* L ($P = 0.0001$); F *vs.* V ($P = 0.0001$)	—
Normal morphology (%)	8.69 ± 0.27	8.88 ± 0.37	16.79 ± 0.54	F *vs.* L ($P = 0.0001$); F *vs.* V ($P = 0.001$)	—
Apoptosis (%)	13.84 ± 1.05	14.00 ± 1.53	7.71 ± 0.13	F *vs.* L ($P = 0.009$); F *vs.* V ($P = 0.011$)	—
Necrosis (%)	17.41 ± 1.12	19.00 ± 2.91	13.64 ± 2.14	—	—
IL-6 (pg/mL)	14.75 ± 2.63	15.41 ± 3.48	1.86 ± 0.49	—	F *vs.* L ($P = 0.0001$); F *vs.* V ($P = 0.004$)
TNF-α (pg/mL)	36.21 ± 3.38	46.59 ± 8.74	11.82 ± 1.57	—	F *vs.* L ($P = 0.0001$); F *vs.* V ($P = 0.002$)

Table 2: Means and standard errors of ghrelin, obestatin, MDA, GSH/GSSG ratio, and catalase assayed in semen samples of 70 men divided into 3 groups according to their clinical diagnoses and controls. Statistical methods are indicated, and the exact values of P are reported.

Variables	Diagnosis			Statistics	
	Leukocytospermia (L no. 32)	Varicocele (V no. 24)	Fertile men (F no. 14)	HDS Tukey's post hoc test (P value)	Games-Howell post hoc test (P-value)
Ghrelin (ng/mL)	0.92 ± 0.03	0.916 ± 0.043	1.12 ± 0.047	F *vs.* L ($P = 0.0001$); F *vs.* V ($P = 0.0001$)	—
Obestatin (ng/mL)	4.00 ± 0.28	4.40 ± 0.33	6.01 ± 0.29	F *vs.* L ($P = 0.0001$); F *vs.* V ($P = 0.006$)	—
MDA (nmol/mL)	8.59 ± 1.27	3.66 ± 0.85	0.55 ± 0.05	—	F *vs.* L ($P = 0.0001$); F *vs.* V ($P = 0.011$); L *vs.* V ($P = 0.008$)
GSH/GSSG (nmol/mg of proteins)	10.38 ± 0.74	9.02 ± 0.73	14.58 ± 0.85	F *vs.* L ($P = 0.003$); F *vs.* V ($P = 0.0001$)	—
CAT (U/mg of proteins)	9.72 ± 1.26	10.21 ± 0.76	2.96 ± 0.46	—	F *vs.* L ($P = 0.0001$); F *vs.* V ($P = 0.0001$)

to those observed in both the leukocytospermia and varicocele groups (Table 1).

With respect to controls, the leucocytospermia and varicocele groups showed significantly increased sperm apoptosis ($P = 0.009$ and $P = 0.011$, respectively), IL-6 ($P = 0.0001$ and $P = 0.004$, respectively), and TNF-α ($P = 0.0001$ and $P = 0.002$, respectively) levels (Table 1).

Table 2 reports the comparisons of ghrelin and obestatin levels, MDA concentration, GSH/GSSG ratio, and CAT activity among the considered groups.

Both the leukocytospermia and varicocele groups showed significantly lower levels of ghrelin ($P = 0.0001$), obestatin ($P = 0.0001$ and $P = 0.006$, respectively), and GSH/GSSG ratio ($P = 0.003$ and $P = 0.0001$, respectively) compared to those measured in the control group.

The concentration of MDA was significantly increased in the leukocytospermia group versus controls ($P = 0.0001$) in the varicocele group versus controls ($P = 0.011$) and in the leukocytospermia group versus the varicocele group ($P = 0.008$). Finally, CAT activity was higher in both the leukocytospermia and varicocele groups ($P = 0.0001$) than that measured in the control group.

Table 3 reports the correlations between the measured variables calculated in the total group of the study participants (no. 70 subjects). As far as the sperm parameters are concerned, sperm concentration, motility, and normal morphology show significant positive correlations among them ($P = 0.0001$).

Sperm concentration positively correlates with ghrelin ($P = 0.012$) and GSH/GSSG ($P = 0.0001$) and negatively with necrosis ($P = 0.012$), TNF-α level ($P = 0.004$), IL-6 level ($P = 0.019$), MDA concentration ($P = 0.050$), and catalase activity ($P = 0.0001$).

Sperm motility shows positive correlations with obestatin ($P = 0.001$) and GSH/GSSG ($P = 0.001$) and negative correlations with apoptosis ($P = 0.004$), necrosis ($P = 0.0001$),

Table 3: Correlations (Spearman's coefficient (rho)) between all considered variables in 70 individuals. The exact values of P are reported in parentheses.

	Sperm ($mL \times 10^6$)	Motility (%)	Normal morphology (%)	A (%)	N (%)	Ghrelin	Obestatin	TNF-α	IL-6	MDA	CAT	GSH/GSSG
Sperm/mL $\times 10^6$	1											
Motility (%)	0.576 (.0001)	1										
Normal morphology (%)	0.509 (.0001)	0.475 (.0001)	1									
A (%)	-0.136 (ns)	-3.41 (.004)	-0.377 (.001)	1								
N (%)	-0.298 (.012)	-0.530 (.0001)	-0.166 (ns)	0.007 (ns)	1							
Ghrelin	0.299 (.012)	0.172 (ns)	0.421 (.0001)	-0.359 (.002)	0.076 (ns)	1						
Obestatin	0.122 (ns)	0.417 (.001)	0.282 (.018)	-0.241 (.045)	0.068 (ns)	0.639 (.0001)	1					
TNF-α	-0.338 (.004)	-0.389 (.001)	-0.553 (.0001)	0.751 (.0001)	-0.001 (ns)	-0.454 (.0001)	-0.396 (.001)	1				
IL-6	-0.324 (.019)	-0.399 (.003)	-0.577 (.0001)	0.370 (.007)	0.309 (.026)	-0.496 (.0001)	-0.422 (.002)	0.546 (.0001)	1			
MDA	-0.257 (.050)	-0.344 (.015)	-0.468 (.001)	0.334 (.019)	0.0168 (ns)	-0.502 (.0001)	-0.506 (.0001)	0.452 (.001)	0.443 (.005)	1		
CAT	-0.732 (.0001)	-0.562 (.0001)	-0.690 (.0001)	0.548 (.0001)	0.194 (ns)	-0.525 (.001)	-0.304 (.050)	0.626 (.0001)	0.540 (.003)	0.491 (.004)	1	
GSH/GSSG	0.515 (.0001)	0.420 (.001)	0.476 (.0001)	-0.096 (ns)	-0.309 (.018)	0.149 (ns)	0.105 (ns)	-0.255 (.050)	-0.315 (.045)	-0.185 (ns)	-0.551 (.0001)	1

Legend: Sperm/mL $\times 10^6$: number of sperm/ml; Motility (%): percentage of progressive sperm motility; Normal morphology (%): percentage of normal sperm morphology assessed with Papanicolaou staining; A (%): percentage of sperm apoptosis assessed with AnV/PI assay; N (%): percentage of sperm necrosis assessed with AnV/PI assays; Ghrelin (ng/mL); Obestatin (ng/mL); TNF-α (pg/mL): tumor necrosis factor-α (pg/mL); IL-6: interleukin 6 (pg/mL); MDA (nmol/mL): malondialdehyde (nmol/mL); GSH (nmol/mg): glutathione (nmol/mg of protein); GSSG (nmol/mg): glutathione oxidized form (nmol/mg of protein); CAT (U/mg): catalase activity (U/mg of protein).

TNF-α level ($P = 0.001$), IL-6 level ($P = 0.003$), MDA concentration ($P = 0.015$), and catalase activity ($P = 0.0001$).

Sperm normal morphology percentage positively correlates with ghrelin ($P = 0.0001$), obestatin ($P = 0.018$), and GSH/GSSG ($P = 0.0001$) and negatively with sperm apoptosis ($P = 0.001$), TNF-α level ($P = 0.0001$), IL-6 level ($P = 0.0001$), MDA concentration ($P = 0.001$), and catalase activity ($P = 0.0001$).

Sperm apoptosis exhibits positive correlations with TNF-α ($P = 0.0001$), IL-6 levels ($P = 0.006$), MDA concentration ($P = 0.019$), and catalase activity ($P = 0.0001$) and negative correlations with ghrelin ($P = 0.002$) and obestatin ($P = 0.045$).

Sperm necrosis positively correlates with IL-6 ($P = 0.026$) and negatively with GSH/GSSG ($P = 0.018$).

Ghrelin presents positive correlations with obestatin and negative correlations with TNF-α level, IL-6 level, MDA concentration ($P = 0.0001$), and catalase activity ($P = 0.001$).

Obestatin shows negative correlations with TNF-α ($P = 0.001$), IL-6 level ($P = 0.002$), MDA concentration ($P = 0.0001$), and catalase activity ($P = 0.050$).

TNF-α level positively correlates with IL-6 level ($P = 0.0001$), MDA concentration ($P = 0.001$), and catalase activity ($P = 0.0001$) and negatively with GSH/GSSG ratio ($P = 0.050$).

IL-6 level exhibits positive correlations with MDA concentration and catalase activity ($P = 0.005$ and $P = 0.003$, respectively) and negative correlations with GSH/GSSG ratio ($P = 0.045$).

Finally, catalase activity is positively correlated with MDA concentration ($P = 0.004$) and negatively with GSH/GSSG ($P = 0.0001$).

4. Discussion

The data presented in this paper are the first to show an interplay among ghrelin, obestatin, cytokines, MDA, GSH/GSSG ratio, and CAT activity in human semen. Some years ago, our group demonstrated that the human semen samples are particularly rich in ghrelin and obestatin with respect to serum samples [19] and that both hormones are produced by different organs and tissues of the male reproductive system [20]. Despite their massive presence, until now, the role of both hormones was unexplored in human semen. In the present study, ghrelin and obestatin positively correlated with semen parameters and each other and negatively with sperm apoptosis, IL-6 and TNF-α levels, MDA, and CAT. Moreover, infertile patients with leukocytospermia or varicocele showed significantly reduced seminal concentrations of obestatin and ghrelin concomitant with increased levels of CAT, MDA, IL-6, and TNF-α with respect to those observed in the control group. Therefore, we may hypothesize a relationship between an oxidizing environment and the levels of ghrelin and obestatin. Leukocytospermia and varicocele are both conditions associated with oxidative stress, as a consequence of inflammatory situations [27–30], and characterized by increased levels of cytokines [29, 31–33]. TNF-α seems to play a role in the pathogenesis of varicocele probably inducing sperm apoptosis [33], which, also in this study, was significantly increased in both groups of infertile patients with respect to controls. In both leukocytospermia [34] and varicocele [27, 30], the involvement of oxidative stress by the increased production of ROS was demonstrated. In this research, we confirmed that leukocytospermia and varicocele are characterized by an impairment of redox status because the GSH/GSSG ratio [35] was decreased and negatively correlated with cytokines, MDA, and CAT. GSH scavenges the excess of ROS and is oxidized to the GSSG form; in addition, GSH reacts with cytotoxic aldehydes, products of lipid peroxidation (LPO), and can protect the sperm plasma membrane [36]. LPO was detected by MDA evaluation and antioxidant buffering capacity by enzymatic CAT activity involved in the decomposition of H_2O_2 into water and oxygen, thus preventing LPO and improving sperm motility [37]. The rise of CAT activity in semen of infertile patients with varicocele and leukocytospermia could be explained as a possible "chronic oxidative stress" [38]. Despite the increased CAT activity in the semen of both groups of infertile patients, MDA levels were still elevated, suggesting that the CAT activity was not able to counteract the peroxide excess. Even though other authors reported a decreased CAT activity in infertile patients with varicocele [39, 40], the results of this study are similar to those obtained in a previous research of ours [35].

The ability of ghrelin to attenuate inflammation and its potent inhibitory effect on the expression of proinflammatory cytokines was recently reported [5] in districts other than the male reproductive system. Despite a quite rich literature on the multiple roles of ghrelin, very few data is available on the function played by obestatin either in animal models or in humans. It has been established that obestatin plays a role in increasing cell survival and proliferation and counteracting apoptotic process and inflammation [41]. Regarding the reproductive system, a protective activity of ghrelin was observed in different researches, performed in animal models, demonstrating that this peptide can decrease the testicular damages [17, 42–44]. Recent studies deal with the defensive role of ghrelin that can exert beneficial activity on redox imbalance for its anti-inflammatory and antioxidant properties also in the male reproductive system [18, 45–47]. In a rat model of varicocele, Asadi et al. [45] demonstrated that ghrelin can decrease the oxidative stress (evaluated by means of MDA) caused by varicocele and enhance the activity of antioxidant enzymes. In addition, ghrelin attenuates the negative effects of cyclophosphamide, a chemotherapeutic and immune-suppressor drug, upon sperm parameters by reducing oxidative stress and LPO of sperm membranes and by enhancing the antioxidant activity [18]. These recent reports are extremely in accordance with our observations made in human semen.

Therefore, it is possible to hypothesize a protective antioxidant role of ghrelin and obestatin also in human semen, even though in this study we did not explore the mechanism of action. In this research, another interesting observation regards the relationship between ghrelin/obestatin and sperm apoptosis. In human semen, decreased levels of ghrelin and obestatin are concomitant with an increase of sperm apoptosis, indicating a possible antiapoptotic activity of both hormones. The antiapoptotic effect of ghrelin is reported in

the literature, for example, in neuronal cells [48] and in the male reproductive system also. Ghrelin showed notable anti-inflammatory and antiapoptotic effects in a rat testicular ischemia reperfusion model by decreasing IL-6 and TNF-α levels [47]; Kheradmand et al. [49] used a rat model of scrotal hyperthermia and demonstrated that ghrelin downregulated Bax expression, a protein involved in apoptotic process, and concomitantly upregulated PCNA, which is involved in cell proliferation.

5. Conclusions

We demonstrated that ghrelin and obestatin concentrations are reduced in patients with leukocytospermia and varicocele and both hormones are correlated with redox imbalance. Obviously, we do not expect that the results of the present study are simply translated to the clinical field. These data can add insights to understand the complexity of the different mechanisms involved in spermatogenesis and functioning of the male reproductive system.

Abbreviations

IL-6: Interleukin-6
TNF-α: Tumor necrosis factor-α
MDA: Malondialdehyde
GSH: Reduced glutathione
GSSG: Oxidized glutathione
CAT: Catalase
WHO: World Health Organization
FSH: Follicle stimulating hormone
LH: Luteinizing hormone
T: Testosterone
PAP: Papanicolaou
FITC: Fluorescein isothiocyanate
AnV: Annexin V
PI: Propidium iodide
ELISA: Enzyme-linked immunosorbent assay
RIA: Radioimmunoassay
HPLC: High-performance liquid chromatography
ROS: Reactive oxygen species
LPO: Lipid peroxidation.

Data Availability

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

Conflicts of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Authors' Contributions

All the authors gave substantial contributions to the research design, or the drafting of the paper or revising it critically, and the approval of the submitted version. Lucia Micheli, in particular, is responsible for the oxidized and reduced glutathione evaluation, catalase activity, data interpretation, and statistical analysis. Giulia Collodel, in particular, is assigned to the patient selection, spermiograms, ghrelin, obestatin determinations, data interpretation, and research design. Daniela Cerretani, in particular, is responsible for the malondialdehyde assessment and data interpretation. Andrea Menchiari, in particular, is also assigned to the statistical analysis. Daria Noto, in particular, is also responsible for the spermiograms and data interpretation. Cinzia Signorini, in particular, determined the ghrelin and obestatin. Elena Moretti, in particular, is assigned to the IL-6 and TNF-α determinations, research design, paper writing, data interpretation, and annexin-propidium iodide assay.

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Research Article

Relationship between Oxidative Stress and Physical Activity in Women with Squamous Intraepithelial Lesions in a Cervical Cancer Control Program in the Brazilian Amazon

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Human papillomavirus (HPV) infection is recognized as the most common sexually transmitted disease in the world, and there is a consensus on its role in the etiology of preneoplastic epithelial changes in the cervix. Through the process of lipid peroxidation, oxidative stress is found in the course of premalignant and malignant changes. Moreover, the level of physical activity can exert an influence on markers of oxidative stress, lowering the serum levels of these markers. *Objective*. To determine the relationship between levels of malondialdehyde (MDA) and the level of physical activity in women with squamous intraepithelial lesion (SIL) of the cervix. *Methods*. A cross-sectional study was conducted with 46 women participating in a cervical cancer control program. The women had been submitted to the cytopathological exam and were divided into two groups: 18 with SIL and 28 controls. MDA concentrations were determined, and the International Physical Activity Questionnaire (IPAQ) was administered on the same day as the gynecological appointment (prior to the Papanicolaou test). *Results*. The SIL group had higher MDA levels than the control group (mean: 47.63 ± 9.57 vs. 9.32 ± 4.79, respectively) and a lower IPAQ score (median: 713.5 vs. 1875, respectively). A weak correlation was found between the MDA level and IPAQ score ($r^2 = -0.34$, $p = 0.018$). *Conclusion*. The women with SIL had higher levels of oxidative stress and were less physically active than the women in the control group. These findings suggest that physical exercise exerts an influence on markers of oxidative stress in the development of intraepithelial squamous lesions.

1. Introduction

Squamous intraepithelial lesions (SIL) are precursors to cervical cancer. The central agent in the pathogenesis of SIL is human papillomavirus (HPV), which is detected in 97% of cases [1, 2]. Although preventable and with a good prognosis, HPV infection and SIL affect 20 to 60% of the female population and can evolve to squamous cell carcinoma of the cervix, especially the malignant type, in women of the Amazon region in Brazil [3].

The role of oxidative stress in the pathogenesis of cancer is widely discussed. The chronic presence of reactive oxygen species (ROS) is thought to favor the integration of the viral oncogene with cellular DNA, leading to the overexpression of proteins HR, E6, and E7 and the consequent formation of tumor cells. Malondialdehyde (MDA) alone is an important cellular mutagenic agent [4, 5]. A direct association has been found between lipid peroxidation expressed by MDA and cervical cancer as well as precursor squamous lesions, although few studies have specifically addressed SIL [6, 7].

The practice of physical activity is considered an important factor in the prevention of diseases, probably due to the improvement in antioxidant defenses [7]. However, little is known regarding the relationship between squamous lesions and the level of physical activity among affected women, as no studies have related these variables.

There are several studies indicating the increased formation of serum oxidative markers in patients with cervical cancer; some have shown that even premalignant lesions may regulate oxidative stress but there is no research indicating that physical activity may be associated with the reduction of serum levels of lipid peroxidation in women with SIL [4–7]. This may indicate a new approach to recommendation and intervention based on regular physical activity in the treatment of all stages of lesions.

Therefore, the aim of the present study was to analyze the association between serum levels of MDA and the level of physical activity in women with SIL at a cervical cancer prevention and control service in the Amazon region of Brazil.

2. Materials and Methods

2.1. Patients and Study Design. A cross-sectional study was conducted with women at the Cervical Cancer Prevention Service of the Center for Tropical Medicine of the Universidade Federal do Pará, Brazil. This service meets the spontaneous demand for care from users of the Brazilian public health system sent from primary care units or through direct referrals from the university as well as from research and extension programs of the university.

All women who underwent the Papanicolaou test at the service between February 20 and June 27, 2018, were asked to participate in the study. Those who met the following inclusion criteria were selected to compose the sample: age 18 years or older and agreement to participate in all steps of the study. Women with cognitive limitations, a diagnosis or undergoing treatment for cancer of any type, innate or acquired immunodeficiency, and making regular use of a corticosteroid and those with autoimmune or infectious-parasitic diseases were excluded from the study.

Eighteen women formed the group with SIL. Twenty-eight women matched for age with the SIL group (±2 years) with a cytopathological exam considered normal or with atypical squamous cells of an undetermined significance (ASCUS) [8] composed the control group. The cervicovaginal material collected and processed for the Papanicolaou test was submitted to cytopathological analysis based on the Bethesda system by experts in the field in accordance with the guidelines of the Brazilian Health Ministry for screening for cervical cancer [8, 9].

2.2. Epidemiological Data. Epidemiological data and reproductive information were acquired from the medical chart of each patient: age, marital status, schooling, number of children, smoking, eating habits, and self-perceived states of stress and/or anxiety at work or home. Information not found on the charts was obtained directly from the patients on the day of the gynecological exam.

2.3. Level of Physical Activity. Prior to the collection of the material for the cytopathological exam, the patients answered the short form of the International Physical Activity Questionnaire (IPAQ). This questionnaire was developed to estimate the level of physical activity among adults between 18 and 65 years of age, but studies have demonstrated its applicability to elderly individuals up to 90 years of age [10]. The IPAQ was designed by a workgroup that involved 14 research centers from 12 countries. Data on its reliability and applicability were published in 2003. The questionnaire is composed of eight items addressing the time spent per week walking, daily activities that involve moderate or intense effort, and time spent on sedentary activities [11]. The questions are listed below:

(1) During the last 7 days, on how many days did you walk for at least 10 minutes at a time?

(2) How much time did you usually spend walking on one of those days?

(3) During the last 7 days, on how many days did you perform moderate activities for at least 10 continuous minutes?

(4) How much total time per day did you spend on these activities?

(5) During the last 7 days, on how many days did you perform vigorous activities for at least 10 continuous minutes?

(6) How much total time per day did you spend on these activities?

(7) How much total time did you spend in a sitting position on a weekday?

(8) How much total time did you spend in a sitting position on a weekend day?

The answers were entered into a Microsoft Excel® program. Predefined formulas were used to calculate the number of calories spent during a week. The respondents were then categorized as sedentary, mildly active, moderately active, or highly active.

2.4. Determination of Serum MDA. Blood samples were collected from the vein of the forearm using a vacutainer and stored in test tubes with anticoagulant and EDTA. The serum was then separated from the plasma for the biochemical analysis. Serum MDA was analyzed by the reaction between MDA and thiobarbituric acid (TBA) in low pH and a high temperature to form the MDA-TBA complex, which has a pinkish color and maximum absorption at 535 nm. Lipid peroxidation was measured by estimating thiobarbituric acid reactive substances (TBARS). The method consisted of the precipitation of the lipoproteins of the samples by the addition of 1% trichloroacetic acid, 1% TBA, and sodium hydroxide. The union of lipid peroxide and TBA was performed by heating in a water bath for 30 minutes. The formed chromogens were extracted in n-butanol and read at 535 nm. Lipid

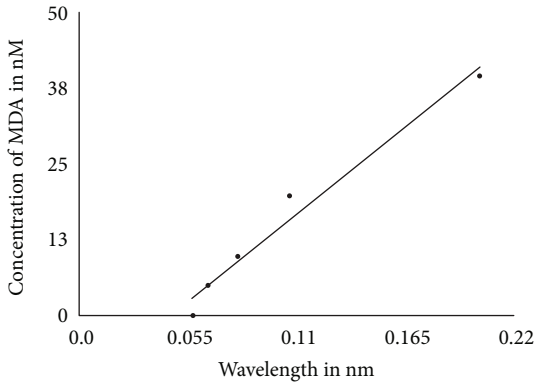

Figure 1: Linear regression curve of standard concentration of MDA (0, 5, 10, 20, and 40 nM) with a correlation coefficient of 0.972 and regression equation of $y = 264.5x - 11.979$.

peroxidation was expressed as nmol/ml of MDA (Isaksson et al., 2009). The calculation was performed using a five-point calibration curve (0, 5, 10, 20, and 40 nM) established from an MDA solution (tetra-hydroxypropane) of 20 nM [12] (Figure 1).

2.5. Statistical Analysis. The data were represented in graphs and tables and analyzed using descriptive statistics (absolute and percentage frequency, mean and standard deviation, and median and interquartile range (25^{th} to 75^{th} percentile)). The D'Agostino test was used to determine the distribution (normal or nonnormal) of the data. Depending on the result, either Student's *t*-test or the Mann-Whitney test was used to compare continuous variables between groups. The chi-square test and Fisher's exact test were employed to compare nominal and categorical variables. Spearman's correlation coefficient was calculated to determine the strength of the correlation between serum MDA and the IPAQ score. All statistical tests were performed with the aid of SPSS version 20.0, with the level of significance set to 5% ($p < 0.05$).

2.6. Ethical Aspects. This study received approval from the Human Research Ethics Committee of the Center for Tropical Medicine of the Universidade Federal do Pará (certificate number 2.051.391) and was conducted in accordance with the determinations of Resolution 466/2012 of the Brazilian National Board of Health. All volunteers agreed to participate by signing a statement of informed consent.

3. Results

3.1. Description of the Groups. During the entire study period, a total of 163 women visited the clinic of the Cervical Cancer Prevention Service; 20 of whom had SIL of the cervix and the rest had either normal results or ASCUS. A problem occurred during the blood collection from two patients in the SIL group. Therefore, these women were excluded from the study, and the case group was composed of 18 patients. The control group was formed using a randomization process: simple lottery with sealed envelopes containing the registration number of patients in the target age range (±2 years) in comparison to the patients in the case group. Thus, 28 age-matched women whose cytopathological exam results were either normal or ASCUS composed the control group.

Mean age was 52.17 years in the case group and 47.96 years in the control group. Chronic noncommunicable diseases were reported in 61% of the case group and 50% of the control group. Smoking was reported in 17.9% of the control group and none of the patients in the SIL group. Self-reported food restriction or dieting was found in 22.2% of the case group and 28.6% of the control group. A total of 61.1% of the women in the SIL group and 39.3% of the women in the control group had more than two children. A total of 50% of the SIL group and 42.9% of the control group reported being either married or in a stable union. Reports of stress in the work or home setting were found in 71.8% of the SIL group and 60.7% of the control group (Table 1). Mean blood sugar was 98.72 mg/dL in the SIL group and 104.32 mg/dL in the control group. Triglyceride levels were 165 mg/mL and 165.19 mg/mL, respectively. Total cholesterol and fraction (HDL, VLDL, and LDL) levels were, respectively, 200, 58, 33, and 110 mg/dL in the SIL group and 196, 61, 30, and 102 mg/dL in the control group. Transaminases (TGO and TGP) were, respectively, 31 and 34 mg/dL in the SIL group and 27 and 31 mg/dL in the control group (Table 2).

3.2. Serum Levels of TBARS and IPAQ Score. A difference was found in the serum MDA concentration between the groups (Figure 2). The women in the SIL group had significantly higher levels of lipid peroxidation compared to those whose test results were normal or revealed nonspecific inflammatory lesions. The IPAQ score was lower in the SIL group, charactering less physical activity in comparison to the control group (Figure 3). A weak, inversely proportional correlation was found between the MDA levels and IPAQ scores (Figure 4), suggesting that the level of physical activity may exert an influence on serum MDA concentrations and consequently affect the oxidative balance of the organism.

4. Discussion

Few studies have analyzed oxidative stress among individuals in cancer screening and prevention programs, and fewer still have correlated levels of oxidative stress in women with SIL with a physical activity assessment tool, such as the IPAQ. In the present study, women with SIL had higher levels of MDA than those with a normal cytopathological exam or ASCUS. Moreover, the women in the SIL group had lower IPAQ scores, characterizing less effort exerted on activities of daily living in comparison to the control group. Indeed, an inverse correlation was found between MDA levels and IPAQ scores.

Many studies have demonstrated that HPV infection is associated with an imbalance between the production of free radicals and the antioxidant response of the organism [13]. Camini and colleagues report that viral infections participate in processes that involve an increase in the production of ROS, which is due to phagocyte activation

Table 1: Epidemiological, reproductive, and social characteristics and self-reported diseases.

Variable	SIL group ($n = 18$)	Control group ($n = 28$)	p value
Age	Mean: 52.17 ± 15.15	Mean: 47.96 ± 14.36	NS*
Schooling			NS**
≤8 years of study	75%	38.5%	
>8 years of study	25%	61.5%	
Declared income			NS**
≤BMMW	91.7%	84.6%	
2-3 × BMMW	8.3%	15.4%	
Number of children			NS**
None	11.1%	14.3%	
≤2	27.8%	46.4%	
>2	61.1%	39.3%	
Self-reported stress at work or home			NS**
Yes	71.8%	60.7%	
No	22.2%	39.3%	
Chronic noncommunicable disease			NS**
Yes	61.1%	50.0%	
No	38.9%	50.0%	
Reported dieting or food restriction			NS**
Yes	22.2%	28.6%	
No	77.8%	71.4%	
Smoking			NS**
Yes	0.0%	17.9%	
No	100%	82.1%	
Marital status			NS**
Single or separated	50.0%	57.1%	
Married or in stable union	50.0%	42.9%	

Source: Cervical Cancer Prevention Service, Center for Tropical Medicine. NS: nonsignificant; BMMW: Brazilian monthly minimum wage. **Chi-square test. *Student's t-test.

Table 2: Biochemical characteristics of participants.

Variable	SIL group Mean ± SD (mg/dL)	Control group Mean ± SD (mg/dL)	p value
Total cholesterol total	200 ± 30	196 ± 49	NS
HDL	58 ± 12	61 ± 18	NS
LDL	110 ± 27	102 ± 34	NS
VLDL	33 ± 17	30 ± 19	NS
TGO	31 ± 33	27 ± 10	NS
TGP	34 ± 51	31 ± 14	NS
Blood sugar	98.72 ± 18.32	104.32 ± 51.78	NS
Triglycerides	165 ± 88	165.19 ± 117.5	NS

Source: Oxidative Stress Laboratory (NMT) and Clinical Analysis Laboratory (ICB). NS: nonsignificant. Student's t-test.

induced by inflammation stemming from the virus. The production of a large amount of ROS plays an important role in the transformation of the cells of the cervix, favoring the integration of viral oncogenes to cellular DNA [14].

Studies report a correlation between the production of ROS and cervical cancer as a concomitant factor and even an etiological factor for the development of the neoplastic process. However, few studies have focused on nonspecific and premalignant inflammatory lesions, the latter of which

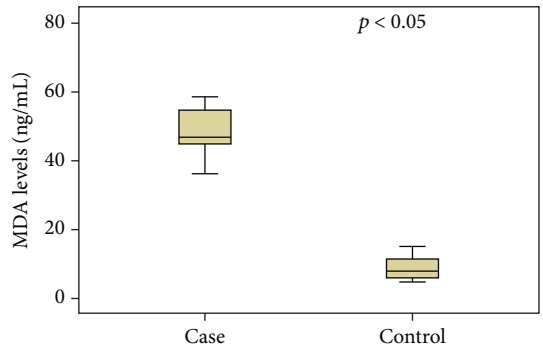

Figure 2: Comparison of serum MDA levels between groups.

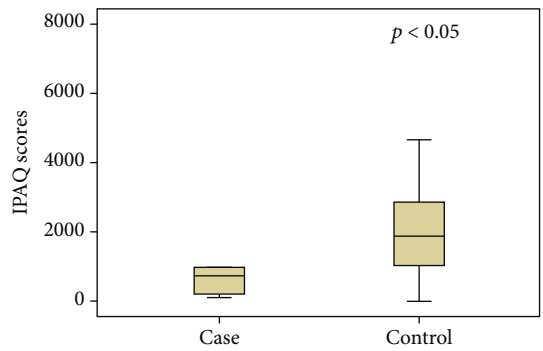

Figure 3: Comparison of IPAQ scores between groups.

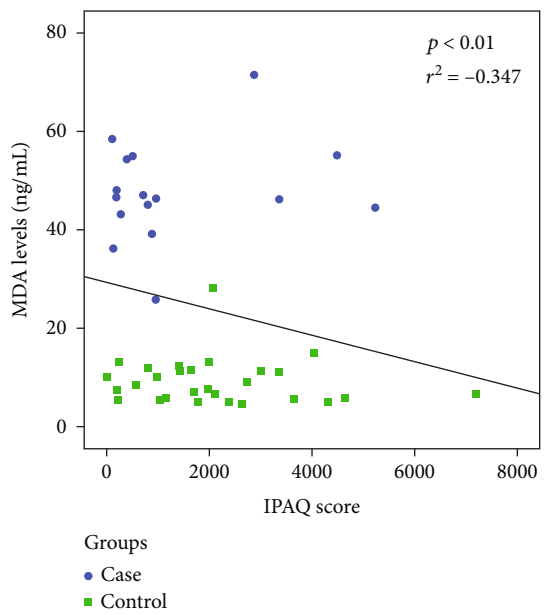

Figure 4: Correlation between MDA levels and IPAQ scores.

may be submitted to either a "wait-and-see" or therapeutic (excision of the affected region) approach, depending on the clinical criteria or results of the cytopathological exam [15, 16].

In the present study, the women with SIL had higher serum levels of MDA in comparison to those whose exam results were normal or revealed nonspecific inflammatory lesions. Gonçalves and colleagues demonstrated that TBARS levels were significantly higher in women with malignant and premalignant lesions compared to the control group and found a positive association between lipid peroxidation and the severity of the lesions; the authors also found that individuals with low-grade SIL also had higher levels than the control group [17]. Studying 202 samples from colpocytopathological exams, Visalli et al. also found that the levels of oxidative stress in patients with more severe SIL were much higher than the levels in the control group and a significant increase was found even among those with low-grade SIL [18].

Questionnaires addressing physical activity can be valuable, and low-cost tools for estimating the degree of physical activity in a sample exhibit good correlations when compared to methods that use physiological markers or movement meters [19, 20]. In the present study, the women with the SIL had lower IPAQ scores compared to the control group, which could mean that the women in the control group were more physically active. Some studies have found that the level of physical activity has a direct impact on the health status of a population and should be part of a nonpharmacological approach to diverse pathological processes [21, 22]. Bauman et al. demonstrated the viability of the administration of the IPAQ in a population of 49,493 individuals from 20 countries. The authors found satisfactory results regarding the classification of the physical activity level of the participants [23], providing further evidence of the usefulness of this simple, accessible, adaptable assessment tool in studies involving heterogeneous samples of patients [23].

An inverse correlation was found between levels of oxidative stress based on serum MDA and the IPAQ score. As all other variables studied were similar between the two groups, this correlation suggests the influence of the quantity and quality of energy expenditure during daily activities on lipid peroxidation.

Analyzing 53 healthy individuals submitted to different exercise programs, Bouzid et al. demonstrated that lipid peroxidation levels were lower among those that adhered to the program in comparison to the control group, which had higher levels of serum TBARS [24]. Likewise, Vincent et al. conducted a study involving 49 obese older adults submitted to regular physical exercise for six months and found that TBARS levels were significantly lower at the end of the program [25]. However, some authors report that the reduction in oxidative stress depends on the intensity of the exercise, with exercises of mild to moderate intensity offering the most benefit [26–28].

There are different biochemical pathways regarding the production of ROS related to physical activity, and different types and levels of activity elicit different organic responses. Therefore, one must consider the different effects of aerobic exercises and activities that predominantly involve the anaerobic metabolism as well as the intensity at which each exercise is performed. Although not fully understood, the adaptive response to exercise demonstrates a clear relationship to such particularities [29].

The present study has limitations that should be considered. Only one marker of oxidative stress was used: serum MDA through the TBARS method involving spectrophotometry. It is possible that more substantial results would have been obtained if other oxidant and even antioxidant markers had been used for a more accurate comparison of the REDOX equilibrium. Another limitation was not having better control over variables regarding the daily lives of the participants, as the analysis was limited to self-reported data on physical activity, eating habits, lifestyle habits, smoking, and alcohol intake. Finally, the sample size was small due to the detection of diagnosed cases during the study.

In conclusion, the present investigation is a pioneering study on the association between physical activity level and oxidative stress in women participating in a cervical cancer prevention program. The findings reveal that women with squamous intraepithelial lesions had higher levels of serum MDA and lower scores on the International Physical Activity Questionnaire in comparison to the control group and an inverse correlation was found between these two variables in the overall sample. These findings pave the way for further studies on the influence of different levels of physical activity on the oxidative response in women with squamous intraepithelial lesions of the cervix, offering novel options regarding prevention, therapy, and health promotion actions for these patients.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interest related to this study.

Acknowledgments

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Supplementary Materials

In the file, there are the score of IPAQ, the values of all the variables shown in Table 2, and the MDA concentration in both groups (caso in Portuguese as the case group in English and controle as the control group). *(Supplementary Materials)*

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Research Article

Relationships between Serum Uric Acid, Malondialdehyde Levels, and Carotid Intima-Media Thickness in the Patients with Metabolic Syndrome

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Oxidative stress is the major cause of atherosclerosis and cardiovascular diseases. This cross-sectional study is aimed at determining if parallel serum markers of oxidative stress are related to carotid intima-media thickness (IMT). We enrolled 134 participants with varied metabolic syndrome (Met-S) scores (zero, $n = 21$; one, $n = 19$; two, $n = 27$; three, $n = 26$; four, $n = 25$; five, $n = 16$). Biochemical profiles and potential oxidative stress biomarkers malondialdehyde (MDA) and uric acid were measured in fasting plasma. We found that carotid IMT positively correlated with both MDA and uric acid levels. Multivariate analysis revealed that both MDA ($p < 0.05$) and uric acid ($p < 0.01$) levels were significantly associated with carotid IMT in participants whose Met-S scores were ≥1 or ≥2. However, only uric acid ($p < 0.01$) levels were positively associated with carotid IMT in patients with metabolic syndrome. Linear regression model analysis revealed that the prediction accuracies for carotid IMT from MDA combined with uric acid and from a combination of MDA, uric acid, and Met-S score were 0.176 and 0.237, respectively. These were better than the predication accuracies from MDA ($r^2 = 0.075$) and uric acid ($r^2 = 0.148$) individually. These results suggest that measuring uric acid levels along with MDA biomarkers and Met-S scores may be a promising step in the development of an effective model for monitoring the severity of carotid IMT and atherosclerosis in the patients with metabolic syndrome.

1. Introduction

Oxidative stress plays a crucial role in the pathophysiological processes of several diseases, including atherosclerosis [1]. Metabolic syndrome is a cluster of conditions that includes abdominal obesity, high blood pressure, high blood sugar, high serum triglycerides, and low high-density lipoprotein cholesterol (HDL-C) levels. Accordingly, each of these conditions including obesity, dyslipidemia, hypertension, and hyperglycemia carries an independent risk for cardiovascular disease and atherosclerosis [2–5]. An increasing number of studies confirm that metabolic dysregulation causes increases in oxidative stress, contributing to the pathogenesis of atherosclerotic changes [6, 7].

Serum uric acid and malondialdehyde (MDA) are two important biomarkers of oxidative stress [8–10]. In particular, MDA is the most frequently used indicator of oxidative damage to cells and tissue in several conditions (e.g., diabetes). Several studies have shown that elevated serum uric acid levels are associated with conditions of metabolic dysregulation such as hyperlipidemia [11], hypertension, and cardiovascular risk-related factors [12, 13]. MDA is a

toxic product of aldehydes from lipid peroxidation. High serum concentration of MDA is associated with metabolic dysregulation of glucose and lipid profiles [6].

Carotid intima-media thickness (IMT), measured non-invasively by ultrasonography, is currently a widely used marker for atherosclerotic disease. Carotid IMT is directly associated with an increased risk of cardiovascular disease [14, 15]. Although previous studies have reported that high levels of MDA and uric acid are individually associated with increased carotid IMT in patients with hypertension and metabolic syndrome [16–18], whether uric acid, or MDA, or their combination may serve as a better predictor of carotid IMT among the patients with metabolic syndrome has not been clarified yet. Therefore, this study was designed to address this issue. Our data show that the prediction accuracies for carotid IMT from MDA combined with uric acid in the cases with higher metabolic syndrome scores were better than those from MDA and uric acid individually.

2. Participants and Methods

2.1. Study Design and Participants. This study was designed as a cross-sectional case-control study, conducted at the Health Management Center at the Changhua Christian Hospital over a two-year period. All participants aged between 45 and 60 years were eligible for inclusion. Exclusion criteria included evidence of hyperthyroidism, hypothyroidism, alcoholism, or viral hepatitis (type B or C). Cases were also excluded if the patients were receiving antidiabetic medication, statins, antioxidants, vitamin C, vitamin E, nonsteroidal anti-inflammatory drugs, or hepatotoxic agents during the study period. Waist circumference and blood pressure were measured in all participants. After an >8-hour overnight fast, venous blood specimens were obtained from all subjects for biochemical profiles as well as high-sensitivity C-reactive protein (hs-CRP), uric acid, and MDA levels; the information of anthropometric measurements and metabolic syndrome-associated profiles were also obtained. The study was conducted in strict accordance with guidelines for research involving human subjects developed by the Taiwan Ministry of Health and Welfare. All study protocols were approved by the Institutional Review Board of the Changhua Christian Hospital (Approval Number 110507). All participants provided written informed consent to participate in the study.

2.2. Definition of Metabolic Syndrome (Met-S) Scores. In this study, metabolic syndrome was defined according to the modified National Cholesterol Education Program (NCEP) criteria with Asian-specific cutoffs [19]. Briefly, metabolic syndrome was diagnosed in patients with ≥ three of the following five components: (1) waist circumference (≥90 cm for men and ≥80 cm for women), (2) blood pressure (systolic ≥ 130 mmHg and/or diastolic ≥ 85 mmHg), (3) triglycerides (≥150 mg/dl), (4) HDL-C (<40 mg/dl in male and <50 mg/dl in female), or (5) fasting glucose (≥100 mg/dl). The participants were initially stratified into Met-S score subgroups according to the number of Met-S components.

2.3. Anthropometric Measures. Blood specimens were obtained from participants in the morning following an overnight fast. The plasma was aliquoted and stored at −80°C, without thawing until assay. A history of cigarette and alcohol use was obtained from each study participant. Height and weight were measured in light clothing without shoes. BMI was calculated as weight (kg)/height^2 (m)^2.

2.4. Carotid Ultrasonography to Measure Intima-Media Thickness of the Common Carotid Arteries. IMT of the common carotid arteries was assessed using high-resolution B-mode ultrasonography (Acuson 128XP, equipped with a 7 MHz linear array transducer). All measurements were conducted by the same experienced sonographer on a day close to the day of blood biochemistry analysis. The IMT value was defined as the mean of 10 IMT measurements on the far wall of the bilateral common carotid arteries about 10 mm proximal to the carotid bifurcation. The lumen/intima leading edge (I-line) to media/adventitia leading edge (M-line) method was used, which is validated anatomically as previously described [20].

2.5. Assays for Plasma MDA and Plasma Superoxide Dismutase. Plasma MDA was assayed with a thiobarbituric acid reactive substances assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. Absorbance of the samples was measured at 532 nm by a microplate reader (Versa Max, Molecular Devices, Sunnyvale, CA, USA). MDA level was determined by using an MDA standard curve. The superoxide dismutase (SOD) is an enzyme protecting lipid from superoxide-induced oxidative stress. The activity (U/ml) of plasma SOD was therefore quantified in order to characterize the antioxidant capability of a biological system using a superoxide dismutase assay kit (Cayman Chemical Company, MI, USA) according to the manufacturer's instructions. The interassay and intra-assay laboratory coefficients of variation for MDA were 5.8% and 8.0%, respectively, and for SOD were 7.2% and 6.5%, respectively.

2.6. Assays for Oxidative DNA Damage. Increased oxidative stress may lead to oxidative DNA damage, which may in turn bring about cell injury and death. 8-hydroxy-2′-deoxyguanosine (8-OHdG) is a biomarker that is widely used to indicate the oxidative stress-induced single nucleotide-based lesions [21]. γ-H2AX is a protein involved in the first step for repairing DNA double-strand breaks and is a sensitive marker of DNA damage and repair [22]. Quantitation of those two proteins (8-OHdG and γ-H2AX) was performed by 8-OHdG assay kit (Wuhan Fine Biotech Co. Hubei, China) and γ-H2AX assay kit (Wuhan Fine Biotech Co. Hubei, China), according to the manufacturer's instructions, respectively. The interassay and intra-assay laboratory coefficients of variation for 8-OHdG were 6.8% and 7.5%, respectively, and for γ-H2AX were 8.5% and 9%, respectively.

2.7. Biochemistry Assays and Other Measures. Serum concentrations of aspartate transaminase (AST), alanine transaminase (ALT), uric acid, fasting blood sugar, hs-CRP, total white blood cell count (WBC), and lipid profiles including

total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL-C), and HDL-C were measured using standard procedures at the Department of Laboratory Medicine, Changhua Christian Hospital. In addition, glycated hemoglobin (HbA1c) levels in venous bloods samples were analyzed by the D-100 HbA1c test (Bio-Rad Laboratories, Inc., CA). The interassay and intra-assay laboratory coefficients of variation for HbA1c varied between 1.1 and 2.3%.

2.8. Statistical Analysis. Data are represented as mean ± standard deviation (SD). The chi-square test was used for categorical comparisons of data, and the ANOVA test was used to measure differences in means of continuous variables between the six Met-S score subgroups (Table 1 and Figure 1). The Jonckheere-Terpstra test was used to test for an ordered alternative hypothesis within six subgroups (Table 1). The Pearson correlation analysis was performed to evaluate the correlation between carotid IMT and oxidative stress biomarkers, as well as Met-S scores (Figure 1). The multiple linear regression model was used to assess the relationships between oxidative stress biomarkers (Tables 2 and 3), and conditions of metabolic dysregulation, as well as carotid IMT (Table 4). A linear regression model was used to calculate the predicted values of carotid IMT (Figure 2). A p value < 0.05 was considered as an indicator of significant statistical difference. All statistical analyses were conducted using the statistical package SPSS (IBM SPSS Statistics, version 20, IBM Corporation, Chicago, IL, USA).

3. Results

3.1. Demographic, Clinical, and Laboratory Data. This study enrolled 134 participants who visited the Health Management Center at the Changhua Christian Hospital for health management reasons over a two-year period. Table 1 shows demographic, clinical, and laboratory data from participants in the six Met-S score subgroups. There were no significant differences in age, gender, alcohol use, or cigarette use among these subgroups. WBC, hs-CRP, average IMT, GOT, and GPT showed graded increasing trends, while HDL-C showed a graded decrease (Table 1).

3.2. Increased Levels of Oxidative Stress Markers and Lower Activity of Antioxidant Enzymes in the Subgroups with Higher Met-S Scores. The plasma levels of uric acid and lipid peroxidation product MDA were significantly increased in parallel with the number of Met-S scores (Table 1). The activity of antioxidant enzyme SOD showed the tendency toward decrease among Met-S subgroups, whereas both 8-OHdG and γ-H2AX levels of oxidative DNA damage showed the tendency toward increase (Table 1). The plasma level of MDA was significantly correlated with 8-OHdG levels ($r = 0.2418$, $p = 0.0089$).

3.3. Correlations between Carotid IMT, Uric Acid, MDA, hs-CRP, and Metabolic Syndrome Scores. We examined the relationships between carotid IMT and the oxidative stress biomarkers, MDA and uric acid, and the inflammatory factor, hs-CRP, in all 134 participants using Pearson's correlation test. The analysis revealed that carotid IMT positively correlated with uric acid ($r = 0.382$, $p < 0.001$) and MDA ($r = 0.274$, $p < 0.001$), but not significantly correlated with hs-CRP ($r = 0.151$, $p = 0.082$) (Figures 1(a)–1(c)). We also found that carotid IMT showed significant correlation with Met-S scores ($r = 0.225$, p trend < 0.001) (Figure 1(d)).

3.4. Relationship of Oxidative Stress Biomarkers (MDA and Uric Acid) with Metabolic Components. Reports have suggested that oxidative stress is associated with conditions of metabolic syndrome and several traditional risk factors [6, 7]. Therefore, we examined which factors are closely associated with MDA and uric acid. After adjusting for the five conditions of metabolic syndrome and traditional risk factors, multivariate analysis revealed that MDA was significantly correlated with fasting blood sugar ($p < 0.001$), Met-S score ($p < 0.001$), and triglycerides ($p = 0.005$) as well as negatively with HDL-C ($p = 0.014$) (Table 2). Uric acid was positively correlated with diastolic blood pressure ($p = 0.001$) and metabolic score ($p < 0.001$) as well as negatively with HDL-C ($p = 0.028$) (Table 3).

3.5. Multivariate Analysis to Evaluate the Associations among MDA, Uric Acid, and Metabolic Syndrome Traits and Carotid IMT. We then examined the role of these two oxidative stress biomarkers in association with carotid IMT in our study participants. Multivariate analysis revealed that Met-S score ($p = 0.044$) and uric acid ($p = 0.013$) were positively associated with carotid IMT among all participants ($n = 134$), after adjusting for traditional risk factors (age, gender, smoking, and alcohol use). However, MDA and hs-CRP were not positively associated with carotid IMT (Table 4). Stratified by Met-S scores, further analysis revealed that both MDA ($p < 0.05$) and uric acid ($p < 0.01$) levels were significantly associated with carotid IMT in participants whose Met-S scores were ≥1 or ≥2. However, only uric acid ($p < 0.01$) was positively associated with carotid IMT in participants with metabolic syndrome, i.e., patients with Met-S scores ≥ 3.

3.6. Uric Acid Combined with MDA and Met-S Score Is Better Predictive of Severity of Carotid IMT. Finally, the linear regression model was used to calculate the predicted values of carotid IMT based on the total variation of carotid IMT. We observed that the prediction accuracy (r^2) of carotid IMT variance for MDA and uric acid was 0.075 and 0.148, respectively. The prediction accuracy (r^2) of carotid IMT variance for MDA combined with uric acid and for a combination of MDA, uric acid, and Met-S score was 0.176 and 0.237, respectively (Figure 2).

4. Discussion

In this study, we evaluated the association between oxidative stress biomarkers and carotid IMT, and whether combination of these biomarkers is a better predictor for carotid IMT in our study participants. We found that participants with higher metabolic syndrome scores, MDA, and uric acid levels had significantly higher levels of carotid IMT (all p values < 0.001 and p trend < 0.001). Multiple regression analysis showed that both MDA and uric acid were

Table 1: Demographic, clinical, and laboratory data of the stratified subgroups by metabolic syndrome score.

	Met − S score = 0	Met − S score = 1	Met − S score = 2	Met − S score = 3	Met − S score = 4	Met − S score = 5	p value	p trend
Sample size, n	21	19	27	26	25	16		
Age (year)	49.67 ± 4.85	50.21 ± 4.42	52.93 ± 5.87	50.88 ± 5.74	51.24 ± 6.08	53.19 ± 6.84	0.264	0.089
Male, n (%)	7 (33.33%)	10 (52.63%)	17 (62.96%)	12 (46.15%)	13 (52%)	10 (62.5%)	0.383	0.214
Smoking, n (%)	1 (4.76%)	1 (5.26%)	3 (11.11%)	3 (11.54%)	4 (16%)	5 (31.25%)	0.189	0.015
Alcohol, n (%)	3 (14.29%)	3 (15.79%)	8 (29.63%)	7 (26.92%)	6 (24%)	4 (25%)	0.792	0.344
BMI (kg/m^2)	22.13 ± 2.78	23.61 ± 1.96	24.84 ± 3.01	25.85 ± 2.7	27.55 ± 3.32	29.44 ± 3.74	<0.001	<0.001
Waist (cm)	74.6 ± 6.28	78.66 ± 6.02	85.39 ± 8.57	84.48 ± 6.57	92.21 ± 8.77	97.88 ± 9.33	<0.001	<0.001
SBP (mmHg)	107.52 ± 8.9	122.74 ± 13.08	125.93 ± 15.35	130.73 ± 17.36	139.04 ± 12.24	145.88 ± 16.3	<0.001	<0.001
DBP (mmHg)	71.14 ± 6.36	78.26 ± 7.85	79.67 ± 8.06	84.92 ± 13.41	88.16 ± 8.54	88.5 ± 7.92	<0.001	<0.001
Uric acid (mg/dl)	5.1 ± 1.4	5.5 ± 1.4	6.05 ± 1.44	6.04 ± 1.07	6.46 ± 1.53	6.78 ± 0.7	0.001	<0.001
MDA (μm/ml)	6.96 ± 1.17	6.98 ± 1.3	7.62 ± 2.11	8.54 ± 2.05	9.09 ± 1.9	13.02 ± 3.78	<0.001	<0.001
SOD (U/ml)	4.06 ± 0.37	3.88 ± 0.31	3.59 ± 0.36	3.26 ± 0.34	2.58 ± 0.32	2.42 ± 0.36	0.0073	<0.001
8-OHdG (ng/ml)	36.97 ± 6.74	55.72 ± 8.36	70.04 ± 8.21	77.46 ± 9.62	81.38 ± 5.01	90.06 ± 6.76	<0.001	<0.001
γ-H2AX (pg/ml)	29.97 ± 4.14	34.53 ± 5.09	36.74 ± 6.029	42.52 ± 5.23	53.23 ± 4.82	54.05 ± 4.02	0.0061	<0.001
hs-CRP (mg/dl)	0.05 ± 0.06	0.06 ± 0.08	0.12 ± 0.13	0.22 ± 0.28	0.25 ± 0.32	0.49 ± 0.4	<0.001	<0.001
Fasting blood sugar (mg/dl)	87.1 ± 5.86	93.74 ± 4.39	93.48 ± 8.53	104.15 ± 31.94	111.56 ± 19.93	160.94 ± 57.86	<0.001	<0.001
Hemoglobin A1c (%)	5.19 ± 0.21	5.37 ± 0.4	5.48 ± 0.53	5.81 ± 1.02	6.12 ± 1.04	8.14 ± 2.33	<0.001	<0.001
Total cholesterol (mg/dL)	190.48 ± 31.01	212.74 ± 32.37	205.11 ± 49.95	205.46 ± 35.44	207.44 ± 38.24	229 ± 68.79	0.189	0.035
HDL-C (mg/dL)	56.86 ± 8.74	54.68 ± 14.11	48.22 ± 10.82	45.54 ± 8.31	39.52 ± 7.95	38 ± 8.07	<0.001	<0.001
Triglyceride (mg/dL)	70.05 ± 21.8	95.95 ± 44.18	126.37 ± 63.75	151.81 ± 63.98	215.96 ± 94.92	233.44 ± 109.11	<0.001	<0.001
LDL-C (mg/dL)	114.99 ± 26.63	134.63 ± 27.91	127.12 ± 34.36	129.35 ± 30.38	125.07 ± 45.09	138.81 ± 35.91	0.356	0.160
Tchol_HDLratio	3.39 ± 0.58	4.06 ± 0.93	4.32 ± 0.9	4.63 ± 1.07	8.94 ± 17.52	6.36 ± 2.81	0.148	0.045
WBC (mm^3)	4.74 ± 1.22	4.83 ± 1.42	5.49 ± 1.32	5.7 ± 1.51	6.81 ± 1.33	7.53 ± 2.56	<0.001	<0.001
AST (U/L)	25.86 ± 9.32	23.47 ± 4.94	27.67 ± 7.68	26.23 ± 7.91	36.36 ± 19.41	34.88 ± 15.2	<0.001	<0.001
ALT (U/L)	21.05 ± 8.84	23.84 ± 7.75	31.26 ± 17.01	29.73 ± 15.53	47.36 ± 28.42	41.19 ± 18.04	<0.001	<0.001
Creatinine (mg/dl)	0.71 ± 0.17	0.76 ± 0.17	0.81 ± 0.16	0.72 ± 0.2	0.78 ± 0.16	0.8 ± 0.18	0.251	0.246
Renal GFR (ml/min/1.73 m^2)	100.07 ± 22.36	99.15 ± 19.18	95.83 ± 15.75	106.86 ± 29.99	93.77 ± 16.03	97.59 ± 27.49	0.384	0.681
avgIMT (mm)	0.62 ± 0.09	0.64 ± 0.07	0.70 ± 0.12	0.74 ± 0.14	0.74 ± 0.12	0.78 ± 0.1	<0.001	<0.001

Data are presented as mean ± SD or n (%) for categorical data. Differences in mean values of variables between the four Met-S-stratified subgroups were tested by one-way ANOVA test. p for trend was calculated by the Jonckheere-Terpstra Test to test for an ordered alternative hypothesis within six subgroups. Met-S: metabolic syndrome; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; WBC: white blood cell; ALT: alanine aminotransferase; AST: aspartate aminotransferase; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; hs-CRP: high-sensitivity C-reactive protein; MDA: malondialdehyde; avgIMT: average of intima-media thickness of both common carotid arteries; SOD: superoxide dismutase; 8-OHdG: 8-hydroxy-2′-deoxyguanosine.

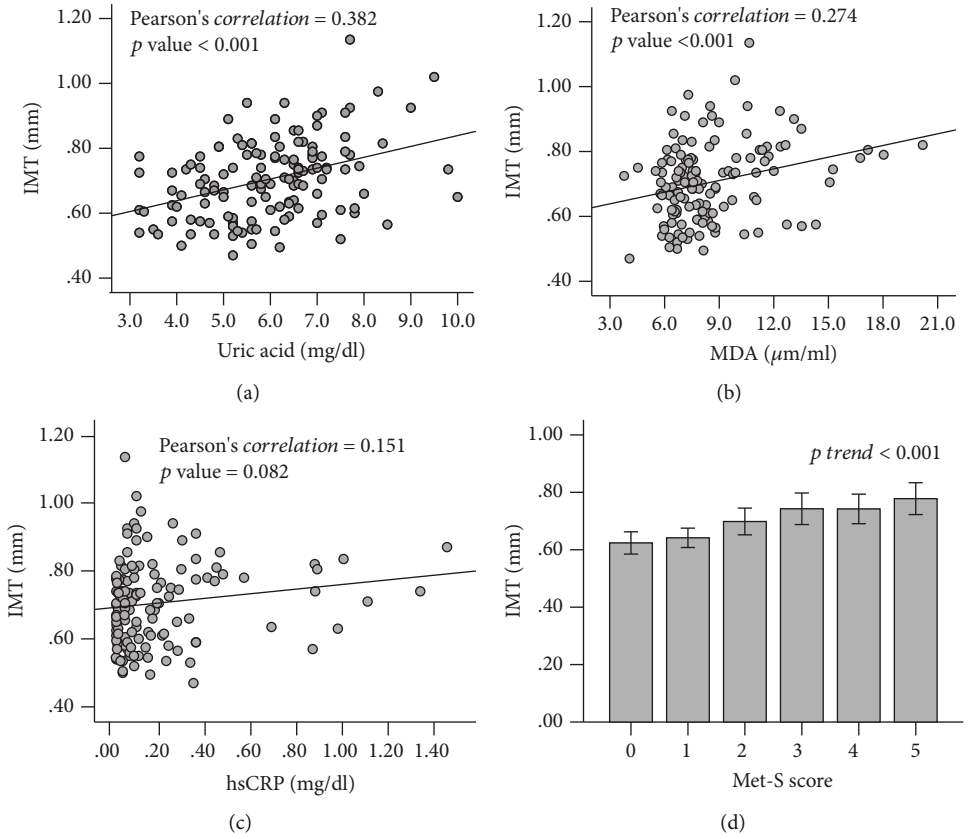

Figure 1: Correlations between carotid IMT and malondialdehyde, uric acid, hs-CRP, and metabolic syndrome scores in all 134 participants. Associations of carotid IMT are positive with uric acid (a), malondialdehyde (MDA) (b), and high-sensitivity C-reactive protein (hs-CRP) (c) using Pearson's correlation test, and with metabolic syndrome (Met-S) scores (d) using one-way ANOVA analysis.

Table 2: Associations of MDA with metabolic components and traditional factors ($n = 134$).

Variables	Model 1		Model 2		Model 3	
	Standardized coefficient beta	p value	Standardized coefficient beta	p value	Standardized coefficient beta	p value
Waist	0.077	0.327				
SBP	0.136	0.193				
DBP	-0.133	0.169				
Fasting blood sugar	0.481	<0.001	0.528	<0.001		
HDL-C	-0.152	0.114	-0.178	0.014		
Triglyceride	0.213	0.040	0.212	0.005		
Total cholesterol	0.011	0.941			0.024	0.830
LDL-C	0.110	0.381	0.114	0.067	0.091	0.401
Met-S score					0.315	<0.001

MDA level was log-transformed (ln) due to nonnormally distribution. Model 1 was the full model with adjusting for waist, LDL-C, TG, HDL-C, total cholesterol, SBP, DBP, and fasting blood sugar. Model 2 was carried out the backward elimination procedure for Model 1. Model 3 was adjusted for Met-S, total cholesterol, and LDL-C. Met-S: metabolic syndrome; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; MDA: malondialdehyde; SBP: systolic blood pressure; DBP: diastolic blood pressure.

significantly associated with carotid IMT in participants with ≤2 Met-S components. Furthermore, in participants with metabolic syndrome, uric acid was an independent factor of carotid IMT. An evaluation of both oxidative stress biomarkers (MDA plus uric acid) or evaluating them in combination with Met-S scores might provide more accurate measurements of carotid IMT.

The present study showed consistent results in the increased levels of oxidative stress products and its related DNA damage markers (e.g., 8-OHdG and γ-H2AX), and in the lower activity of antioxidant enzyme (e.g., SOD), supporting the increase of oxidative stress in metabolic syndrome and its component pathologies [6, 7, 12, 22, 23]. However, the mechanism underlying oxidative stress in metabolic

Table 3: Associations of uric acid with metabolic components and traditional factors ($n = 134$).

Variables	Model 1		Model 2		Model 3	
	Standardized coefficient beta	p value	Standardized coefficient beta	p value	Standardized coefficient beta	p value
Waist	0.154	0.129				
SBP	-0.063	0.637				
DBP	0.293	0.020	0.275	0.001		
Fasting blood sugar	-0.098	0.285				
HDL-C	-0.174	0.162	-0.204	0.028		
Triglyceride	0.168	0.208	0.171	0.064		
Total cholesterol	-0.040	0.842			-0.047	0.745
LDL-C	0.117	0.470			0.118	0.413
Met-S score					0.415	<0.001

Uric acid level was log-transformed (ln) due to nonnormally distribution. Model 1 was the full model with adjusting for waist, LDL-C, TG, HDL-C, total cholesterol, SBP, DBP, and fasting blood sugar. Model 2 was carried out the backward elimination procedure for Model 1. Model 3 was adjusted for Met-S, total cholesterol, and LDL-C. Met-S: metabolic syndrome; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure.

syndrome remains unclear. We revealed the significant correlation of total WBC counts with serum MDA ($r = 0.3511$, $p < 0.0001$, Pearson's test) and uric acid ($r = 0.1944$, $p = 0.0244$, Pearson's test), respectively. Given evidence indicates that phagocytic NADPH oxidases in leukocytes become overactive in the metabolic syndrome patients, which has been considered as the primary source of reactive oxygen species (ROS) involved in atherosclerosis [7, 24]. Since phagocytic NADPH oxidases are predominantly expressed in the innate immune cells [25], total WBC count might serve as a nontraditional potential biomarker for lipid peroxidation and atherosclerosis in the patients with metabolic disorders [25–27].

Uric acid is the end product of purine metabolism in human beings and higher primates. Although it has been suggested that uric acid can act as an antioxidative scavenger, providing powerful free radical scavenging capacity in plasma [28, 29], it may also act as a prooxidant to trigger oxidative stress in cells and contribute to endothelial dysfunction and damage via triggering oxidative and endoplasmic reticulum stress and inducing mitochondrial dysfunction and mitochondrial DNA damage [30, 31]. Despite the reported beneficial role of uric acid [28, 29], this study suggests that higher levels of uric acid are correlated with carotid IMT in participants with various conditions of metabolic dysregulation (Table 4) [32].

Due to a molecular structure that is abundant with reactive double bonds, lipids are susceptible targets of oxidation [33, 34]. MDA is one of the main products of lipid peroxidation. It is also a toxic molecule with oxidized low-density LDL. Therefore, it promotes atherosclerosis [35, 36]. Consistent with previous studies [37, 38], our study suggests that elevated levels of MDA are correlated with carotid IMT. We further reveal that this association was closely observed in subjects with fewer metabolic syndrome components (Table 4). This might imply that more complicated mechanisms are involved in advanced stages of atherosclerosis, although higher MDA levels tend to be associated with having more conditions within the metabolic syndrome.

A number of studies have shown that MDA and uric acid are individual risk factors for increase of carotid IMT and atherosclerosis [39–41]. However, few studies have been conducted to show how both parallel biomarkers simultaneously contribute to carotid atherosclerosis and its progression [42]. Our study, to the best of our knowledge, is the first to report that the presence of circulating uric acid combined with MDA in participants with varied metabolic syndrome scores significantly increased the risk of carotid IMT (Figure 2). Interestingly, our study showed that MDA and uric acid had different relationship profiles with carotid IMT, metabolic syndrome conditions, and traditional risk factors. Lipid peroxidation MDA level was predominantly associated with lipid disorders (e.g., high LDL-C), high blood sugar levels, and metabolic scores (Table 2). Although glucose is not a lipid, it can be converted from the glycerol component of triglycerides, in particular, in patients with metabolic syndrome and diabetes. In contrast, uric acid was associated with hypertension, TGs, and metabolic scores (Table 3). The comparative relationships of uric acid and MDA to carotid IMT might suggest that a diagnostic model comprised of Met-S score and those two oxidative stress serum biomarkers may provide an effective strategy for detecting and monitoring the development of atherosclerosis.

There are several limitations in this study. Firstly, this study was a cross-sectional study. Therefore, it did not allow for determination of causal relationships. Secondly, the study had a relatively small sample size, which may have reduced its statistical power. Longitudinal studies with a larger sample size are needed to establish cause-effect relationships in the future. Thirdly, the information of alcohol and cigarette use, which are considered as common causes of oxidative stress for cardiovascular disease, was obtained by self-reported questionnaire. It might be underestimated for alcohol and cigarette use in our participants.

5. Conclusion

We found that oxidative stress biomarkers, increased levels of MDA and uric acid, were positively associated with increased carotid IMT in cases with multiple metabolic syndrome conditions. Specifically, we found that the combination of Met-S

Table 4: Multivariate linear regression analysis of selected risk factors associated with carotid IMT.

Variables	All patients ($N = 134$)		Met − S score = 0 ($N = 21$)		Met − S score ≥ 1 ($N = 113$)		Met − S score ≥ 2 ($N = 94$)		Met − S score ≥ 3 ($N = 67$)		Met − S score ≥ 4 ($N = 41$)	
	Standardized coefficient beta	p value	Standardized coefficient beta	p value	Standardized coefficient beta	p value	Standardized coefficient beta	p value	Standardized coefficient beta	p value	Standardized coefficients beta	p value
Main effect terms												
Uric acid	0.221	0.013	-0.007	0.978	0.292	0.003	0.3295	0.0013	0.3583	0.0016	0.4107	0.0079
MDA	0.115	0.188	0.053	0.830	0.180	0.043	0.1861	0.0478	0.0970	0.3033	0.1239	0.3708
hs-CRP	-0.023	0.771	-0.048	0.843	0.020	0.807	-0.0126	0.8880	-0.0478	0.6080	0.0303	0.8270
Met-S score	0.197	0.044										

Multivariate linear regression model was adjusted for age, gender, smoking, alcohol, uric acid, Met-S score, MDA, and hs-CRP. Met-S: metabolic syndrome; hs-CRP: high-sensitivity C-reactive protein; MDA: malondialdehyde.

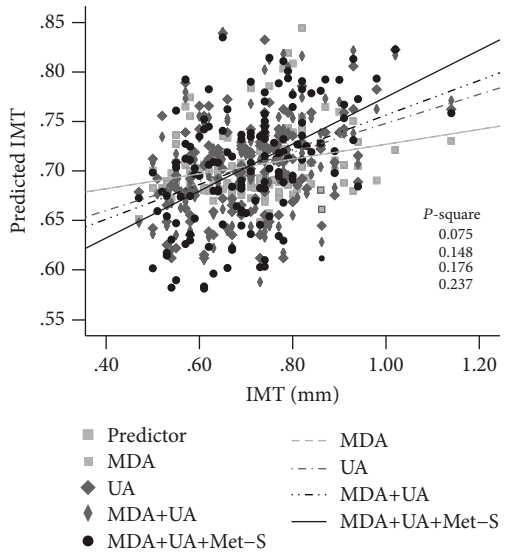

Figure 2: The prediction accuracy (r^2) of carotid IMT variance for malondialdehyde (MDA), uric acid, and metabolic syndrome (Met-S) scores in all 134 participants by using a linear regression model.

scores with uric acid and MDA was the most accurate measure for predictive IMT values. A diagnostic model comprising of the clinical features, metabolic components, and serum oxidative stress biomarkers may provide an effective strategy for detecting and monitoring the development of atherosclerosis. Longitudinal studies are needed to support our novel findings.

Data Availability

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

Conflicts of Interest

The authors declare no competing financial interests.

Authors' Contributions

HMW and SSW conceived and designed the experiments. TYC, KHL, KLS, and WWS performed the experiments. CTK analyzed the data. HMW, SSW, and CTK drafted the paper. All authors read and approved the final manuscript. Shun-Sheng Wu and Chew-Teng Kor contributed equally to this work.

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Research Article

Peroxiredoxin III Protects Tumor Suppressor PTEN from Oxidation by 15-Hydroperoxy-eicosatetraenoic Acid

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Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid and protein phosphatase that coordinates various cellular processes. Its activity is regulated by the reversible oxidation of an active-site cysteine residue by H_2O_2 and thioredoxin. However, the potential role of lipid peroxides in the redox regulation of PTEN remains obscure. To evaluate this, 15-hydroperoxy-eicosatetraenoic acid (15s-HpETE), a lipid peroxide, was employed to investigate its effect on PTEN using molecular and cellular-based assays. Exposure to 15s-HpETE resulted in the oxidation of recombinant PTEN. Reversible oxidation of PTEN was also observed in mouse embryonic fibroblast (MEF) cells treated with a 15s-HpETE and Lipofectamine mixture. The oxidative dimerization of thioredoxin was found simultaneously. In addition, the absence of peroxiredoxin III aggravated 15s-HpETE-induced PTEN oxidation in MEF cells. Our study provides novel insight into the mechanism linking lipid peroxidation to the etiology of tumorigenesis.

1. Introduction

Lipoxygenases (LOX) are a heterogeneous family of enzymes that catalyze the insertion of molecular oxygen into polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA) and linoleic acid (LA), into the corresponding hydroperoxyl derivates, which can be potent inflammatory and prooxidant mediators [1, 2]. 15-Lipoxygenase (15-LOX), a member of the LOX family, is widely expressed in different organisms [3–8]. 15-LOX metabolizes AA to form 15(s)-hydroperoxyeicosatetraenoic acid (15s-HpETE), the oxidative precursor of 15-hydroxyeicosatetraenoic acid (15s-HETE). 15s-HpETE, 15s-HETE, and many of their analogous metabolites have important physiological functions. However, the end-products of lipid peroxidation have demonstrated mutagenicity [2, 9], providing further evidence that inflammation plays an important role in carcinogenesis via its ability to increase cellular oxidative stress. Increased levels of lipid peroxides have also been linked to the pathogenesis of a variety of human diseases through cellular oxidative damage, including neurodegeneration, atherosclerosis, type II diabetes, metabolic disorders, solid tumors, and hematologic malignancies [3, 10–12]. The redox status is also altered in cancer cells, which may result from increased levels of lipid peroxides [12]. Emerging evidence has suggested that 15s-HpETE-induced membrane lipid peroxidation and free radical generation [9] may exert proinflammatory properties and contribute to endothelial cell injury [13]. Both 15s-HpETE and 15s-HETE were shown to inhibit the growth of cultured human chronic myelogenous leukemia K562 cells by a mechanism associated with reactive oxygen species (ROS) [11, 14, 15]. 15s-HpETE and 15s-HETE formed during

inflammation have divergent effects on angiogenesis [16]. 15s-HETE promoted pulmonary artery inflammation by activating the NF-κB pathway [17]. Intradermal injection of 15s-HpETE induced inflammatory symptoms, such as plasma exudation, in rabbits [18]. Although 15s-HpETE has been implicated in the pathogenesis of multiple chronic diseases, its specific molecular target remains unclear.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a member of the protein tyrosine phosphatase (PTP) superfamily. It is a potent tumor suppressor gene, frequently lost in a variety of human sporadic cancers [19], diabetes, and inherited syndromes, such as Cowden disease and Lhermitte-Duclos syndrome [20]. The primary cellular substrate of PTEN is phosphatidylinositol-3,4,5-triphosphate (PIP3), a lipid second messenger molecule generated by the action of phosphatidylinositol-3-kinase (PI3K). PI3Ks can be activated by a series of stimuli, including insulin, cytokines, neurotransmitters, peptide growth factors, and hormones [21–24]. PIP3 activates serine-threonine kinase protein kinase B (PKB/Akt) and 3-phosphoinositide-dependent kinase (PDK) [25]. By hydrolyzing PIP3, PTEN blocks PI3K signaling activities, such as membrane recruitment and the activation of Akt, thereby inhibiting cell proliferation, growth, survival, and metabolism [26–28]. Functional loss of PTEN results in cancer susceptibility and favors tumor progression. PTEN is negatively regulated through oxidation of its active-site cysteine. Numerous studies have demonstrated that the catalytic activity of PTEN was modulated by ROS, resulting in its catalytic inhibition [29, 30]. H_2O_2-oxidized PTEN forms a disulfide bond between cysteine residues Cys^{124} and Cys^{71}. Oxidized PTEN can be reversibly converted back to its reduced form by intracellular-reducing systems, such as the thioredoxin (Trx) and glutaredoxin (Grx) systems [30–32]. Trx is a highly conserved antioxidant protein, which comprises the Trx system with selenoprotein thioredoxin reductase (TrxR) and NADPH. Trx maintains the thiol-related redox balance status and plays a pivotal role in the regulation of redox signaling [33, 34]. Oxidative stress-mediated dimerization of Trx can delay the reduction of its active-site disulfide by TrxR, resulting in inactivation of the Trx system [35].

We previously reported that exogenous organic peroxides and hydroperoxides can cause the irreversible oxidation of PTEN by impairing the cellular Trx system [34, 36]. The ability of lipid peroxides to oxidize PTPs has been reported previously [37], as well as the PTEN oxidation by unidentified arachidonic acid metabolites [38]. As a member of the PTP superfamily, PTEN may be a preferential molecular target of lipid peroxides. Thus, the objective of this study was to investigate the effect of lipid peroxide on the redox state of PTEN using an endogenous lipid peroxide 15s-HpETE as a model.

2. Materials and Methods

2.1. Materials and Reagents. Recombinant wild-type PTEN was purified as described previously [30]. 15s-HpETE and 15s-HETE were purchased from Cayman Chemical (Ann Arbor, MI, USA). NAP-5 Sephadex G25 columns were purchased from GE Healthcare Life Sciences (Little Chalfont, UK). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Lipofectamine 2000 transfection reagent, and anti-actin antibody were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The PTEN and Trx antibodies were prepared as described previously [39, 40]. Anti-rabbit IgG horseradish peroxidase-conjugated antibody was purchased from Ab Frontier (Daejeon, Korea).

2.2. Cell Culture and Treatment. Mouse embryonic fibroblasts (MEFs) were prepared at embryonic day 13.5 from embryos obtained by mating Prdx $III^{+/-}$ mice. All cells were cultured in DMEM supplemented with 10% FBS and maintained in a humidified 5% CO_2 incubator at 37°C. The cells were seeded into 6-well plates and cultured in complete medium supplemented with 10% FBS to reach 80% confluence. After rinsing three times with phosphate-buffered saline (PBS), the cells were maintained in FBS-free DMEM for 30 min. Lipofectamine 2000 transfection reagent was used to assist lipid peroxide transfer across the cell membranes. After 10 μM 15s-HpETE was mixed with Lipofectamine 2000 transfection reagent, the mixture was added to the culture plate, followed by incubation for the indicated times. The reactions were stopped by removal of the culture medium. The cells were washed three times with cold PBS.

2.3. Oxidation of Recombinant PTEN. Recombinant PTEN was oxidized during the course of purification. It was prereduced with 1 mM DTT for 2 h and then passed through a NAP-5 Sephadex G25 column preequilibrated with PTEN assay buffer (100 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 0.1% BSA) to remove DTT before treatment with 15s-HpETE. PTEN assay buffer was deoxygenated under a stream of argon gas before use. Prereduced PTEN was exposed to 5 μM 15s-HpETE for the indicated times or to varying concentrations for 30 min at room temperature. The reactions were terminated with 2 mM NEM. NEM was used to prevent artificial redox reactions by blocking the thiol groups.

2.4. Analysis of Redox Status of PTEN by Immunoblotting. Oxidative modifications of PTEN specifically involved in the formation of intramolecular disulfide bonds were readily identified by nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis, as described previously [39]. After incubation, cells were washed three times with ice-cold PBS and lysed with NP-40 lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% glycerol, 0.1% NP-40, 1 mM phenylmethylsulfonylfluoride, and protease inhibitor cocktail) containing 10 mM NEM. Protein concentrations were measured with the BCA protein assay kit (Thermo Fisher Scientific). The samples were subjected to nonreducing electrophoresis gel-loading buffer (60 mM Tris (pH 6.8), 25% glycerol, 2% SDS, and 0.5% bromophenol) or reducing gel-loading buffer, followed by immunoblotting for PTEN.

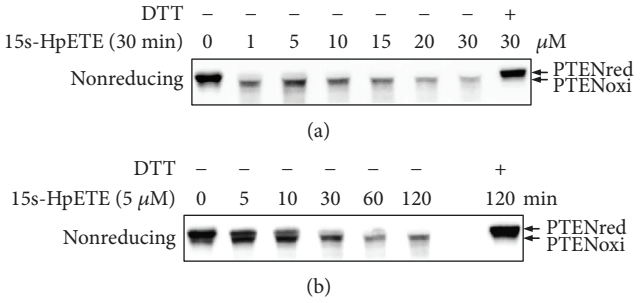

Figure 1: Effects of 15s-HpETE on the redox state of recombinant PTEN. Recombinant PTEN was prereduced with 1 mM DTT for 120 min, followed by passing through a NAP-5 column to remove excess DTT and exposure to various concentrations of 15s-HpETE for 30 min (a) or 5 μM 15s-HpETE for the indicated times (b). The reaction was quenched by 2 mM NEM to block free thiols for 10 min. The samples were then treated with or without 1 mM DTT for reduction and subjected to nonreducing SDS-PAGE, followed by immunoblotting for PTEN.

3. Results

3.1. 15s-HpETE-Induced Oxidation of Recombinant PTEN. To investigate the effects of 15s-HpETE on PTEN, purified recombinant PTEN was first assayed. Prereduced recombinant PTEN was incubated with increasing concentrations of 15s-HpETE for 30 min (Figure 1(a)) or increasing periods of time with 5 μM 15s-HpETE (Figure 1(b)) at room temperature. After incubation, NEM was added to block free sulfhydryls to quench further reactions. After exposure of recombinant PTEN to 15s-HpETE, the faster migrating bands in nonreducing SDS-PAGE were increased compared to those in the absence of 15s-HpETE. We previously demonstrated that the faster migrating bands correspond to oxidized PTEN [30]. However, the appearance of PTEN was decreased by 15s-HpETE treatment in a concentration-dependent manner (Figure 1(a)). The loss of immunoblot signal might be due to the loss of sticky protein during oxidation or conformational changes of the PTEN molecule. The addition of DTT to oxidized PTEN (far right lane) resulted in a nearly complete recovery of immunoblot band intensity, suggesting that conformational alteration occurred. PTEN can form homodimers at the plasma membrane and in the solution [41, 42]. Homodimerized PTEN is in an active conformation and exerts lipid phosphatase capability on PIP3 [41]. 15s-HpETE-induced PTEN oxidation was increased with increasing incubation times (Figure 1(b)). These observed results showed that PTEN was oxidized by 15s-HpETE and that the thiol group might be involved in the oxidation because the PTEN oxidized by 15s-HpETE was reduced by DTT.

3.2. Reversible Oxidation of PTEN by 15s-HpETE in MEF Cells. To substantiate the findings obtained from recombinant protein, various cell lines, including C2C12, HeLa, and HT22 cells, were further treated with 15s-HpETE to analyze whether endogenous cellular PTEN was an oxidation target. Confluent monolayers of cells were rinsed with PBS and incubated in serum-free growth medium containing 10 μM

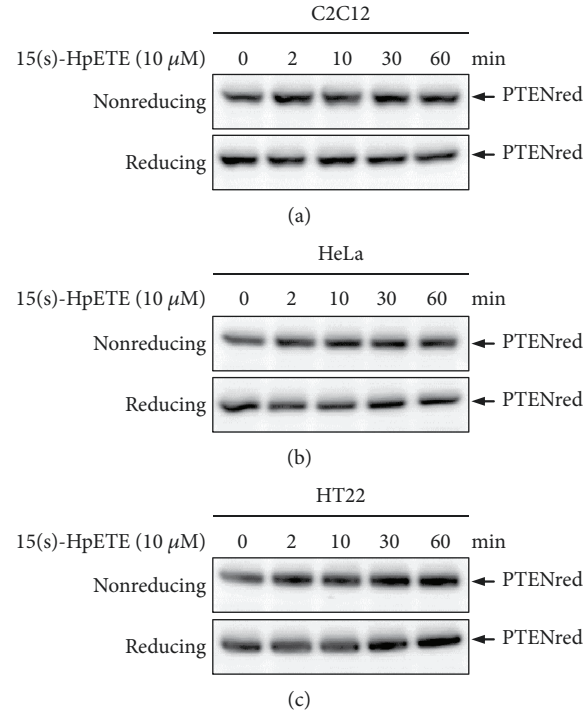

Figure 2: Effects of 15s-HpETE on the redox state of PTEN in C2C12, HeLa, and HT22 cells. The cells were treated with 10 μM 15s-HpETE for the indicated times. Cellular protein extracts were then alkylated with 10 mM NEM and subjected to nonreducing or reducing SDS-PAGE, followed by Western blot analysis using antibodies to PTEN.

15s-HpETE for increasing periods of time (0, 2, 10, 30, and 60 min). The redox status of PTEN was then monitored. Surprisingly, 15s-HpETE treatment was unable to induce PTEN oxidation in the tested cell lines (Figure 2). This was presumably due to the inability of 15s-HpETE to penetrate the cell membranes [9] because of the negative charge imparted by its carboxylate group and the phospholipid-like structure of the cell membrane. Even if a small amount of 15s-HpETE entered the cells, it might be scavenged and degraded rapidly by cellular antioxidants before exerting its effect.

We further used Lipofectamine transfection reagent to assist 15s-HpETE penetration in cell membranes. HeLa cells were treated with different ratios (v/v) of 10 μM 15s-HpETE and Lipofectamine reagent for 5 min, followed by Western blotting using the PTEN antibody. The data presented in Figure 3(a) shows that PTEN was rarely oxidized in the HeLa cells when treated with the mixture of 15s-HpETE and Lipofectamine reagent. We next tested the effects of 15s-HpETE on PTEN oxidation in MEF cells. As shown in Figure 3(a), more than 60% of PTEN was apparently oxidized, irrespective of the different ratios of 15s-HpETE and Lipofectamine reagent used. The observed changes in PTEN oxidation might come from injurious action of 15s-HpETE to cultured MEFs; therefore, we determined whether cell death occurred during treatment with the mixture of 10 μM 15s-HpETE and Lipofectamine reagent. The cytotoxic assay results revealed that 10 μM 15s-HpETE had a negligible effect on cell viability

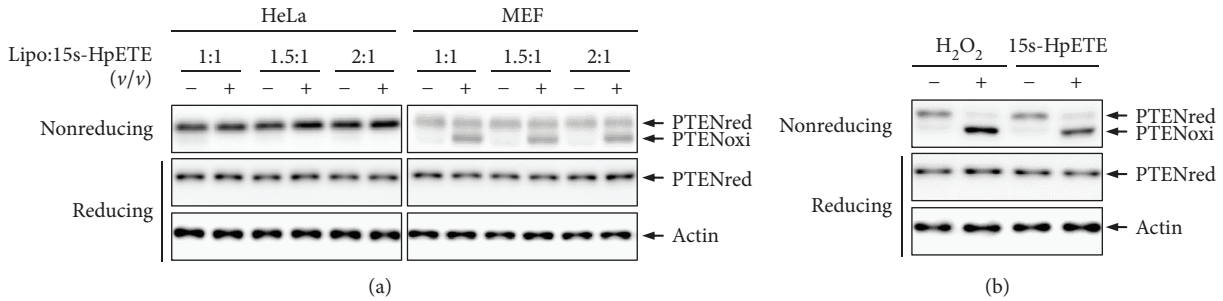

Figure 3: Effects of 15s-HpETE on the redox state of PTEN in HeLa and MEF cells. (a) HeLa and MEF cells were treated with different ratios (*v*/*v*) of 10 μM 15s-HpETE and Lipofectamine 2000 transfection reagent for 5 min. (b) MEFs were incubated with 1 mM H_2O_2 or the mixture of 10 μM 15s-HpETE and Lipofectamine (1 : 1 ratio) for 5 min. Cellular protein extracts were then alkylated with 10 mM NEM and subjected to nonreducing or reducing SDS-PAGE, followed by Western blot analysis using antibodies to PTEN or actin. "-" indicates Lipofectamine 2000 transfection reagent only; "+" indicates the mixture of 15s-HpETE and Lipofectamine 2000 transfection reagent or H_2O_2.

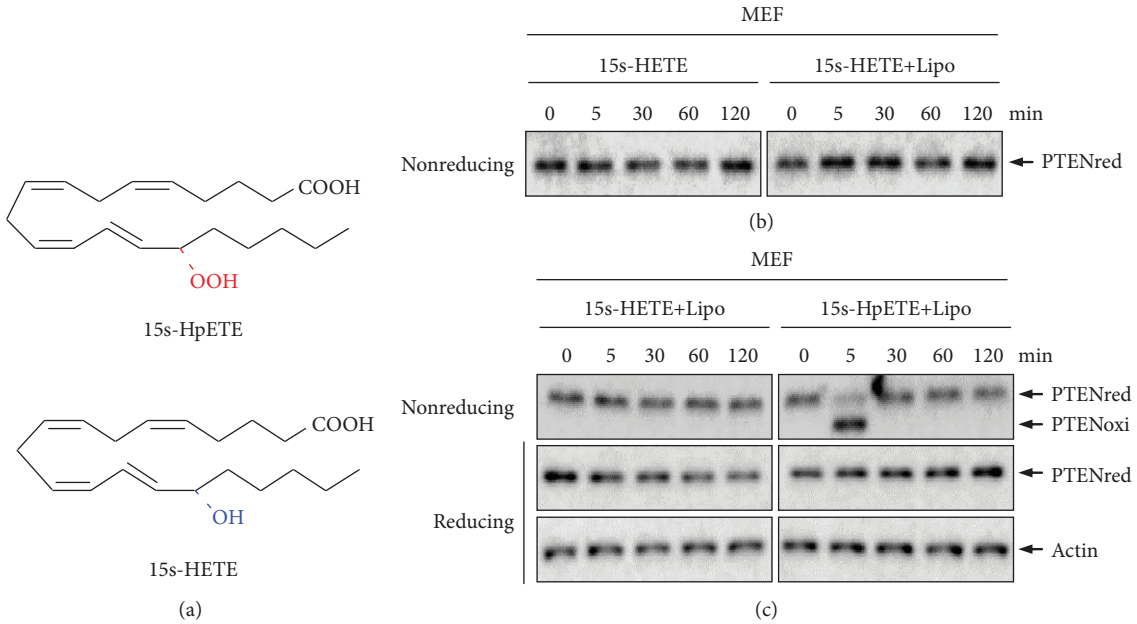

Figure 4: Effects of 15s-HETE and 15s-HpETE on the redox state of PTEN in MEF cells. (a) Chemical structures of 15s-HpETE and 15s-HETE. (b) MEF cells were treated with 10 μM 15s-HETE or the mixture of 10 μM 15s-HETE and Lipofectamine 2000 transfection reagent for the indicated times. (c) MEF cells were treated with the mixture of 10 μM 15s-HETE and Lipofectamine 2000 transfection reagent or the mixture of 10 μM 15s-HpETE and Lipofectamine 2000 transfection reagent for the indicated times. Cellular protein extracts were then alkylated with 10 mM NEM and subjected to nonreducing or reducing SDS-PAGE, followed by Western blot analysis using antibodies to PTEN or actin.

(data not shown). Therefore, a 1 : 1 *v*/*v* ratio of 10 μM 15s-HpETE and Lipofectamine reagent was used in further experiments. It is well established that PTEN is predominantly modified into the oxidized form, with an intramolecular disulfide bridge between Cys^{124} and Cys^{71} residues, upon treatment with H_2O_2. To substantiate whether an identical intramolecular disulfide bond of PTEN was formed after 15s-HpETE treatment, lysates from MEFs treated with H_2O_2 or the mixture of 15s-HpETE and Lipofectamine were fractionated on nonreducing gels and probed with PTEN antibody. Faster migrating bands, similar to those seen in our previous studies [30, 34, 36, 39], were detected following H_2O_2 and a mixture of 15s-HpETE and Lipofectamine treatment (Figure 3(b)).

15s-HpETE is short lived in cells and is rapidly reduced to 15s-HETE by glutathione peroxidase 4 (GPx 4) [43, 44]. Previous reports have suggested that exogenous organic peroxides and hydroperoxides caused irreversible oxidation of PTEN [34, 36]. We, therefore, sought to characterize whether endogenous eicosanoids 15s-HpETE and 15s-HETE exert the same effects on the redox regulation of PTEN. As was to be expected showing in Figure 4(b), exposure of MEF cells with 15s-HETE or to the mixture of 15s-HETE and Lipofectamine reagent was unable to induce PTEN oxidation. MEF cells were then treated with the mixture of 15s-HETE and Lipofectamine reagent or the mixture of 15s-HpETE and Lipofectamine reagent for the indicated time points (0, 5, 30, 60, and 120 min). As depicted in Figure 4(c), PTEN was

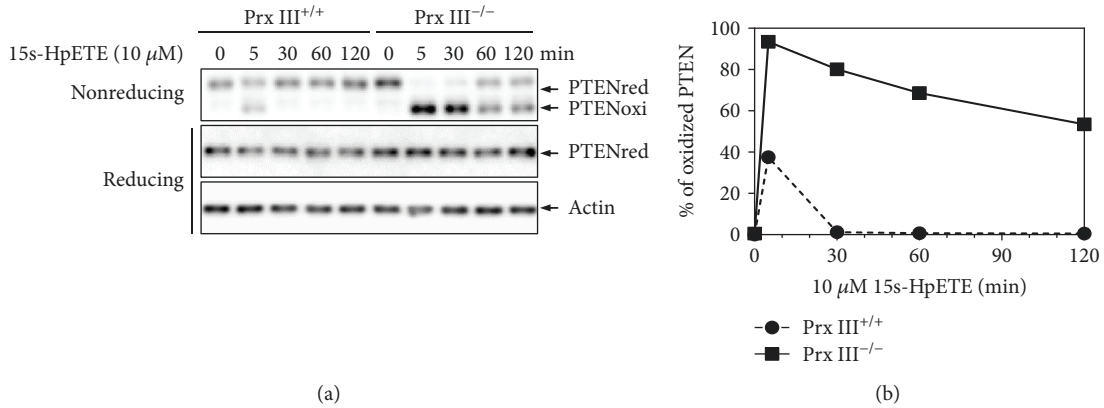

Figure 5: Effects of 15s-HpETE on the redox state of PTEN in Prx III$^{+/+}$ and Prx III$^{-/-}$ MEF cells. (a) MEF cells were treated with the mixture of 10 μM 15s-HpETE and Lipofectamine 2000 transfection reagent for the indicated times. Cellular protein extracts were alkylated with 10 mM NEM and subjected to nonreducing or reducing SDS-PAGE, followed by Western blot analysis using antibodies to PTEN or actin. (b) The intensity of oxidized PTEN was quantitated using ImageJ software.

oxidized by 15s-HpETE in MEFs at 5 min after treatment and the oxidized PTEN was completely converted to the reduced form by cellular antioxidants after 30 min of treatment. Peroxiredoxins (Prx) are a superfamily of small nonseleno peroxidases that catalyze the reduction of H_2O_2, organic hydroperoxides, and peroxynitrite. Prxs play critical roles in protecting cellular components from oxidative damage [45]. Treatment of Prx III$^{-/-}$ MEFs with 15s-HpETE enhanced PTEN oxidation at a higher level compared to that in Prx III$^{+/+}$ MEFs (Figures 5(a) and 5(b)). In Prx III$^{-/-}$ MEFs, approximately 90% of the PTEN was oxidized by 15s-HpETE at 5 min after incubation and the band intensity of oxidized PTEN decreased when the incubation time was extended, indicating that the oxidized PTEN was reduced by cellular antioxidants.

3.3. 15s-HpETE Inhibits Reduction of Oxidized PTEN by Inducing Trx Dimerization. Our previous studies revealed that oxidized PTEN induced by H_2O_2 was reversibly converted back to the reduced form by intracellular-reducing systems, predominantly by the Trx system. To test whether Trx was involved in the PTEN oxidation induced by 15s-HpETE, MEF cells were treated with the mixture of 10 μM 15s-HpETE and Lipofectamine for the indicated times and the Trx status was analyzed using nonreducing SDS-PAGE (Figure 6). Trx dimers started to accumulate in Prx III$^{+/+}$ MEFs after 5 min of incubation with 15s-HpETE. The dimeric forms protein completely converted to the monomeric forms after 30 min of incubation, which was reminiscent of the reduction kinetics of 15s-HpETE-oxidized PTEN. When Prx III$^{-/-}$ MEFs were challenged with 15s-HpETE, dimerization of Trx was evident within 60 min; then the dimerization converted to the monomeric form after 120 min of treatment. In addition, high-molecular weight proteins and its dimer cooccurred after incubation with 15s-HpETE. These high-molecular weight bands may represent the oligomeric forms of Trx. It has been reported that Trx is functional as a monomer in redox reactions [46]. The accumulation of dimeric and high-molecular weight

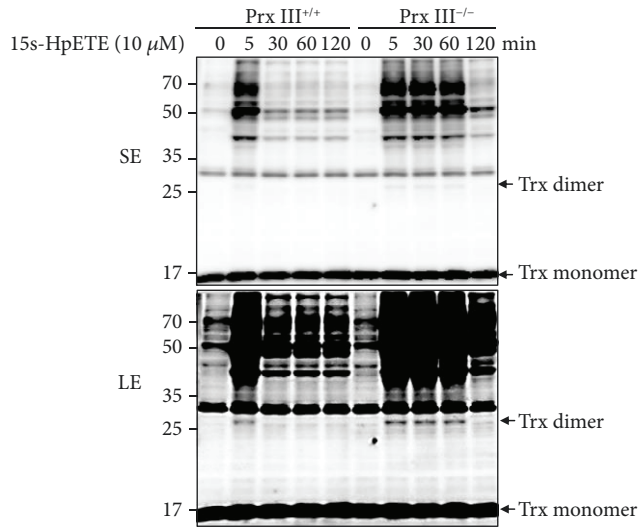

Figure 6: Effects of 15s-HpETE on the mobility of thioredoxin in Prx III$^{+/+}$ and Prx III$^{-/-}$ MEF cells. MEF cells were treated with the mixture of 10 μM 15s-HpETE and Lipofectamine 2000 transfection reagent for the indicated times. Cellular protein extracts were alkylated with 10 mM NEM and subjected to nonreducing SDS-PAGE, followed by Western blot analysis using an antibody to thioredoxin. Trx: thioredoxin; SE: shorter exposure; LE: longer exposure.

protein bands after incubation with 15s-HpETE could be attributed to impairment of the Trx-reducing system.

Based on these results, it could be concluded that endogenous lipid peroxide 15s-HpETE could inhibit tumor suppressor PTEN by reversible oxidation and simultaneous decrease of Trx activity by dimerization. In addition, Prx III played a critical role in protecting PTEN from lipid peroxide-induced oxidative inactivation.

4. Discussion

Our results demonstrated a previously unrecognized ability of endogenous lipid peroxides in the redox regulation of

tumor suppressor PTEN. Lipoxygenases can catalyze the production of HpETE from arachidonic acid. These HpETEs are subsequently reduced and transformed to produce eicosanoids, important signaling molecules in immune responses and other physiological processes. A recent study reported that active lipid hydroperoxides formed by lipoxygenases can lead to diseases through cellular oxidative damage [47]. Oxidative stress and carcinogenesis are closely related; however, their specific molecular targets, especially the underlying mechanism involved in the promotion of prooncogenic signaling pathways, are scarce. PTEN, a member of the protein tyrosine phosphatase (PTP) family, is involved in the regulation of various cellular processes. PTEN deficiency is a hallmark of a variety of human tumors [19, 48]. It is accompanied by increased cell proliferation, decreased cell apoptosis, and enhanced Akt activity. The PTEN redox status is intimately linked to its enzymatic activity. Reversible oxidation of its catalytic Cys^{124} after hydrogen peroxide treatment can lead to the formation of a disulfide bond with Cys^{71} and inactivation of PTEN's phosphatase activity. Exposure of recombinant PTEN to 15s-HpETE resulted in increases of faster migrating bands in nonreducing SDS-PAGE, and such effects were completely reversed by treatment with DTT. 15s-HpETE-mediated cellular PTEN oxidation was identical to oxidation by H_2O_2, suggesting that an identical intramolecular disulfide bond was formed after 15s-HpETE treatment. 15s-HpETE was unable to induce cellular PTEN oxidation in C2C12, HeLa, or HT22 cells. This might be due to the fact that 15s-HpETE cannot cross phospholipid bilayers or that it was scavenged by cellular antioxidants. Even with the assistance of Lipofectamine, oxidized PTEN was not observed after 5 min of 15s-HpETE treatment in HeLa cells. This might be attributable to abnormal lipid metabolism in the transformed cells and warrants further study. Treatment of MEF cells with the premixture of 15s-HpETE and Lipofectamine induced oxidation of endogenous PTEN, further suggesting that 15s-HpETE could specifically mediate PTEN-related cellular responses. It is noteworthy that the concentration of 15s-HpETE (10 μM) used to achieve substantial cellular PTEN oxidation was much higher than the physiological range [49]. Further studies should explore the intracellular levels of 15s-HpETE after treatment with a mixture of 15s-HpETE and Lipofectamine.

Prxs play key functions in the control of H_2O_2, organic hydroperoxides, and peroxynitrite reduction. Specifically, mitochondrial Prx III showed significant inhibition of PTEN oxidation induced by 15s-HpETE, suggesting that Prx III protected cells from oxidative damages induced by lipid peroxide. Human 15-LOX has two isoforms, 15-LOX-1 and 15-LOX-2. 15-LOX-1 is a dual-specificity enzyme that metabolizes AA principally to 15-HpETE and to far smaller amounts of 12-HpETE. 15-LOX-2 metabolizes AA to 15-HpETE and has little or no ability to metabolize AA to 12-HpETE. Mouse Alox15 metabolizes AA predominantly to 12-HpETE. We also investigated the effect of 12s-HpETE on the redox regulation of PTEN using MEF cells. 12s-HpETE showed a similar capacity of 15s-HpETE to oxidize and inactivate PTEN in MEFs (data not shown). Approximately 80% of PTEN was oxidized after 5 min of treatment with the mixture of 10 μM 12s-HpETE and Lipofectamine, and the oxidized protein was converted to the reduced form within 30 min. Prx III deficiency enhanced 12s-HpETE-induced PTEN oxidation, and the oxidized PTEN was completely reduced after 60 min of incubation. However, the significance and mechanisms involved in 12/15s-HpETE-mediated PTEN oxidation need to be further elucidated.

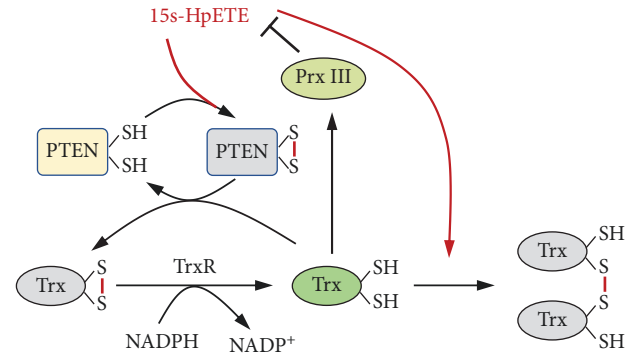

Figure 7: A schematic model of 15s-HpETE on the redox regulation of the tumor suppressor PTEN by the Trx system and Prx. Trx and Prx III play vital roles in the control of endogenous lipid peroxide-induced redox regulation of PTEN. 15s-HpETE inhibits the Trx redox system by inducing dimerization of Trx, resulting in the delayed reduction of oxidized PTEN and oxidized Prx. Prx III prevents 15s-HpETE-mediated PTEN oxidation by catalyzing the reduction of lipid peroxide. TrxR: thioredoxin reductase; Trx: thioredoxin; Prx: peroxiredoxin.

Oxidized PTEN is converted back to the reduced form by cellular-reducing agents, predominantly by the Trx system. The Trx system, which consists of Trx, NADPH, and TrxR, maintains redox homeostasis in cells by catalyzing the conversion of protein disulfide to dithiol. The oxidative stress-mediated dimerization of Trx results in inactivation of the Trx system [35]. We previously reported that dimers and oligomers of Trx were increased after longer exposure times to organic peroxides and hydroperoxides, leading to the irreversible oxidation of PTEN [34, 36]. In the present study, Trx dimerization was not observed after 5 min of incubation upon exposure to 15s-HpETE in Prx $III^{+/+}$ MEFs, consistent with PTEN oxidation. Trx dimerization was not observed after 60 min of incubation upon exposure to 15s-HpETE in Prx $III^{-/-}$ MEFs. Oxidized PTEN was not reduced after 120 min of incubation in Prx $III^{-/-}$ MEFs, indicating that Prx III might play a critical role in protecting PTEN, as well as the Trx system, from oxidation by lipid peroxide 15s-HpETE.

In this study, we showed that lipid peroxide 15s-HpETE could cause reversible oxidation of PTEN. Trx and Prx III were also involved in the 15s-HpETE-mediated redox regulation of PTEN (Figure 7). Taken together, our results unveil a new mechanism whereby lipid peroxides contribute to the etiology of tumorigenesis.

Data Availability

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

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Ying Zhang and Jiyoung Park contributed equally to this work.

Acknowledgments

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Research Article

Patients with Incompetent Valves in Chronic Venous Insufficiency Show Increased Systematic Lipid Peroxidation and Cellular Oxidative Stress Markers

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Chronic venous insufficiency (CVI) is a disease that impacts cellular homeostasis. CVI may occur with a valvular destruction process known as venous reflux or valvular incompetence. One of the cellular processes that may be triggered as a consequence of these events is the production of reactive oxygen species (ROS), which may trigger the production of different cellular markers and cell damage processes, such as lipid peroxidation. Therefore, the present study performed an observational, analytical, and prospective cohort study by reviewing 110 patients with CVI, and the activities and plasma levels of iNOS, eNOS, NOX1, and NOX2 were determined using immunohistochemistry and RT-qPCR. Lipid peroxidation (MDA) was also measured. Patients were distributed according to the presence or absence of valvular incompetence-venous reflux, which was diagnosed clinically as the absence of venous reflux (NR = 29) or presence of venous reflux (R = 81). Each group was divided according to age, with a cutoff point of fifty years (NR < 50 = 13, NR ≥ 50 = 16, R < 50 = 32, and R ≥ 50 = 49). The results showed that R patients exhibited significantly increased plasma MDA levels, and R < 50 patients exhibited the highest statistically significant increase. iNOS, NOX1, and NOX2 exhibited the highest gene and protein expression in R patients. The increased expression was maintained in the R < 50 patients. Our data suggest that young patients with valvular incompetence (venous reflux) show higher levels of lipid peroxidation and oxidative stress, which reflects the characteristics of an aged patient.

1. Introduction

Chronic venous insufficiency (CVI) is a disorder of the venous system that prevents the return of blood to the heart [1]. In general, IVC is not a serious pathology, but it occurs with a high incidence in the population [2, 3]. Currently available pharmacological treatments are not effective, and surgery is the treatment of choice when the disease progresses. In fact, these patients represent one of the most common consultations to vascular surgeons [4]. Different epidemiological studies performed worldwide reveal that CVI is a chronic pathology that occurs with high incidence

and prevalence in the population [5, 6]. One of the main risk factors for developing CVI is age because of the progressive deterioration of the venous wall and increased pressure at the level of the superficial venous system. Other factors that influence the development of CVI are gender, family history, ethnicity, number of pregnancies, obesity, and risk professions [7–11]. CVI is associated with a wide variety of signs and symptoms, but it seems likely that all of the symptoms are related to venous hypertension. Venous hypertension often occurs due to reflux caused by incompetent venous valves [12]. These valves decrease the venous pressure, which favors the return of blood to the heart, and tolerate high pressures for limited periods of time. Therefore, events that modify the structure of these valves will trigger valvular incompetence and generate a blood reflux that progressively increases the venous pressure in the leg [13].

Reactive oxygen species (ROS) are physiologically produced in a regulated manner from the incomplete reduction of oxygen in the vascular wall. An imbalance between the production of ROS and the antioxidant defense mechanisms creates an oxidative stress that produces lipid peroxidation, oxidation of DNA, RNA, protein, and the inactivation of some enzymes [14–16]. Numerous authors demonstrated that the roles of nitric oxide (NO) and nitric oxide synthase (NOS) in vascular diseases are prominent in ROS activity [1, 17, 18].

The present study examined the process of valvular incompetence (venous reflux) and measured the differential expression of cellular oxidative stress markers (iNOS, eNOS, NOX1, and NOX2) according to patient age and how these conditions change the profile of lipid peroxidation as quantified using malondialdehyde (MDA). The aim of this study is to demonstrate how the oxidative stress that occurs at the tissue level has systemic consequences in correlation with age.

2. Patients and Methods

2.1. Study Population. This study was an observational, analytical, and prospective cohort study that reviewed patients with chronic venous insufficiency. Patients were divided according to age (cutoff point at 50 years of age) and the presence (R) or absence (NR) of incompetent valves (venous reflux). There were a total of 110 patients [NR = 29, 51.51 ± 14.04 years (NR < 50 = 13, 38.53 ± 6.21 years, NR ≥ 16, 62.06 ± 8.54 years), R = 81, 50.09 ± 15.91 years (R < 50 = 32, 62.06 ± 8.54 years, R ≥ 49, 59.98 ± 11.81 years)]. The study cohort was selected according to the following criteria. *Inclusion criteria:* women and men diagnosed with CVI and with and without venous reflux in the great saphenous vein; BMI ≤ 25; informed consent signed; and commitment to follow-ups during the pre- and postoperative periods plus tissue sample collection. *Exclusion criteria:* patients with venous malformations or arterial insufficiency; patients who did not provide their clinical history; patients with pathology affecting the cardiovascular system (e.g., infectious diseases, diabetes, dyslipidemia, hypertension); patients with toxic habits; and patients who doubted that they could complete the full follow-up.

Each patient underwent an exploratory examination using a M-Turbo Eco-Doppler (SonoSite) transducer of 7.5 Mz. The examination of the lower limbs was performed in a standing position with the explored leg in external rotation and support on the contralateral leg. The examination included the greater saphenous axis from the inguinal region to the ankle and femoral vein. A distal compression maneuver was performed. Valsalva maneuvers were performed in the present study. Pathological reflux was considered when this was greater than 0.5 sec. NR patients had a compressive syndrome as the indication for surgery. Patients were classified according to CEAP international criteria [18].

Saphenectomy of the vein was produced, and the total of the arch of the greater saphenous vein was taken. These fragments were introduced into two different sterile tubes: one tube contained minimum essential medium (MEM) with 1% antibiotic/antimycotic (both from Thermo Fisher Scientific, Waltham, MA, USA) and the other tube contained RNAlater® solution (Ambion, Austin, TX, USA). Blood samples are taken from the study population via puncture of the superficial vein of the elbow fold, after placement of a tourniquet on the arm. One tube (Vacutest® Kima, Piove di Sacco, Italy) of blood sample was collected from each study subject. The tube contained heparin to obtain blood serum.

The present study was performed in accordance with the basic ethical principles, autonomy, beneficence, nonmaleficence, and distributive justice, and its development followed Good Clinical Practice standards and the principles enunciated in the last Declaration of Helsinki (2013) and the Convention of Oviedo (1997). Patients were duly informed, and each was asked to provide written informed consent.

2.2. RT-qPCR. RNA was extracted from the samples collected in RNAlater® using the guanidine-phenol-chloroform isothiocyanate method of Chomczynski and Sacchi (1987). RNA samples (50 ng/μl) were used to synthesize complementary DNA (cDNA) via reverse transcription. Each sample (4 μl) was mixed with 4 μl of an oligo-dT solution (15) 0.25 μg/μl (Thermo Fisher Scientific) and incubated at 65°C for 10 minutes in a dry bath (AccuBlock™, Labnet International, Inc., Edison, NJ, USA) to denature the RNA, following the protocol of Ortega et al. [3]. The amount of cDNA in each sample of the following genes of interest was quantified using qPCR. De novo primers or specific primers were designed for all of the genes studied (Table 1) using the Primer-BLAST online application [19] and AutoDimer [20]. The constitutively expressed genes of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to formalize the results. Gene expression was normalized using GAPDH as reference gene. The qPCR was performed in a StepOnePlus™ System (Thermo Fisher Scientific), and the relative standard curve method was used. For this, 5 μl of each sample was mixed 1/20 with 10 μl of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 1 μl of forward primer, 1 μl of reverse primer (reverse primer), and 3 μl of DNase and RNase-free water in a MicroAmp® 96-well plate (Thermo Fisher Scientific), for a total reaction volume of 20 μl. Fluorescence detection was performed at the end of each repetition cycle (amplification) and at each step of the dissociation curve. The data

Table 1: The primers used in RT-qPCR, the sequence, and the binding temperature (Temp).

Gene	Sequence fwd (5′→3′)	Sequence rev (5′→3′)	Temp
GADPH	GGA AGG TGA AGG TCG GAG TCA	GTC ATT GAT GGC AAC AAT ATC CAC T	60°C
eNOS	AAG AGG AAG GAG TCC AGT AAC ACA GA	ACG AGC AAA GGC GCA GAA	60°C
iNOS	CCT TAC GAG GCG AAG AAG GAC AG	CAG TTT GAG AGA GGA GGC TCC G	61°C
NOX1	GTT TTA CCG CTC CCA GCA GAA	GGA TGC CAT TCC AGG AGA GAG	55°C
NOX2	TCC GCA TCG TTG GGG ACT GGA	CCA AAG GGC CCA TCA ACC GCT	60°C

Table 2: Primary and secondary antibodies used in the immunohistochemical studies performed, showing the dilutions used and the specificities in their protocol.

Antigen	Species	Dilution	Provider	Protocol specifications
eNOS	Rabbit	1:100	Abcam (ab66127)	Citrate tampon in heat (pH = 6)
iNOS	Rabbit	1:500	Abcam (ab95866)	—
NOX1	Rabbit	1:250	Abcam (ab78016)	EDTA (pH = 9) before incubation with blocking solution
NOX2	Goat	1:500	Abcam (ab111175)	—
Anti-rabbit IgG	Mouse	1:1000	RG-96 (Sigma-Aldrich)	—
Anti-goat IgG	Mouse	1:100	A5420 (Sigma-Aldrich)	—

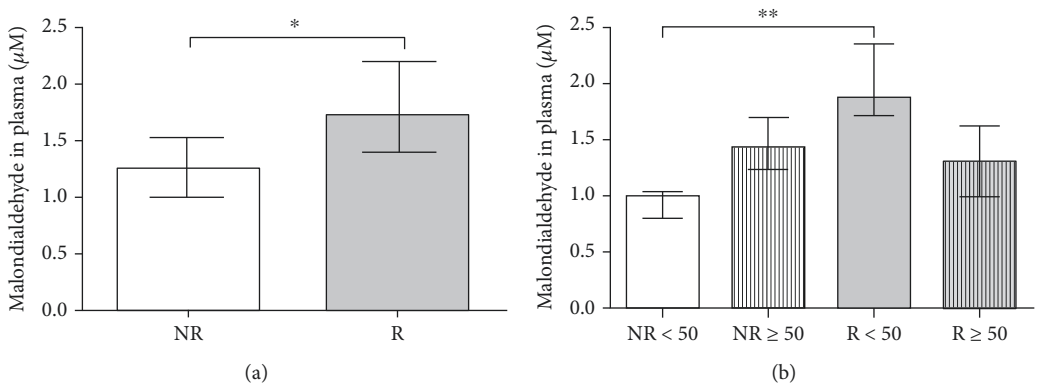


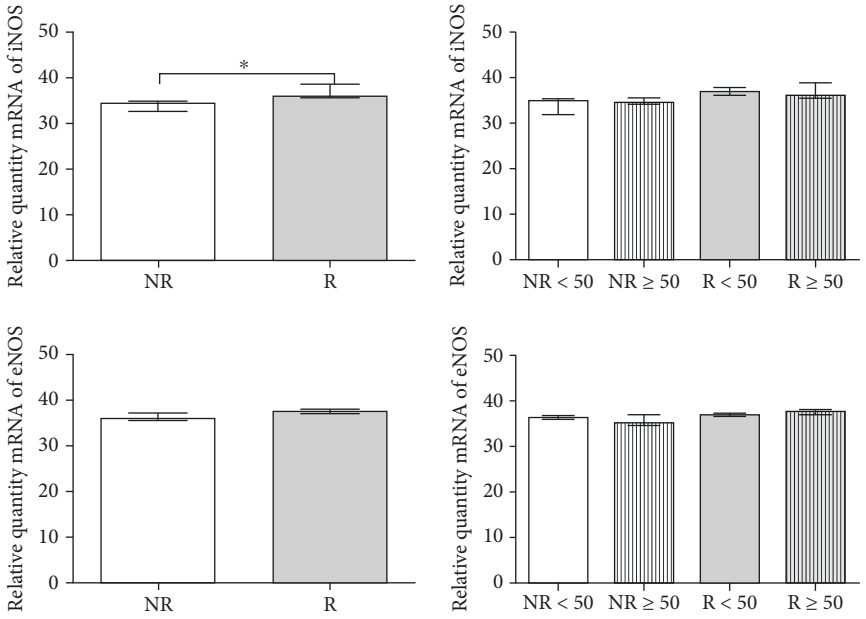
Figure 1: (a) Diagram showing the levels of malondialdehyde (MDA) in μM in the plasma of patients without reflux (NR) and with reflux (R). $*p < 0.05$. (b) Diagram showing the μM levels for malondialdehyde (MDA) in the plasma of patients without reflux less than fifty years of age (NR < 50), without reflux greater than or equal to fifty years of age (NR ≥ 50), with reflux less than fifty years of age (R < 50), and with reflux greater than or equal to fifty years of age (NR ≥ 50). $**p < 0.005$.

obtained from each gene were interpolated using a standard curve created from serial dilutions of a mixture of the study samples that was included in each plate. Results are expressed as arbitrary units. All tests were performed in duplicate.

2.3. Immunohistochemistry. Samples destined for immunohistochemical studies were processed using standardized protocols [3, 21]. Samples were embedded in paraffin and sectioned using a microtome into 5 μm thick sections. Sections were deparaffinized and hydrated. The different study molecules were detected using commercial primary and secondary antibodies (Table 2). Sections of the same tissue were used as negative controls in all immunohistochemical studies, in which the primary antibody was replaced with blocking solution. Detection of the antigen-antibody reaction was performed using the ABC method (avidin-biotin complex) (DAB Kit, SK-4100, Vector, Burlingame, CA, USA), which used the chromogen avidin-peroxidase ExtrAvidin®-Peroxidase (Sigma-Aldrich, St. Louis, MO, USA) at a 1 : 200 dilution in PBS.

Histological samples of the patients were stratified as negative (0) or positive (1). For each of the patients of the established groups, 5 sections and 5 random fields per section were examined. Patients were described as positive when the average of the test sample marked for each study subject was greater than or equal to 5% of the total [22].

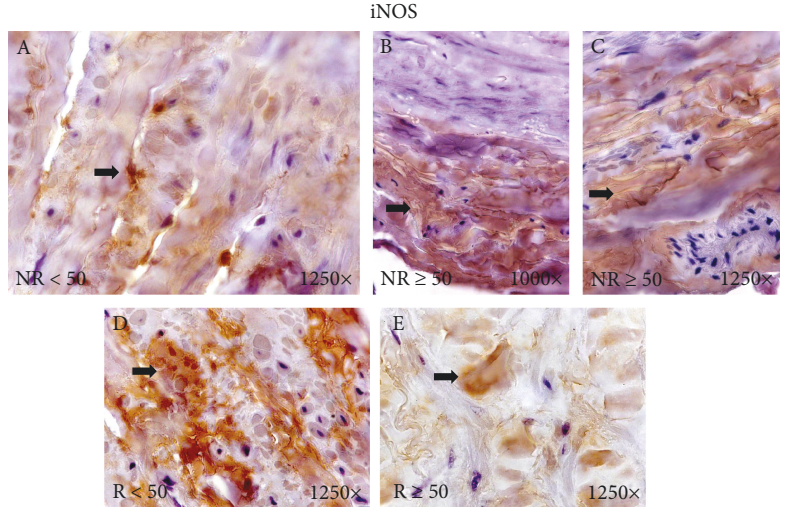
2.4. Oxidative Stress Determination. MDA production is proportional to polyunsaturated fatty acid degradation of lipid peroxidation. Therefore, MDA concentration was measured to determine the oxidative stress in patient plasma. The lipid peroxidation assay kit (ab118970) is a suitable method for the sensitive detection of the malondialdehyde of the sample. The MDA present in the sample reacts with thiobarbituric acid (TBA) to generate an MDA-TBA adduct, which



(a)

% positive expression (n)					
	iNOS			eNOS	
NR	34.48 (10)	NR < 50	15.38 (2)	44.83 (13)	15.38 (2)
		NR ≥ 50	50.00 (8)		68.75 (11)
R	48.15 (39)	R < 50	84.37 (27)	87.65 (71)	90.62 (29)
		R ≥ 50	24.49 (12)		42.86 (21)

(b)



(c)

Figure 2: Continued.

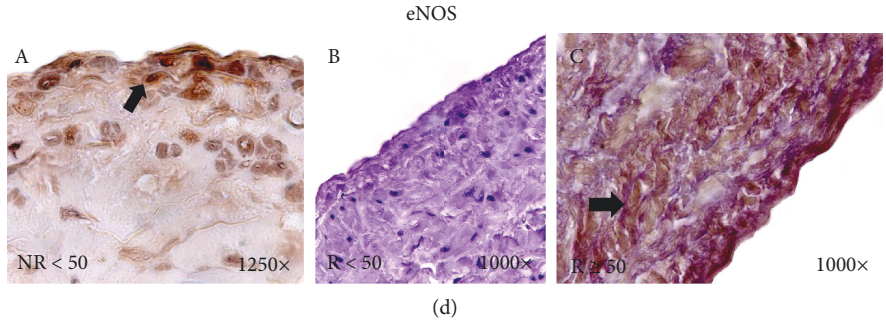


Figure 2: (a) Levels of mRNA for iNOS and eNOS quantified using RT-qPCR in patients without reflux (NR) and with reflux (R) and by their ages. Results are expressed as arbitrary units. $*p < 0.05$. (b) Distribution of the percentage of patients with positive protein expression for iNOS and eNOS in patients without reflux (NR) and with reflux (R) and by age, n = number of patients. (c) Protein expression images of iNOS in NR < 50 (A), NR ≥ 50 (B, C), R < 50 (D), and R ≥ 50 (E) patients. (d) Protein expression images of eNOS in NR < 50 (A), R < 50 (B), and R ≥ 50 (C) patients. The arrows are the brown coloration indicating the specific precipitate that correlates with the expression of the said protein.

is easily quantified using colorimetry. The sensitivity of this method was 0.1 nmol MDA/well.

2.5. Statistical Analysis. GraphPad Prism 5.1 software was used for statistical analyses, and the Mann-Whitney U test was used. Data are expressed as the means ± standard deviation. The significance was set at $p < 0.05$ (∗), $p < 0.005$ (∗∗), and $p < 0.001$ (∗∗∗).

3. Results

3.1. Study of Lipid Peroxidation Levels: Malondialdehyde. Lipid peroxidation levels were determined using malondialdehyde levels in the plasma of the study cohort. Patients with venous reflux (R) exhibited a significant increase compared 1to the NR subjects ($p < 0.05$) (Figure 1(a)). The mean malondialdehyde levels were $1.306 \pm 0.116\,\mu$M in nonreflux patients and $1.745 \pm 0.142\,\mu$M in patients with reflux. A clear differential distribution was found in relation to the age factor, which significantly increased the levels of malondialdehyde in R < 50 patients compared to NR < 50 patients ($0.952 \pm 0.067\,\mu$M, NR < 50 versus $1.966 \pm 0.142\,\mu$M, R < 50), $p < 0.005$ (Figure 1(b)). No significant differences were observed between groups greater than or equal to fifty years of age ($1.508 \pm 0.124\,\mu$M, NR ≥ 50 versus $1.303 \pm 0.175\,\mu$M, R ≥ 50).

3.2. iNOS and eNOS. The gene expression measure of iNOS was 34.168 ± 1.424 in NR versus 36.665 ± 2.314 in R, which was significantly different ($p < 0.05$). The distributions of gene expression by age were 34.209 ± 2.113 in NR < 50 versus 34.127 ± 0.773 in NR ≥ 50 and 36.536 ± 1.977 in R < 50 versus 36.730 ± 2.758 in R ≥ 50. No significant differences in eNOS were obtained (36.090 ± 1.164 in NR versus 37.703 ± 0.889 in R) (Figure 2(a)).

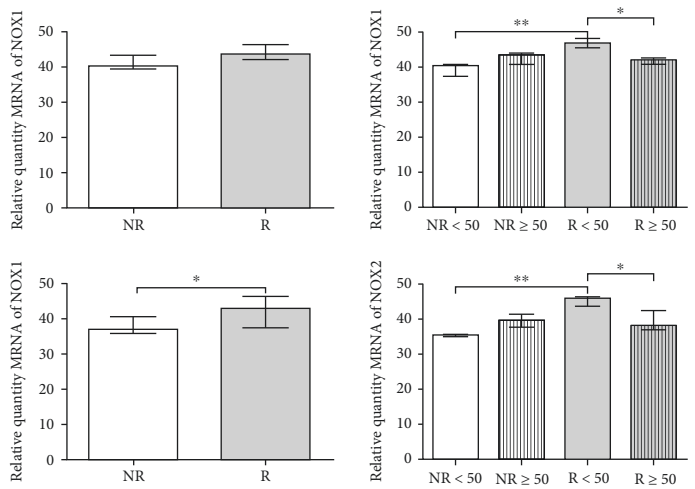
The study patients exhibited differential protein expression of iNOS and eNOS (Figure 2(b)). These markers represented 34.48% and 44.83% in NR patients, respectively. These values were 48.15% and 61.73%, respectively, in R patients. There was a marked increase in the number of R patients who exhibited positive protein expression.

When the age factor was considered, the values of iNOS were 15.38% in NR < 50 and 50.00% in NR ≥ 50 patients. These values were 84.37% for R < 50 and 24.49% for R ≥ 50 patients. The expression of eNOS was 15.38% in NR < 50 and 68.75% in R ≥ 50. At reflux, eNOS was 90.62% in R < 50 compared to 42.86% in R ≥ 50. These results show that NR ≥ 50 and R < 50 patients exhibited the highest percentage of positive expression for iNOS and eNOS.

iNOS expression showed that marker differences were established in the different layers of the human vein according to patient age (Figure 2(c)). iNOS protein was clustered in the three tunicas of NR patients. However, NR ≥ 50 patients exhibited a greater intensity of protein expression that was located more intensely in the adventitial tunica (Figure 2(c), B and C). NR < 50 patients exhibited large accumulations along the entire length of the vein wall, which was very intense in the middle tunica (Figure 2(c), D and C). The expression of eNOS was differentially maintained in the endothelium of NR < 50 patients, and it was especially intense in the adventitial tunica of R ≥ 50 patients (Figure 2(d), A–C).

3.3. NOX1 and NOX2. NOX1 gene expression analysis did not reveal any significant differences between the study groups (40.704 ± 2.534 in NR versus 43.943 ± 2.842 in R). The distributions of gene expression by age were 38.942 ± 1.792 in NR < 50 versus 42.465 ± 1.879 in NR ≥ 50 and 46.408 ± 1.140 in R < 50 versus 41.479 ± 0.821 in R ≥ 50 ($p < 0.05$). Statistically significant differences were established between NR < 50 and R < 50 patients ($p < 0.005$) (Figure 3(a)).

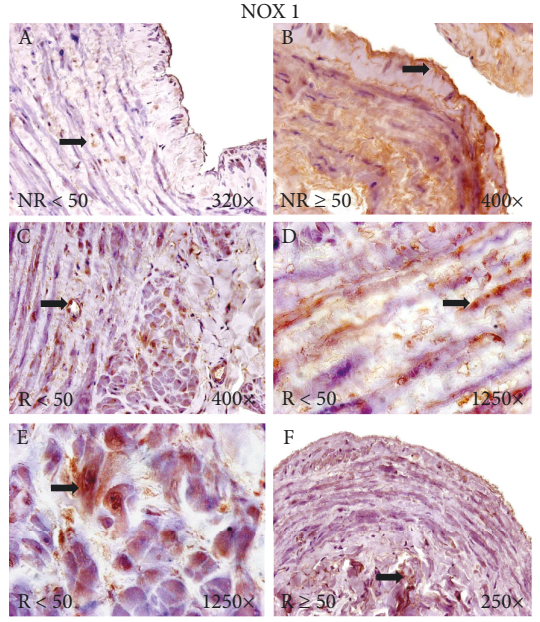
An increase in NOX2 gene expression was observed in patients with venous reflux (37.686 ± 2.643 in NR versus 42.015 ± 4.011 in R) ($p < 0.05$). The distributions of gene expression by age were 35.022 ± 0.296 in NR < 50 versus 39.018 ± 2.125 in NR ≥ 50 and 45.136 ± 1.582 in R < 50 versus 38.894 ± 2.015 in R ≥ 50 ($*p < 0.05$) (Figure 3(a)).



(a)

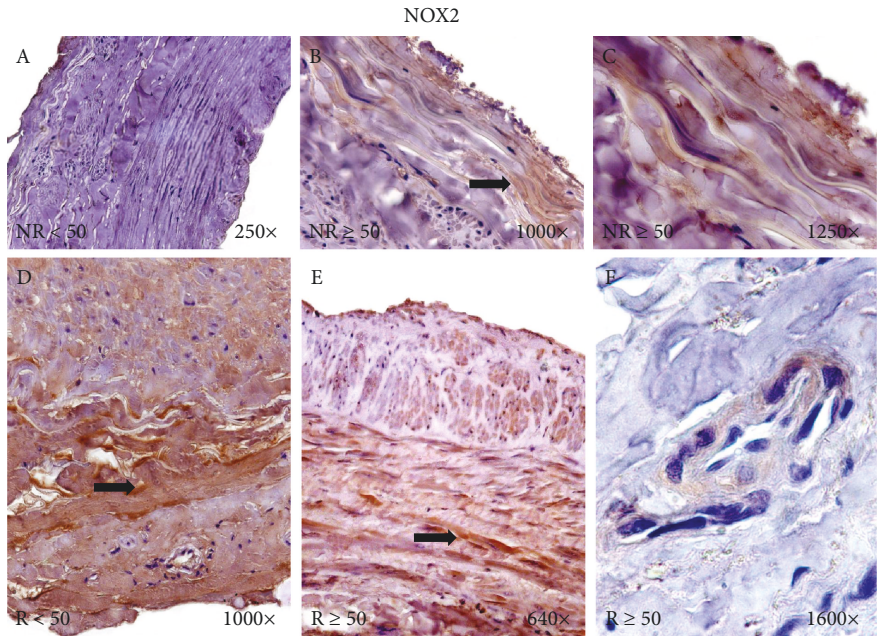
			% positive expression (*n*)			
	NOX1			NOX2		
NR	58.62 (17)	NR < 50	30.77 (4)	44.83 (13)	NR < 50	23.08 (3)
		NR ≥ 50	81.25 (13)		NR ≥ 50	62,50 (10)
R	85.18 (69)	R < 50	93.75 (30)	87.65 (71)	R < 50	96.87 (31)
		R ≥ 50	79.59 (39)		R ≥ 50	81.63 (40)

(b)



(c)

Figure 3: Continued.



(d)

Figure 3: (a) RNA levels of NOX1 and NOX2 quantified using RT-qPCR in patients without reflux (NR) and with reflux (R) and by the ages of the same groups. Results are expressed as arbitrary units. $*p < 0.05$, $**p < 0.005$. (b) Distribution of the percentage of patients with positive protein expression for NOX1 and NOX2 in patients without reflux (NR) and with reflux (R), and by age, n = number of patients. (c) Protein expression images of NOX1 in NR < 50 (A), NR ≥ 50 (B), R < 50 (C, D, E), and R ≥ 50 (F) patients. (d) Protein expression images of NOX2 in NR < 50 (A), NR ≥ 50 (B, C), R < 50 (D), and R ≥ 50 (E, F) patients. The arrows are the brown coloration indicating the specific precipitate that correlates with the expression of the said protein.

NOX1 and NOX2 proteins were differentially expressed in the established groups. For NOX1 expression, 58.62% of NR patients were positive compared to 85.18% of R patients. NOX2 expression was positive with 44.83% and 87.65%, respectively (Figure 3(b)). When studying the distribution of this expression as a function of age, it was observed that R < 50 patients exhibited the highest percentages of NOX1 and NOX2 (93.75% and 96.87%, respectively).

The study of the distribution of expression in the different layers of the human vein revealed important data on histological compression. NR ≥ 50 and R < 50 patients exhibited higher NOX1 protein expression in the intima, media, and adventitia layers of the human vein, and these differences were statistically significant (Figure 3(c), A–F).

NOX2 protein expression was increased in R patients compared to NR patients in the intima, media, and adventitia layers of the vein. R < 50 patients showed a greater intensity of expression in the three tunicas of the venous wall (Figure 3(d), A–F).

4. Discussion

The multitude of mechanisms involved in the progression of CVI made it difficult for the scientific community to identify the factors that trigger this disease. Some studies related reflux with weakening of the venous walls [23], which may be due to an imbalance in the content of collagen and elastin in the vein [24]. Other studies focused on chronic inflammation as the main factor for the onset of the pathology [25]. Krzysciak and Kózka [26] showed that oxidative stress increased the risk of damage to the vascular endothelial wall and DNA and caused a remodeling of the tissue and the consequent progression of the pathology. Therefore, one of the events involved in valvular incompetence is oxidative stress.

Krzysciak and Kózka [26] mentioned that ROS promotes reflux that generates a hypoxic environment in endothelial cells. These events favor the adhesion of leukocytes and other inflammatory mediators that release angiotensin II, which exerts a vasoconstrictive action directly on the smooth muscle and is capable of increasing the expression of growth factors, matrix metalloproteinases (MMPs), and collagen [1, 27]. Overexpression of MMPs was also observed in fibroblasts, endothelial cells, and smooth muscle cells in patients with CVI [28]. Therefore, an alteration in cell balance may cause degenerative damage that compromises cell structure, the content of collagen and elastin, and the contraction and relaxation properties of the smooth muscle of the venous wall [29].

Therefore, ROS plays a decisive role in the progression of chronic venous insufficiency. Our results showed that R < 50 patients exhibited the highest concentrations of MDA in plasma. Krzysciak and Kózka [26] measured MDA concentrations in samples of saphenous veins of patients with CVI before and after development of the disease. These results showed a relationship between oxidative stress and chronic venous insufficiency at the tissue level and the systemic level beginning in the first years of the disease. Mikuła-Pietrasik et al. [30] showed that the sera of varicose

patients increased cell proliferation, expression of the senescence marker SA-β-Gal, and ROS production in the endothelial cells of human umbilical veins (HUVECs) compared to the sera of healthy individuals. This result suggests that the presence of oxidative stress at a systemic level is the main factor triggering the progression of the pathology.

Angiotensin II also activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and enhances the production of superoxide anion (O_2^-) due to endothelium wall stress-dependent stimulation [26]. In addition to being a vasoconstrictor substance, it promotes inflammation, hypertrophy, and fibrosis, and it is implicated in vascular damage and remodeling in cardiovascular diseases [31]. A recent study by Zhang et al. [31] showed that an increase in the expression of NOX1 and NOX2 occurred after the stimulation with angiotensin II in HUVECs. Our results showed the event of oxidative stress in relation to NOX1 and NOX2 and the existence of a differential expression based on the age of the patients. These results should make us consider the implication of an accelerated aging process that leads to greater oxidative and inflammatory stress in the valvular incompetence (venous reflux). In fact, numerous authors noted the correlation of oxidative stress with age, but an accelerated aging process was not mentioned in young patients [22, 32]. On the other hand, we wanted to further develop the implications of iNOS and eNOS in chronic venous insufficiency because many authors mentioned the role that these molecules play in vascular diseases [33].

eNOS is expressed primarily in endothelial cells. Therefore, our immunohistochemistry images of the low expression of eNOS in the tunica intima of the veins of patients with reflux stand out compared to patients without reflux. By providing a baseline level of NO in the vein and neutralizing ROS, it makes sense that patients with low eNOS expression are more susceptible to endothelial deterioration and develop valvular incompetence (venous reflux). The low expression of eNOS may be related to CVI and any disease in which the mechanism involves endothelium dysfunction, as indicated by Mikuła-Pietrasik et al. [30]. However, the expression of eNOS in the tunica adventitia suggests that it is reactive and remains functionally active. Our studies found differences in the iNOS isoform in the adventitia and middle vein tunicas. NR $\geq$ 50 patients tended to exhibit an increase in iNOS expression in the adventitia tunica, likely in response to age-induced stress. Notably, the expression of iNOS in patients with reflux never reached the expression detected in NR $\geq$ 50 patients, despite the oxidative stress generated in these patients. The low expression of eNOS and iNOS decreases the bioavailability of NO in the vein, which makes it more susceptible to oxidative stress. However, the increase in iNOS expression is related to other cardiovascular pathologies [34]. The decrease in the expression of iNOS and eNOS suggests the existence of a suppressive mechanism of expression, perhaps at the level of protein transcription because both proteins are encoded by different genes but share a 50-60% homology in amino acid sequence [35]. Our results support a role for oxidative stress as a mechanism involved in the development of valvular incompetence (venous reflux) in CVI. The present study showed the existence of an oxidative environment in human veins with chronic venous insufficiency and how the different molecular components that participate in CVI were differentially expressed in correlation with the age of the patients. Our study presents some limitations, since to observe the tissue response it would be necessary to develop *in vitro* experiments of the endothelial and muscle cells of the saphenous vein. In this line, another limitation of our study is to observe if this profile of protein and gene expression is the same in other venous territories of the lower limb. However, our study is the first to show how valvular incompetence has important consequences and there is a different profile depending on age.

The importance of this study lies in demonstrating how venous disease produces a tissue change with systemic consequences. Venous disease is a common pathology in the general population that produces great disabilities, knowing its pathophysiology and its systemic consequences will help the development of specific therapies. Future studies should be aimed at discovering possible therapeutic targets at the tissue level that prevent systemic change and its consequences.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Melchor Álavrez-Mon and Julia Buján shared senior authorship in this work.

Acknowledgments

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Review Article

Features of Oxidative and Nitrosative Metabolism in Lung Diseases

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Respiratory diseases are accompanied by intensification of free radical processes at different levels of the biological body organization. Simultaneous stress and suppression of various parts of antioxidant protection lead to the development of oxidative stress (OS) and nitrosative stress (NS). The basic mechanisms of initiation and development of the OS and NS in pulmonary pathology are considered. The antioxidant defense system of the respiratory tract is characterized. The results of the NS and OS marker study in various respiratory diseases are presented. It is shown that NS and OS are multilevel complex-regulated processes, existing and developing in inseparable connection with a number of physiological and pathophysiological processes. The study of NS and OS mechanisms contributes to the improvement of the quality of diagnosis and the development of therapeutic agents that act on different pathogenetic stages of the disease.

1. Introduction

Most diseases of the respiratory tract (RT) are accompanied by the intensification of free radical processes at different levels of the biological organization of the body with simultaneous stress and subsequent oppression of various links of antioxidant protection, which leads to the development of oxidative stress (OS)—imbalance in the reactive oxygen species (ROS) and antioxidant protection of the body [1–3].

Over the past decade, much attention has been paid to studying the molecular mechanisms of the development of both oxidative stress and nitrosative stress (NS) in lung diseases, as well as the identification of prognostic and diagnostic markers in various biological media and the elucidation of the possibilities of therapeutic influence on OS and NS. These processes are inherently associated with the development and course of inflammatory and other physiological and pathophysiological mechanisms that are pathogenetic links in the development of the disease. The initiation of OS and NS can occur by an exogenous and/or endogenous pathway [2, 4–7].

2. Activation of Oxidative and Nitrosative Stress in the Respiratory Tract

For the respiratory tract, the exogenous pathway of the OS and NS initiation is the most relevant. So, about 8000 liters of air containing various gases (oxygen and volatile oxides), infectious agents (bacteria, viruses, and fungi), pollutants, and allergens, which have prooxidant effects, passes through the lungs every day. The main air pollutants of the urban atmosphere are particulate matters (PM), which are a variable composition of organic and inorganic compounds with a carbon core. OS-induced air pollutants and damage to the

respiratory tract occur with the participation of metals of variable valency, trace amounts of which are part of PM. In addition to the initiation of the OS and NS by prooxidants, free radicals can be also in significant amounts in the inhaled air. The gas phase of tobacco smoke contains about 1015 free radicals in one puff, including superoxide anion and hydroxyl radicals. Among the exogenous factors of the OS initiation, short-wave electromagnetic radiation (UV, X-rays, etc.) should also be considered [8, 9].

The endogenous pathway of the OS and NS initiation is represented by a wide variety of mechanisms. The redox reactions accompany a huge number of biochemical processes in vivo. One of the main intracellular sources of free radicals is mitochondrial respiration: 1-2% of electrons can "leak" from the respiratory chain [9]. Radicals and other highly active oxidants are formed in various ways. There are so-called primary radicals which are formed by an enzymatic way: superoxide anion radical and nitric oxide. These radicals give rise to such two pools of highly active groups of molecules as reactive oxygen species (ROS) and reactive nitrogen species (RNS).

The division into ROS and RNS is rather conditional since, in biochemical processes, the radical and nonradical forms of these compounds react with each other. Primary radicals, interacting with various compounds from their microenvironment, form secondary radical, tertiary radical, and so on; highly active nonradical forms; and stable products (Figure 1). ROS includes superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), peroxyl radical ($HO_2^{\cdot}$), and alkoxy radical (RO). During the reaction, ROS derivatives are formed, such as hydrogen peroxide (H_2O_2) and lipoperoxides (ROOH). RNSs include nitric oxide (NO), other higher nitrogen oxides, nitrites, and peroxynitrite ($ONOO^{-}$). Oxidases are involved in the generation of superoxide anion radical: NADPH oxidase, xanthine oxidase, cytochrome P-450 oxidase, etc. [2, 10]. The formation of NO occurs with the help of NO synthase enzymes (NOS) in the NO cycle and with the participation of nitrite/nitrate reductase systems [11].

The physiological role of NO in the respiratory tract (Figure 2) is also widely known. It includes regulation of the basal tone and vascular permeability, modulation of bronchial reactivity, and antimicrobial protection. NO is able to regulate the secretion of bronchial mucus produced by glands located in the submucosal layer of the bronchi. Nagaki et al. studied the effect of inhibitors of NO-synthase L-NAME and L-NMMA on the secretion of mucin glycoproteins by determining glycoconjugates precipitated with trichloroacetic acid in the explants and isolated human submucosal glands [12]. NO synthase inhibitors have been shown to have no direct effect on the secretion of glycoproteins, suppressing the secretion of methacholine and bradykinin in isolated glands. In addition, isosorbide dinitrate, as a source of NO, contributed to a significant increase in mucin secretion. The results of this study suggest a stimulating effect of endogenous NO on the mucin production by the submucosal glands of the respiratory tract.

NO synthase inhibitors slow down the frequency of ciliary beats of respiratory epithelial cells of cows stimulated

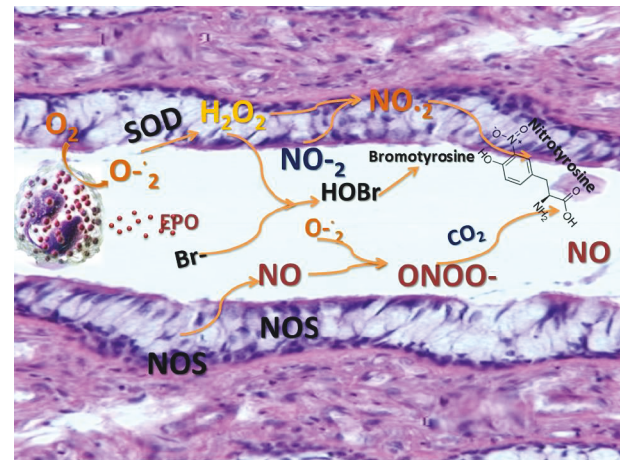

Figure 1: ROS and RNS formation in the respiratory tract.

with isoproterenol, bradykinin, and substance P. This effect is completely reversible when adding the precursor of NO L-arginine, which indicates the NO-dependent mechanism of ciliary motor stimulation by the above-named compounds. Ciliary motility is also activated by TNFα and IL-1β, produced by alveolar macrophages (inducible NO synthase pathway). This stimulating effect is blocked by L-NMMA and restored with the addition of L-arginine, confirming the regulatory role of inducible NO-synthase in its implementation [12].

In addition to the activity of ciliary motility, the effectiveness of mucociliary clearance is determined by the properties of the airway surface liquid (ASL), the composition and volume of which, in turn, depends on the transport of electrolytes. The functional activity of ion channels is also highly susceptible to the modulating effect of NO. The NO molecule activates both the apical anion channels and the basolateral potassium channels along the cGMP-dependent pathway, acting as a physiological regulator of transepithelial ion exchange [12, 13].

The ability of endogenous NO to modulate bronchial hyperreactivity (BHR) induced by various mediators has been experimentally confirmed. Nijkamp et al. revealed histamine-induced bronchoconstriction in guinea pigs by the inhibition of NO synthase in vivo, as well as a dose-dependent reduction under the action of histamine of the smooth muscles of the tracheal tube of the guinea pig in vitro [13]. Ricciardolo et al. showed NO-dependent regulation of bronchoconstriction induced by bradykinin, citric acid, selective NK1 tachykinin agonist, and protease-activated receptor 2 in guinea pigs [14, 15].

Intraluminal perfusion of the intact tracheal tube of guinea pigs with bradykinin, endothelin-1, substance P, adenosine, and calcitonin with a gene-bound protein led to dose-dependent relaxation [14, 15]. At the same time, the addition of the NO synthase inhibitor was accompanied by a tracheal tube contraction, which confirms the NO-dependent mechanism of respiratory tract relaxation. The same effect was reproduced by removing the respiratory epithelium. Consequently, the respiratory epithelium is the main source of endogenous NO, which prevents

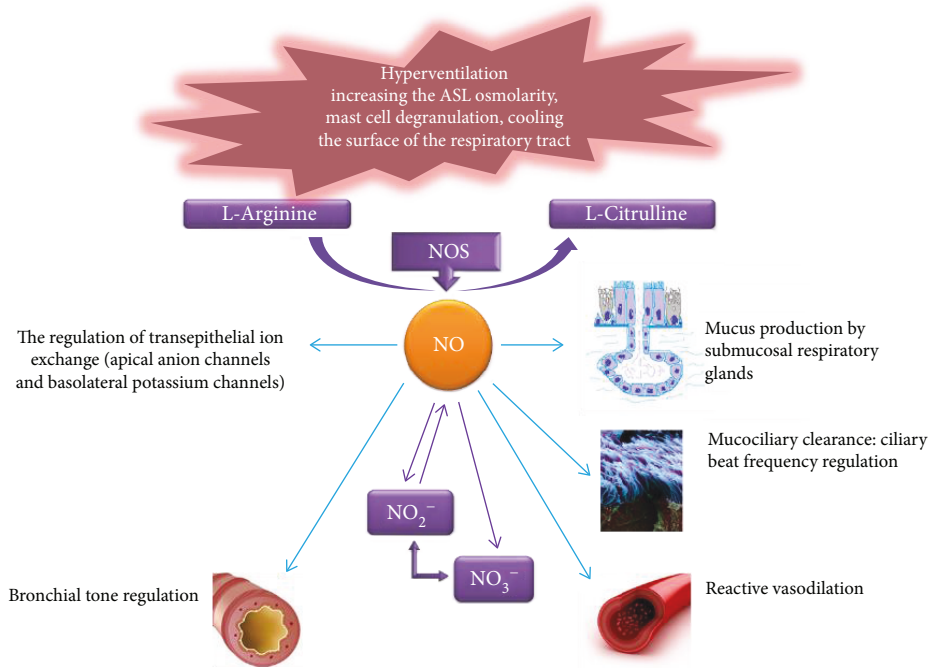

Figure 2: Bronchoprotective NO properties. ASL: airway surface liquid.

bronchoconstriction under the influence of various triggers. The results of the study emphasize the significant role of the respiratory epithelium in the BHR regulation. It is not just a physiological barrier between bronchoconstrictive stimuli and smooth myocytes but also a modulator of the bronchial tone through the release of epithelial relaxation factors.

Further research demonstrated a rapid (within 2 seconds) release of NO in the respiratory epithelium of guinea pigs induced by bradykinin. This phenomenon was absent in the subepithelial layer, free from calcium ions [14, 15]. Therefore, the endogenous bronchoprotective NO release occurs with the participation of calcium-dependent constitutive NO synthase.

An additional mechanism NO bronchoprotective effect in the airways is the cGMP-dependent property of smooth bronchial myocytes. Thus, bradykinin-induced increase in cGMP production in the guinea pig airways was demonstrated. This effect was blocked by the addition of NO synthase inhibitors, which indicates the role of cGMP as a final mediator of NO-dependent epithelial bronchoprotection [14, 15].

In vitro and in vivo studies have shown that BHR, caused by allergen exposure, does not increase with the preliminary addition of NO synthase inhibitors. The BHR-induced virus is completely blocked during exposure to L-arginine, which demonstrates the interrelation of this syndrome with the lack of endogenous NO. It has also been established that a deficiency in constitutive NO synthase production in guinea pigs leads to the BHR progression as part of an early allergic reaction (4-6 hours after allergen exposure), and the restoration of the NO level with inducible NO synthase contributes to the reverse BHR development during later timing (24-48 hours). These conclusions were made based on ineffective inhalation of a specific inhibitor of inducible NO synthase aminoguanidine on histamine-induced BHR after an early allergic reaction and significant BHR decrease after inhalation during the late allergic response [14, 15].

Moreover, Toward and Broadley found that lipopolysaccharide inhalation by guinea pigs suppressed respiratory NO production, which followed by an increase in histamine-induced hyperreactivity (one hour after exposure). 48 hours after inhalation, BHR to histamine decreased simultaneously with an increase of NO metabolites in bronchoalveolar lavage, suggesting a resumption of NO synthesis when activating the NF-κB-dependent expression of the inducible NO synthase [16].

The above NO-dependent mechanisms of bronchodilation, activation of mucociliary clearance, and bronchoprotective properties of NO become crucial during exercise with increase minute ventilation.

In addition to direct registration in exhaled air, the production of NO in RT can be determined from the concentration of its more stable metabolites, such as nitrate and nitrite anions, 3-nitrotyrosine, and nitrosothiols in the exhaled breath condensate (EBC). Nitrate and nitrite anions are the most stable of these metabolites. The metabolism of nitric oxide and oxygen radicals has common points of contact; therefore, it is necessary to emphasize the importance of simultaneously evaluating several indicators of molecular metabolism in EBC for a more objective interpretation and interpretation of results.

In recent decades, considerable evidence about the contribution of NO and its metabolites to the pathogenesis of many diseases of the respiratory tract has been accumulated. So, the total concentration of nitrates and nitrites (TNN) in EBC is significantly increased in patients with uncontrolled and controlled bronchial asthma compared with healthy

children [17, 18]. In addition, in patients with absolute and complete control of bronchial asthma who had not received therapy by glucocorticosteroids, the total content of nitric oxide metabolites in EBC was significantly higher than in patients receiving this therapy. There was established a significant relationship between the total content of nitric oxide metabolites in EBC and the level of control of bronchial asthma in patients treated with the same type of therapy (including glucocorticosteroids).

The EBC concentration of 3-nitrotyrosine (3-NT) is higher in patients with controlled mild asthma not receiving corticosteroids, but it is lower compared with the moderate and severe asthmatics, using inhaled corticosteroids. The level of 3-NT correlates with the level of fractional exhaled NO (FeNO) only in controlled mild asthma [17, 19]. The level of nitrosothiols was studied in patients with mild and moderate asthma. An increase in this parameter was noted in patients with moderate asthma compared with controls and patients with mild asthma [17, 20].

The results of the studies of the exhaled NO level in chronic obstructive pulmonary disease (COPD) are contradictory. Kubysheva et al. demonstrated that the severity of the progression of COPD was linked with an increase in the concentrations of metabolites of nitric oxide in blood and in exhaled breath condensate [21, 22]. For the patients with COPD, the associations between the lung function parameters and the levels of $\Sigma NO_2^-/NO_3^-$ were determined.

However, it was found that smoking and disease severity are the most important factors affecting NO production [23]. Active smokers with severe COPD (especially in combination with a pulmonary heart) show lower levels of exhaled NO compared with former smokers with a nonsevere COPD. An increase in FeNO has been reported in patients hospitalized with exacerbation of COPD. FeNO of patients receiving systemic corticosteroids returned to control values only months later after discharge from hospitals. This result confirms various mechanisms of inflammation in COPD and steroid-sensitive asthma. Acidosis, which often accompanies acute ventilatory respiratory failure associated with an exacerbation of COPD, may also contribute to an increase in exhaled NO [24].

Other disorders associated with the activation of NS include bronchiectasis, active pulmonary sarcoidosis, active pulmonary fibrosis, and the transplant rejection reaction of the lungs [24].

In cystic fibrosis (CF) remission, there is an increase in EBC nitrite anion [25], in contrast to the FeNO level [26]. Formanek et al. [25] showed an increase in nitrate anion and 3-NT with normal FeNO levels in sputum of patients with CF. Increased production of NO and superoxide anion radical may not be accompanied with increase NO in EBC since the constant for the reaction of superoxide with NO is higher than the constant for its reaction with SOD [27, 28].

The role of human microbiota in the nitric oxide cycle, the role of significant components of nitrite and nitrate-reductase systems in the nitric oxide cycle, and the mechanisms of their activation and deactivation (participation of enzymes, cofactors, homeostatic indicators, etc.) are determined under various conditions, which allows detailed control mechanisms of the NO cycle for targeted exposure to therapeutic agents [11].

Recently published data demonstrate the potentially positive role of NOS inhibitors in the treatment of asthma and COPD [29–32]. Inflammation and oxidative stress contribute to the reaction of superoxide anion with available NO, rather than its endogenous neutralizer superoxide dismutase, thus increasing the formation of peroxynitrite in tissues [29, 33]. Consequently, the use of iNOS inhibitors may restore the preferred pathway of superoxide radical detoxification, via superoxide dismutase [34]. Moreover, oxidative and nitrosative stress contribute to arterial stiffness pathogenesis due to oxidative damage to lipids, proteins, and DNA in endothelial cells and uncoupling of NO synthase, leading to endothelial dysfunction. Thus, another target for NOS inhibitors in COPD is arterial stiffness elevation by nitrosative stress, which accompanies structural local and systemic changes in those patients [30]. During rhinovirus-induced COPD exacerbation, high levels of reactive nitrogen species induce nitrosylation of histone deacetylases-2 (HDAC2) and reduced HDAC2 activity in macrophages. This is believed to be a key mechanism of corticosteroid resistance in COPD and can be modified by NOS inhibitors as well [31].

There is evidence of the potential role of the NO donor in the treatment or modulation of asthma and COPD in viral exacerbations [35–38]. Rhinovirus-infected epithelial cells are known to produce chemotactic cytokines, which attract inflammatory cells to airways of patients with asthma and COPD [38]. At the same time, Sanders et al. [5, 39] showed that respiratory epithelial cells also produce nitric oxide (NO), which can play an important role during the antiviral response. NO can inhibit both the replication of the rhinovirus and the rhinovirus-induced production of cytokines in human respiratory epithelial cells [39]. In addition, there is evidence that the addition of chemical NO donors or the induction of NOS causes inhibition of a wide range of viruses, including both DNA and RNA viruses [40]. In vitro studies showed the ability of NO to inhibit human rhinovirus- (HRV-) induced production of IFN-gamma-inducible protein 10 (CXCL10) by inhibiting viral activation of nuclear factor kappa B (NF-kappaB) and of interferon-regulatory factors (IRF), including IRF-1, through a cGMP-independent pathway [41]. Sanders et al. also showed that NO donor 3-(2-hydroxy-2-nitroso-1-propyl-hydrazino)-1-propanamine (NONOate) inhibits the HRV-induced GM-CSF mRNA levels [42]. Thus, NO may play an important antiviral role, and nitric oxide donors may represent a new therapeutic approach for viral exacerbations of asthma and COPD.

3. Markers of Oxidative and Nitrosative Stress in Lung Diseases

The study of RNS and ROS concentrations in in vivo and in clinical practice, in particular, is very problematic, due to the specificity of the detected compounds. The lifetime of most ROS and RNS is hundredths of a second or less. Accordingly, a number of requirements of practical

properties, characterized by ease of use and reproducibility of the results, are imposed on both the methods and the biological media in which the dynamic concentration of oxidants is monitored. In this regard, noninvasive techniques and biological media have the advantage of studying ROS and RNS in the respiratory tract: exhaled breath condensate (EBC) and exhaled air itself. EBC is a liquid formed as a result of cooling and subsequent condensation of exhaled air; therefore, its composition is determined by the composition of exhaled air. To determine the respiratory, ROS use indicators in the blood, which is, as a rule, a reflection of systemic changes in the redox status in the body. EBC and exhaled air markers help to identify the tension of oxidative and nitrosative status directly in the respiratory tract and the airway surface liquid, which is essentially the first line of lungs and whole-body protection from exogenous oxidants. Noninvasive methods of ROS and RNS studying can optimize diagnosis and treatment, as well as help clarify the molecular mechanisms of the pathogenesis of lung diseases [2, 14, 43].

Among the identified OS markers, conditionally stable RNS and ROS and their metabolism products, other oxidation products of RNS and ROS, and ions of variable valency are the most popular in clinical practice [2, 14, 43].

Despite the difficulties in determining the concentration of certain compounds in the EBC, researchers are now increasingly interested in identifying molecules involved in the reactions of the OS and NS, which are key elements in the development of most pulmonary pathologies, allows assessing noninvasively the state of the respiratory tract. Practically, all these molecules are united by the fact that their concentrations in EBC vary from micromolar to nanomolar, and this, in turn, requires the use of highly sensitive determination methods. Despite the high variability of studied parameters, by increasing the samples, statistically significant differences between groups are achieved in diseases such as asthma, COPD, and CF. Among the most intensively studied molecules, a special place is occupied by the markers of OS and NS: ROS and RNS. However, taking into account the specifics of EBC collection, namely, the duration of the procedure from 10 to 20 minutes and the possible change in the concentration of the molecules being determined during this time, the most promising role is assigned to stable metabolites of oxygen and nitrogen [2, 14, 43].

The most studied form of the ROS is hydrogen peroxide (H_2O_2). Hydrogen peroxide in vivo is the product of the superoxide anion radical (O_2^-) dismutation. The sources of such radicals are reactions involving xanthine oxidase and mitochondrial and microsomal electron transfer chains. The concentration of H_2O_2 in the inflammatory regions is especially high, due to which a change in the H_2O_2 level in biological fluids is one of the inflammatory markers [17]. However, the final concentration of H_2O_2 in tissue depends on many parameters. Thus, the concentration of any substance in the body at a given time, including hydrogen peroxide, is the sum of the synthesis rate and the rate of decomposition of this compound. When H_2O_2 is formed in the dismutation reaction of superoxide with the participation of SOD, the reaction rate constant is lower than during the interaction of superoxide and nitric oxide, which indicates the competition between NO and SOD [44]. Therefore, in the presence of NO, the content of which increases in the focus of inflammation according to many studies; the concentration of hydrogen peroxide in the tissue may decrease. For example, the work of Latzin and Griese [45] revealed a negative significant correlation between the levels of atmospheric NO, and in FeNO, and the content of hydrogen peroxide in EBC in 102 healthy children. The presence in the environment of metal ions of variable valency, such as iron, copper, and manganese, can also reduce the H_2O_2 content even at high rates of its formation. This is due to the high rate of decomposition of H_2O_2 in the Fenton reaction with the formation of an extremely reactive hydroxyl radical [17].

Changes in the H_2O_2 concentration can also be observed when the antioxidant (AO) status changes, namely, medium levels of catalase, peroxidase, peroxiredoxins, and other enzymes that have a high affinity for hydrogen peroxide. Such a high dependence on the concentration of other molecules present in the medium makes it necessary to measure along with hydrogen peroxide a number of parameters: the concentration of metal ions of variable valency (iron, copper, and manganese), the level of nitric oxide (NO), and AO status of the organism [17].

Currently, the dynamics of changes in H_2O_2 in EBC has been studied in many pathologies of the respiratory tract. In asthma, an increase in the concentration of H_2O_2 in EBC was shown to correlate with an increase in the level of sputum eosinophils and serum content of eosinophilic cationic protein, as well as with a decrease in FEV1 [46, 47]. It is reported that a significant increase in the H_2O_2 content in EBC, observed in patients with moderate and severe asthma, can serve as an informative marker of the inflammation severity, unlike the FeNO level, which is highly dependent on the therapy (in particular, corticosteroids) [47]. The possibility of monitoring the asthma therapy efficacy by the estimation of the H_2O_2 level in the EBC is shown. In other inflammatory diseases of the respiratory tract, such as COPD [48–50] and bronchiectasis [51], an increase in the H_2O_2 level in EBC is also observed. Increased H_2O_2 concentrations have also been observed in patients with pneumonia. A positive significant correlation was noted between the level of H_2O_2 and the content of thiobarbituric acid (TBA) in EBC on days 1 and 3 of the disease, as well as between the level of H_2O_2 in EBC and the concentration of C-reactive protein and the level of blood leukocytes on day 1. During the therapy, a decrease in OS parameters in EBC was revealed [52].

Those H_2O_2 level dynamics in EBC with these diseases may indicate an increase in the superoxide generation by cells involved in the inflammation process [53, 54] and/or a decrease in AO activity of cells and tissues with the progression of these pathologies. However, in the group of patients with stable CF, no statistically significant change in this parameter was found in comparison with the healthy control group [55].

F2-isoprostanes (F2-IsoPs) are recognized as reliable markers of oxidative stress in lung diseases [56, 57]. IsoPs have been elevated in plasma, urine, bronchoalveolar lavage

Table 1: Respiratory biomarkers and their role in asthma and COPD.

Markers	Specimens	Role in asthma	Role in COPD
FeNO	Exhaled air	Increased in asthma patients and could be used as a potentially valuable tool for assessing the severity of asthma [69].	Elevation in COPD and the association between exacerbated COPD [70].
H_2O_2	Exhaled air, EBC	Higher values in uncontrolled asthma [71].	Correlate with COPD health status as measured by the COPD assessment test [72].
8-Isoprostane	EBC, induced sputum	Increased in adult asthmatic and its concentration is related to asthma severity [73].	Increased during exacerbation of COPD [74].
3-NT	EBC, induced sputum	Increased in allergic asthmatics [75].	High levels in COPD [76].
MPO	Induced sputum	Increased in severe asthma patients, associated with neutrophilic inflammation [77, 78].	Increased in stable COPD patients, especially pronounced during exacerbations [79].
EPO	Induced sputum	Elevated amounts of EPO correspond with the increased numbers of eosinophils [80, 81].	
MMPs	Induced sputum	Increased in asthma, associated with airway remodeling [82].	Contribute to the development of emphysema and small airway fibrosis in COPD [83].
MDA	EBC, induced sputum	Increased in acute asthma attacks [84].	Elevated in COPD [76].

Abbreviations: FeNO: fractional exhaled nitric oxide; 3-NT: 3-nitrotyrosine; MPO: myeloperoxidase; EPO: eosinophil peroxidase; MMPs: matrix metalloproteases; MDA: malondialdehyde.

in patients with mild and severe asthma [58, 59], interstitial lung diseases [60], cystic fibrosis [61], and chronic obstructive pulmonary disease [62].

Development of sensitive and special radioimmunoassay (RIA) allowed measuring the content of 8-isoprostane, which is an isomer of prostaglandin-F2A, in the exhaled air condensate [63]. An increase IsoP concentrations in EBC was also recorded in children with asthma and persistent allergic rhinitis [64], in smokers with COPD [62], and in healthy volunteers after exposure to ozone [65].

IsoPs are not only markers of oxidative stress but also play an important role in lung pathophysiology. In the respiratory tract, isoprostanes are able to regulate cellular processes that affect the tone of pulmonary vessels and smooth muscles of the airways [66] and to stimulate the adhesion and function of macrophages [67]. Arezzini et al. demonstrated the profibrotic role of F2-IsoPs in the pathogenesis of pulmonary fibrosis. In this study, it was shown that F2-IsoPs can activate fibroblasts in myofibroblasts and participate in the synthesis of collagen [68]. These findings provide evidence of the role of isoprostanes in airway inflammation and, thus, identify a potential therapeutic goal for the treatment of pulmonary diseases.

The main airway biomarkers of the oxidative and nitrosative stress in asthma and COPD are summarized in Table 1.

4. Respiratory Antioxidant Protection System

Due to the huge number of reactions occurring in cells and the formation of RNS, ROS, and other highly active compounds, antioxidant (AO) systems exist to regulate the redox balance. The components of these systems are variously distributed both in the cell and at the organ-tissue level. The respiratory tract, which is the first line of body defense against the effects of atmospheric pollutants [2], contains a large number of AO systems. Antioxidant protection of the lungs and airways is carried out by many low molecular weight antioxidants; however, AO enzymes play the main role in protecting the trachea, bronchi, and alveoli epithelium from oxidative damage. The most important AO enzymes include superoxide dismutases (SODs), catalase, glutathione peroxidase (GPx), glutathione S-transferases (GSTs), glutamylcysteine synthases (GCSs), glutaredoxins (Grxs), and thioredexins (Trxs). In humans, all these AO enzymes have been shown to be expressed in the airways [2, 85, 86].

Superoxide dismutases (SODs) are one of the main AO enzymes that are expressed in almost all cells of the human body [87]. The proteins of this family implement the dismutation reaction of $O_2^{\cdot-}$ to H_2O_2. The active center of SOD contains a transition metal ion. For the cytoplasm of eukaryotes, these are copper and zinc ions (Cu/Zn-SOD) [88, 89]. Mitochondria, for example, contain another type of SOD, which includes manganese (MnSOD) in the active center [90]. The rate of enzymatic dismutation of $O_2^{\cdot-}$ is very high (the rate constant is about 10^9 M^{-1} s^{-1}); it is necessary for the rapid control of the $O_2^{\cdot-}$ formation. However, the $O_2^{\cdot-}$ dismutation reaction can proceed more slowly and spontaneously without the participation of the enzyme. Excessive $O_2^{\cdot-}$, at the same time, can react with a variety of cellular targets and impair their functions. For example, the enzyme of the tricarboxylic acid cycle aconitase is inactivated by $O_2^{\cdot-}$, which can cause significant changes in cell metabolism [91]. So, generated H_2O_2 as a result of the $O_2^{\cdot-}$ dismutation reaction, like other ROS, can be toxic to cells and its concentration in cells is also controlled by AO systems.

Catalases are heme-containing enzymes that catalyze the decomposition of H_2O_2 to H_2O and molecular oxygen. Glutathione peroxidase (GPx) is another group of proteins involved in the removal of H_2O_2. The reduction of H_2O_2 to H_2O, carried out by this group of enzymes, is associated with the oxidation of glutathione. In addition to H_2O_2, GPx can interact with other peroxides in cells [92].

The thioredoxin system includes thioredoxin (Trx) itself and thioredoxin reductase (TrxR). Thioredoxins form a family of small-sized proteins with oxidoreductase activity. Trx restores oxidized disulfide peptides under OS. The

reduction of oxidized Trx, in turn, is carried out by NADPH-dependent thioredoxin reductases (TrxRs) [93]. In addition to thioredoxin, TrxR can reduce a large number of other compounds. Thus, TrxR1 is involved in the reduction of H_2O_2 and lipid hydroperoxides formed in high concentrations at OS. The functions of the thioredoxin system overlap with the glutaredoxin-dependent system, which is also of great importance for AO protection. The component of this system is glutaredoxin (Grx), which is involved in thiol/disulfide exchange reactions. The oxidized Grx is reduced nonenzymatically by the reduced glutathione (GSH) pool. Oxidized glutathione (GSSG) restores a special enzyme glutathione reductase (GR). GR has some similarities with TrxR and is a FAD-containing enzyme that uses reducing equivalents of NADPH to restore GSSG [94].

Peroxiredoxins, a family of AO proteins with peroxidase activity, are also involved in H_2O_2 degradation. They were first found in yeast and then in other organisms [85, 86]. In mammals, there are 6 types of peroxiredoxins (Prx1-Prx6), and most of them have been identified in databases recently. In the cell, the peroxiredoxins are found mainly in the cytosol, as well as in the mitochondria, peroxisomes, and chloroplasts. The two isoforms of peroxiredoxins (Prx4 and Prx6) are secretory proteins [85]. The concentration of peroxiredoxins in many cells is unusually high—from 0.1 to 1% of the total water-soluble cellular protein, depending on the type of tissue, making them a redox buffer that controls the level of intracellular peroxides. Peroxiredoxins catalyze the reduction of H_2O_2 and organic peroxides to water and alcohol, respectively. In addition, some isoforms of peroxiredoxins can destroy peroxynitrite. Peroxynitrite reductase activity was first detected in bacterial peroxiredoxins and then confirmed for eukaryotic peroxiredoxins [85]. Neutralization of peroxides and peroxynitrite with peroxiredoxins occurs according to the same catalytic mechanism. The expression level of genes of various types of peroxiredoxins is significantly increased in many pathological conditions accompanied by OS. This correlation indicates that cells increase AO protection with peroxiredoxins to neutralize the increased content of ROS. Peroxiredoxin 6 (Prx6) is one of the types of peroxiredoxins that performs a special function in the respiratory tract (Figure 3). The secretory water-soluble Prx6 was first isolated from the rat olfactory epithelium at the Institute of Biophysics of the Cell of the Russian Academy of Sciences [85, 86]. Biochemical studies have shown that Prx6 in the presence of some thiols has the ability to neutralize both organic and inorganic peroxides and its protective activity is determined mainly by peroxidase activity. Immunohistochemical studies conducted at the light and electron microscopic levels, and in situ hybridization experiments have shown that Prx6 is mainly localized in the olfactory epithelium, bronchi, and epidermis. In the trachea and bronchi, it is synthesized by the goblet cells and the Clara cells and secreted into the mucus, where it is major among the water-soluble mucus proteins. Finally, it was shown that the contribution of Prx6 to the neutralization of ROS in the trachea and bronchi reaches 70% [85, 86].

The important role of Prx6 in the protective mechanisms of the bronchial epithelium which primarily include the

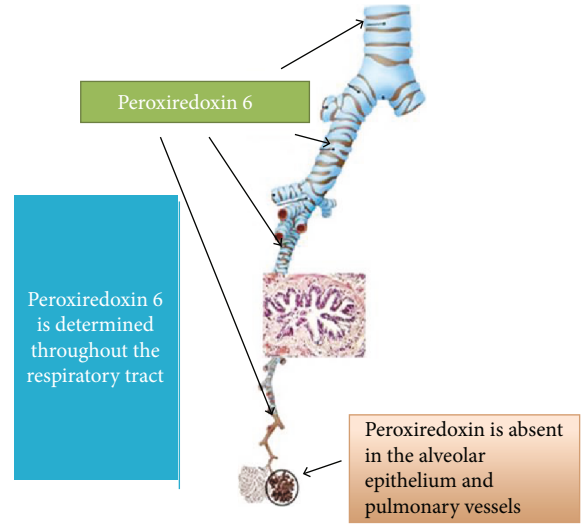

Figure 3: Localization of peroxiredoxin 6 in the lungs.

activation of the Prx6 expression under various pathological conditions led to the assumption that the use of exogenous Prx6 can significantly accelerate the recovery of the affected respiratory epithelium. This hypothesis was tested on models of the acute inflammatory process in the trachea of the rat, caused by bacterial endotoxins, and thermal burns of the upper respiratory tract [85, 86].

The study of AO enzymes in human lung pathology is hampered by a number of objective factors, namely, the inability to assess in vivo protein expression by immunohistochemical methods, the absence of large molecules in noninvasive biological media, the invasiveness of procedures for obtaining biopsy tissue of the lung, the lack of standardization for the evaluation of AO enzymes in the BAL, and induced sputum. The study of the role of enzyme AO systems is currently being evaluated in the blood, in model systems, and in animals. Due to the fact that the work of the AO systems often occurs site-specific (directly at the site of the development of the OS or NS), the evaluation of the AO systems in blood gives conflicting results.

Various low molecular weight substances play the role of antioxidants. These include, in addition to glutathione, ascorbic acid, α-tocopherol, and some others [95–98].

The α-tocopherol molecule (vitamin E) consists of a benzene core with a hydroxyl group (capable of giving an electron, performing the AO function) and a side wick chain that performs the hydrophobic interaction of the antioxidant with membrane structures. Vitamin E is able to quench ROS, interact with the hydroxyl radical, and restore the lipid radicals of the structure $R^{\cdot}$ and $ROO^{\cdot}$. The most active in the lipid bilayer α-tocopherol restores peroxyl radicals. The resulting α-tocopherol radical is relatively little active due to the delocalization of the unpaired electron along the aromatic ring. It is believed that in the presence of water-soluble antioxidants, such as the reduced form of ascorbic acid, vitamin E is able to restore its AO potential through direct recycling. Retinol (vitamin A) in combination with α-tocopherol is also involved in the protection of biological membranes from damage by their prooxidants [95–98].

Ascorbic acid (AA) is an important representative of water-soluble antioxidants. The presence of two-phenolic groups in the AA molecule structure allows it to participate in redox transformations, acting as a donor and acceptor of electrons and protons. AA has an extremely wide range of AO properties related to hypohalides, $O_2^{\cdot-}$, $HO_2^{\cdot}$, $RO_2^{\cdot}$, $1O_2$, $HO^{\cdot}$, $NO^{\cdot}$, $ONOO^{-}$, and nitrosamines. It also restores the oxidized form of α-tocopherol. In the presence of metal cations of variable valency, AA becomes a powerful prooxidant, which is a reliable regulation of the transition metal ion concentration [95–98].

N-Acetylcysteine (NAC), an acetylated amino acid L-cysteine, inactivates free radicals and reactive oxygen species by directly reacting with them (direct antioxidant effect) and also supplying cysteine and promotes the synthesis of glutathione (indirect antioxidant effect). In turn, glutathione is an important component of the detoxification system of xenobiotics, peroxide compounds, and free radicals, which has a protective effect at the cellular level [99, 100].

Of the three amino acids involved in the structure of glutathione (glutamate, glycine, and cysteine), cysteine has the lowest intracellular concentration. At the same time, the main mechanism of glutathione replenishment is de novo synthesis. Consequently, cysteine efficiency may limit the rate of glutathione synthesis under conditions of oxidative stress.

NAC has been used in clinical practice for over 50 years. The positive effect of NAC has been demonstrated on conditions characterized by decreased production of glutathione (GSH) or activation of lipid peroxidation (POL): tobacco smoking, cardiovascular diseases [101], acetaminophen (paracetamol) [102] and heavy metal poisoning, HIV infection, etc. [99, 100]. Preliminary studies demonstrate the effectiveness of NAC as a chemoprophylactic agent in chemotherapy of malignant tumors. The additional roles of the drug are the eradication of Helicobacter pylori and the prevention of gentamicin-induced hearing loss in patients on hemodialysis [103–105].

The prevention of glutathione reserves depletion under the NAC action, and the direct neutralizing effect of the drug in relation to ROS and RNS (OH, H_2O_2, $ONOO^{\cdot}$, and $O_2^{\cdot-}$) helps to reduce the intensity of reactions of OS and NS, which leads to the suppression of inflammation in chronic obstructive pulmonary disease (COPD), influenza, and idiopathic pulmonary fibrosis [100, 101].

COPD is the leading cause of death and morbidity worldwide and is characterized by persistent airflow restriction, hypersecretion and an increase in sputum viscosity, OS, chronic respiratory inflammation, and extrapulmonary manifestations. Currently, a significant evidence base has been collected on the positive impact of NAC on the course of COPD. The antioxidant and anti-inflammatory properties of the drug are associated with its ability to regulate the redox status, as well as the activity of the nuclear transcription factor NF-κB [106–108].

A progressive decrease in inspiratory capacity (IC) during exercise reflects dynamic hyperinflation and is a significant marker of physical training in patients with COPD. The NAC demonstrated the ability to modify small-diameter airways and the associated processes of pulmonary hyperinflation. In a randomized, double-blind, and placebo-controlled trial by Stav and Raz in 24 patients with moderate and severe stable COPD, there was an increase in IV and forced vital capacity (FVC) after a 6-week course of NAC therapy at a daily dose of 1200 mg. An increase in the ratio of residual lung volume (RV) to the total lung capacity (TLC) was also noted. The distance covered in the exercise test was also significantly greater in the NAC group compared with the placebo group [106–109].

The effect of NAC on the severity of COPD symptoms was evaluated in a systematic review of randomized clinical trials. A statistically significant decrease of the disease symptom intensity in the NAC groups was found in all reviewed studies, in comparison with patients taking the placebo. As a result of a meta-analysis, it was concluded that in 26 out of 100 patients with COPD, NAC therapy reduces the severity of clinical manifestations (number needed to treat(NNT) = 3.8) [110].

Decramer et al. demonstrated that N-acetylcysteine at a dose of 600 mg had no effect on the rate of decline in lung function and the frequency of exacerbations in COPD. However, the analysis in subgroups revealed that the frequency of exacerbations when using NAC was reduced in patients who did not receive inhaled corticosteroids [111].

5. Conclusion

NS and OS are multilevel processes, existing and developing in inseparable connection with a number of physiological and pathophysiological processes. They accompany almost all diseases of the respiratory tract, and the study of their subtle mechanisms contributes to improving the quality of diagnosis and the development of therapeutic approaches.

Conflicts of Interest

The authors declare that there is no potential conflict of interest associated with this manuscript.

Authors' Contributions

Each of the authors contributed equally to the manuscript writing.

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Research Article

Higd1a Protects Cells from Lipotoxicity under High-Fat Exposure

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Hypoxia-inducible gene domain family member 1A (Higd1a) has recently been reported to protect cells from hypoxia by helping to maintain normal mitochondrial function. The potential induction of Higd1a under high-fat exposure and whether it could protect cells from oxidative stress attracted our attention. Initially, 0.4 mM oleic acid and 0.2 mM palmitate were added to the growth media of HepG2 and LO2 cells for 72 hours. We discovered increased Higd1a expression, and knocking down Higd1a impaired mitochondrial transmembrane potential and induced cell apoptosis. We then identified that elevated reactive oxygen species (ROS) is responsible for increased Higd1a expression. Furthermore, we found that ROS promoted Higd1a expression by upregulating HIF-1a and PGC-1a expressions, and these two proteins could exert synergistic effects in inducing Higd1a expression. Taken together, these data suggest that Higd1a plays positive roles in protecting cells from oxidative stress, and ROS could induce Higd1a expression by upregulating PGC-1a and HIF-1a expressions.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most prevalent liver diseases and is characterized by a wide range of alterations, including simple steatosis at early stages and steatohepatitis in advanced stages, in which fatty liver is accompanied by inflammation, hepatocyte ballooning, liver fibrosis, and disrupted glucose homeostasis and insulin resistance [1–3]. Due to the increasing prevalence of obesity and type II diabetes, the incidence of NAFLD is increasing dramatically, and NAFLD constitutes a global health concern, affecting not only adults but also children [4, 5]. Mitochondria play a central role in nutrient metabolism and provide energy required for a myriad of cell functions. In NAFLD patients, the rates of fatty acid oxidation (FAO) exceed the tricarboxylic acid cycle (TCA) capacity, resulting in mitochondrial fatty acid overload and leading to incomplete FAO and accumulation of reactive oxygen species (ROS) that contribute to mitochondria dysfunction and cell damage [6, 7]. Impaired mitochondrial β-oxidation and defective mitochondrial respiratory chain subsequently contribute to hepatic steatosis [8, 9]. Thus, protecting mitochondria from oxidative stress is one of the key aspects in treating NAFLD.

Hypoxia-induced gene domain protein-1a (Higd1a) (also known as HIG1 or HIMP1-a) is a 10.4 kDa mitochondrial inner membrane protein and is predicted to have two transmembrane domains oriented in an "N-terminal outside-C-terminal outside and loop inside" conformation [10, 11]. Higd1a is induced by hypoxia and ischemia and may aid in cell survival in the context of severe stress by protecting mitochondria [12–16]. Higd1a could also be induced during central nervous maturation. Under this circumstance, Higd1a may facilitate cell apoptosis and promote tissue remodeling [17]. Thus, the exact functions of Higd1a depend on the developmental stage and cellular microenvironment. In the present study, we found that Higd1a expression is increased under high-fat exposure and protects cells from oxidative stress. Moreover, we found that ROS promotes

the expression of Higd1a by upregulating HIF-1a and PCG-1a. These results may help us to better understand the protective response of cells under oxidative stress.

2. Materials and Methods

2.1. Cells and Transfections. HepG2 and LO2 cells were purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Medical Sciences (Shanghai, China). These cells were maintained at 37°C in DMEM with 10% fetal bovine serum (Gibco, Invitrogen, USA). Higd1a siRNA (50 nM), HIF-1a siRNA (50 nM), PGC-1a siRNA (50 nM), pcDNA HIF-1a, pcDNA PGC-1a, and corresponding negative controls were designed and synthesized by RiboBio Co. (Guangzhou, China). Cell transfection was performed using Lipofectamine 3000 (Invitrogen, USA) according to the supplier's protocol. All sequences are provided in Supplementary Table 1.

2.2. Patients. Liver tissue specimens were obtained from patients who underwent liver resection for benign liver disease (polycystic liver or hepatic hemangioma), and the specimens were obtained at least 2 cm away from the liver lesion. NAFLD was identified by pathological examination. Specimens were stored in liquid nitrogen within 30 minutes after removal from patients during surgery. Informed consent forms were signed by all patients.

2.3. Animals. C57BL/6 mice were housed in sterilized isolators at 22 ± 1°C with 55-65% relative humidity and a 10 h light/14 h dark cycle. Mice were fed a standard diet (SD, composed of 20%, 70%, and 10% calories from protein, carbohydrate, and fat, respectively; Research Diets D12450B, Research Diets Inc., USA) or a high-fat diet (HFD, composed of 20%, 20%, and 60% calories from protein, carbohydrate, and fat, respectively; Research Diet D12492, Research Diets Inc., USA). Six weeks after feeding, adenovirus expressing Higd1a shRNA or control shRNA (Vigen Biosciences, Shandong, China) (the sequences are shown in Supplementary Table 1) at a titer of $4 * 10^9$ was injected to the caudal vein of HFD-fed mice. All mice were sacrificed and analyzed 7 days after virus injection. The Animal Care and Use Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, approved the animal protocol. The animal experiments adhered to the NIH *Guide for the Care and Use of Laboratory Animals*.

2.4. Preparation of FFAs. Sodium oleate and sodium palmitate were dissolved separately in ethanol at a concentration of 100 mM. Then, BSA solution (20%, *W*/*V*) was heated to 45°C, and oleate and palmitate solutions were added to the BSA solution to obtain a mixed FA stock solution (5 mM; oleate and palmitate at final ratio of 2 : 1). We also prepared single fatty acid stock solutions to which only oleate or palmitate solutions were added to the BSA solution. Stock solutions were then diluted in incubation medium containing 5 mg/ml of fatty acid-free BSA (Boehringer Mannheim GmbH, Mannheim, Germany) to desired concentrations. HepG2 and LO2 cells at 80% confluence were exposed to the FFAs for 72 hours.

2.5. Oil Red O Staining. Treated cells were washed three times with iced PBS and fixed with 4% paraformaldehyde for 30 minutes. After fixation, cells were washed three times and stained with Oil Red O solution (Shenggong, Shanghai, China) for 15 minutes at room temperature. Cells were then washed with PBS three times to remove redundant staining and observed under a microscope.

2.6. Inhibitor Studies. The antioxidant *N*-acetyl-L-cysteine was purchased from Sigma and was added to the growth media at a concentration of 1 mM.

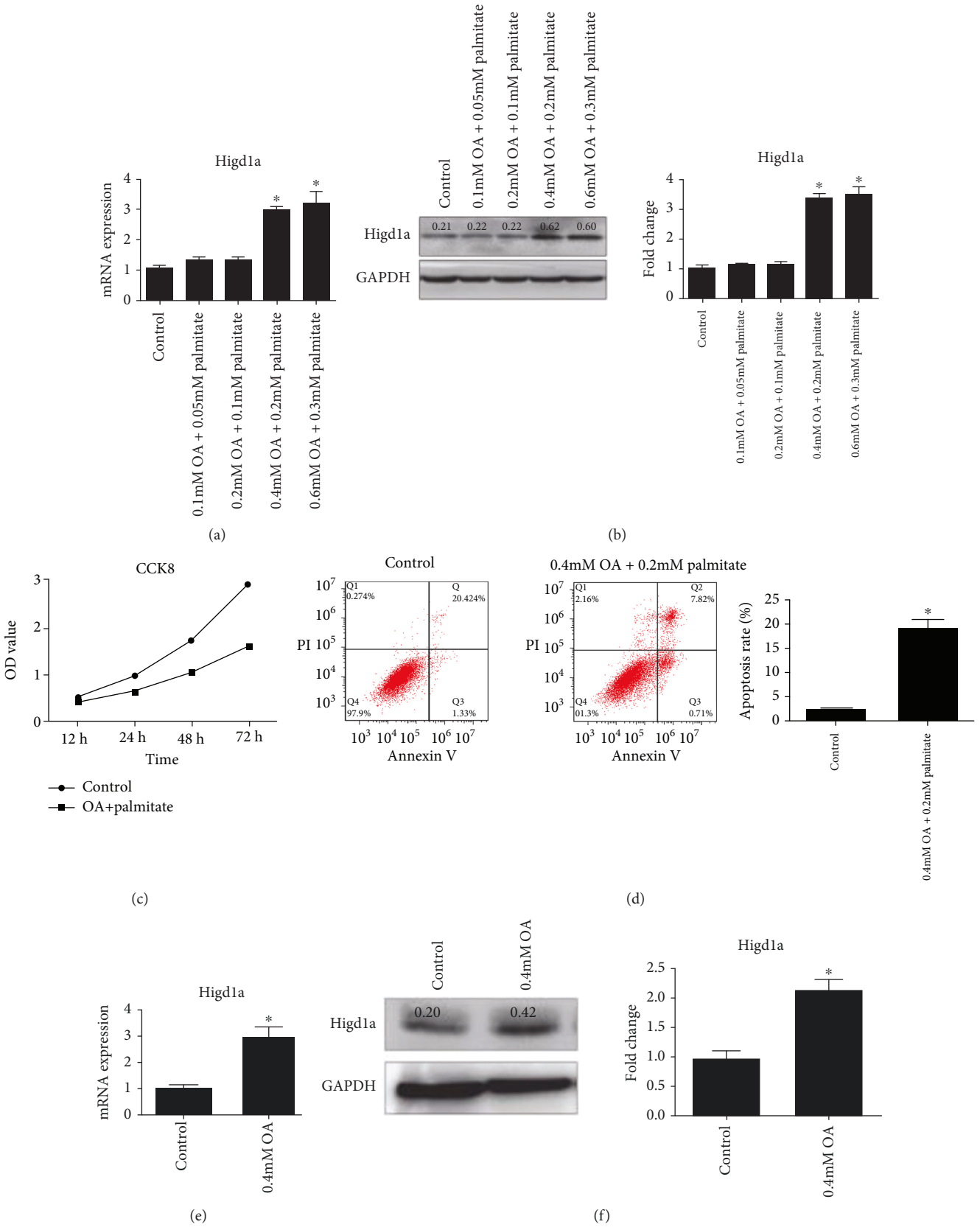
2.7. Western Blotting and Antibodies. Total cell proteins were lysed in RIPA buffer with phosphatase inhibitors and a protease inhibitor cocktail (Sigma, USA). The following antibodies for western blotting were purchased from Santa Cruz (USA): mouse anti-Higd1a (1 : 1000), rabbit anti-HIF-1a, rabbit anti-PGC-1a (1 : 1000), and rabbit anti-GAPDH (1 : 1000). The rabbit/mouse secondary antibodies (1 : 3000) were obtained from Proteintech, China. Proteins were separated by 10% SDS-polyacrylamide gels and transferred to PVDF membranes. After blocking, blots were probed with specific antibodies at room temperature for 2 hours and then incubated with the appropriate secondary antibody. The protein bands were detected using a Bio-Rad imaging system (Bio-Rad, USA). All experiments were repeated at least three times, and the representative bands are indicated in the figures.

2.8. qRT-PCR. Total RNA was extracted from cells/liver tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using a reverse transcription kit (Takara). Quantitative real-time reverse transcription PCR (qRT-PCR) was conducted using the quantitative SYBR Green PCR kit (Takara). Targets were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All qRT-PCR reactions were performed in triplicate. The qRT-PCR primers are presented in Supplementary Table 2.

2.9. Measurement of Mitochondrial Transmembrane Potential. Mitochondrial transmembrane potential (MMP) assay kit with JC-1 (Beyotime, China) was used to assess the MMP. Briefly, cells were incubated with JC-1 working fluid at 37°C for 20 minutes. After incubation, cells were washed twice with JC-1 dyeing buffer. Fluorescence was then detected using a fluorescence microscope (Olympus, Japan).

2.10. Measurement of Reactive Oxygen Species Production. After treatment of FFAs (or FFAs plus NAC) for 72 hours, 5 μM DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate, Beyotime, China) was added to the cells and incubated for 30 minutes. Following the incubation period, the cells were washed with PBS and analyzed using flow cytometry.

2.11. Cell Proliferation Assay. A total of 5×10^3 cells per well were seeded in 96-well plates. Seventy-two hours after FFA treatment, cell viability was determined using the CCK8 kit (Dojindo Laboratories, Japan) according to the



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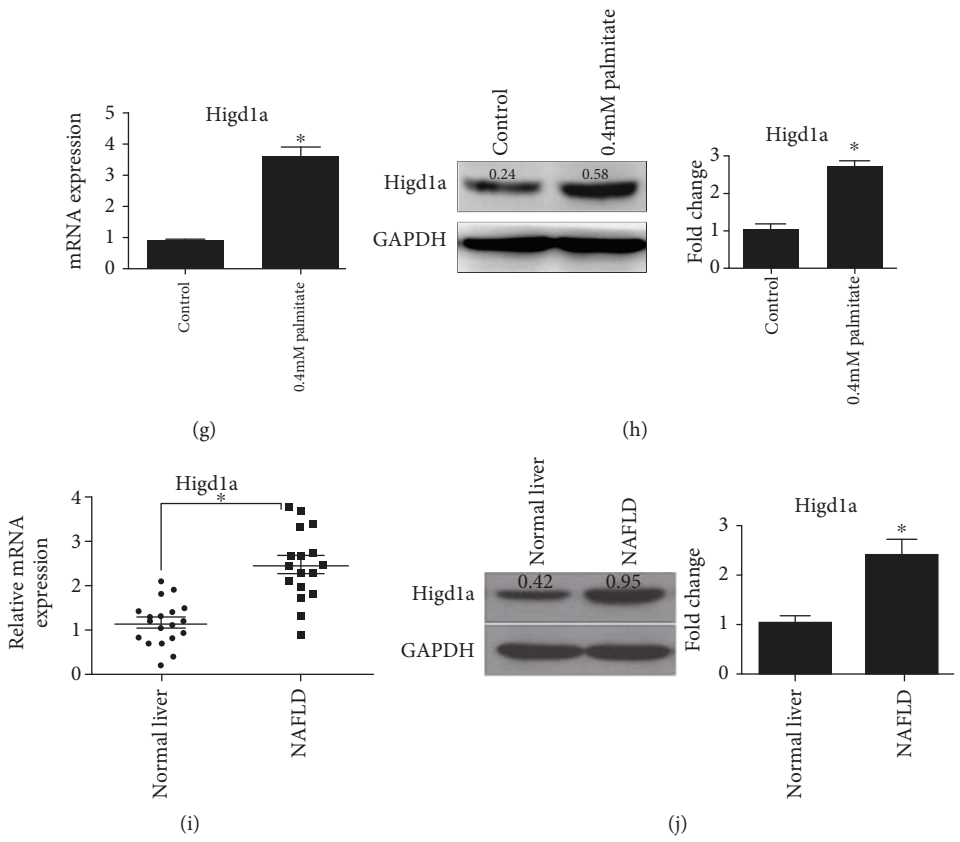


Figure 1: Higd1a expression is elevated under high-fat exposure. (a) LO2 cells were treated with various concentrations of oleic acid and palmitate (2 : 1) for 72 hours, and qRT-PCR and western blot (b) revealed that Higd1a expression was increased significantly upon exposure to 0.4 mM OA and 0.2 mM palmitate. (c) Cells were treated with 0.4 mM OA + 0.2 mM palmitate or untreated, and CCK8 assay revealed inhibition of cell proliferation in the FFA group. (d) Cell apoptosis was measured by flow cytometry using Annexin V/PI staining in the abovementioned groups, and more apoptosis was induced in the FFA group; LO2 cells were treated with 0.4 mM OA (e, f) or 0.4 mM palmitate (g, h) alone, and qRT-PCR and western blot were used to detect Higd1a expression in each group; Higd1a mRNA and protein expression in the livers of NAFLD patients was measured by qRT-PCR (i) and western blot (j) ($^*P < 0.05$ compared with the control group).

manufacturer's protocol. The data were normalized to cells without drugs in each group.

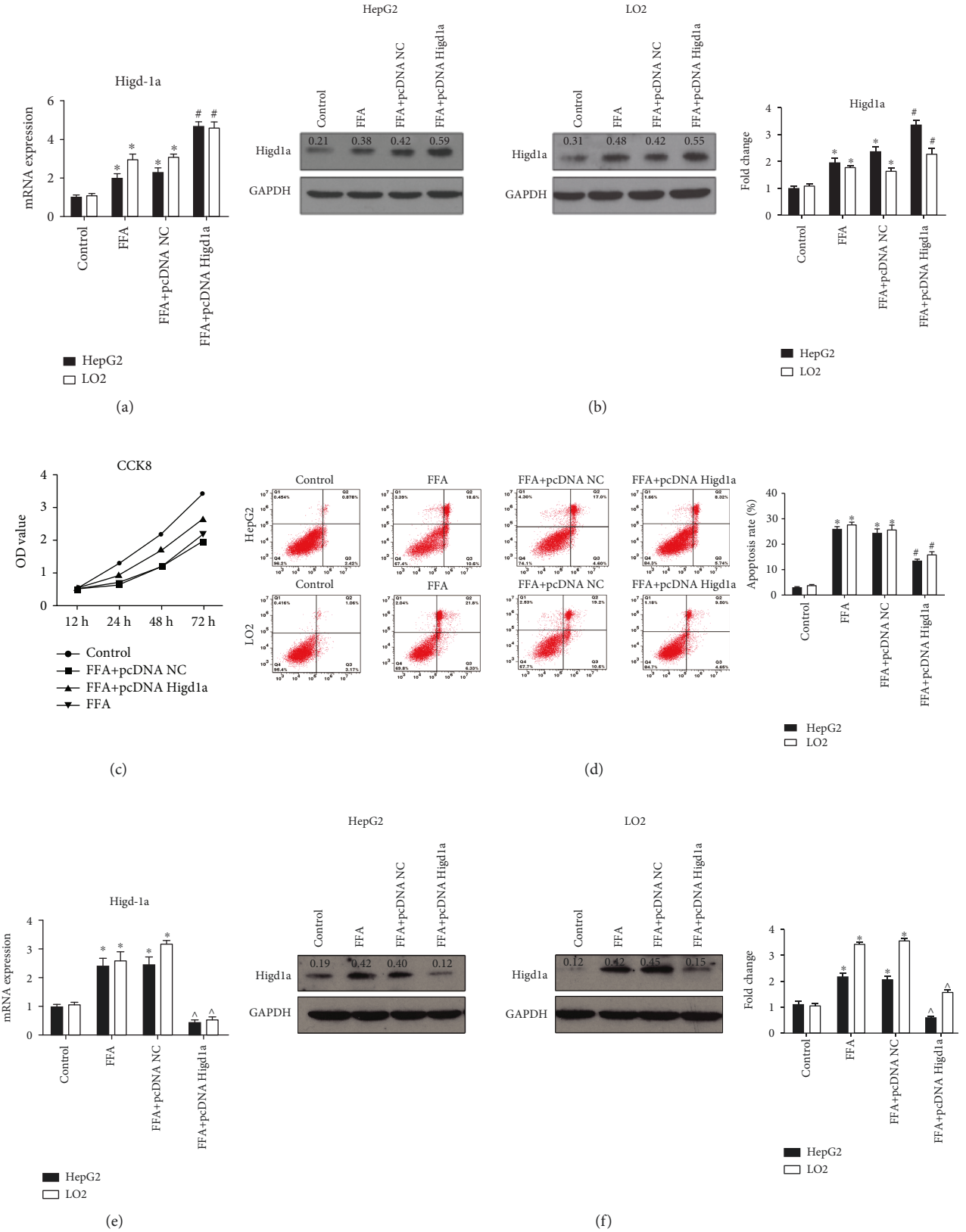
2.12. Cell Apoptosis Assay. Cell apoptosis was assessed 72 hours after FFA treatment. Annexin V/propidium iodide staining was performed to quantify cell apoptosis. Cells were resuspended in 500 μl of binding buffer, stained with Annexin V-FITC (5 μl) and propidium iodide (10 μl) in the dark for 15 minutes, and then analyzed using flow cytometry.

2.13. Statistical Analysis. Data are presented as the mean ± SE of three to four experiments. In appropriate cases, comparisons between groups were analyzed by ANOVA with post hoc analysis using Dunnett's multiple comparison tests. P values < 0.05 were considered statistically significant.

3. Results

3.1. Higd1a Expression Is Increased under High-Fat Exposure. Cells were treated with oleic acid (OA) and palmitate at different concentrations for 72 hours. Higd1a expression was significantly increased with 0.4 mM OA and 0.2 mM palmitate (Figures 1(a) and 1(b)). Oil Red staining revealed lipid droplets in the FFA group (0.4 mM OA + 0.2 mM palmitate) (Supplementary Figure 1). CCK8 assay and flow cytometry using Annexin V/PI staining were used to detect cell proliferation and apoptosis, respectively. Cell proliferation was inhibited in the FFA group, and more cell apoptosis had been induced (the apoptosis rate was 2.8 ± 1.2% in the control group versus 18.5 ± 4.5% in the FFA group) (Figures 1(c) and 1(d)). In addition, we found that OA (Figures 1(e) and 1(f)) or palmitate (Figures 1(g) and 1(h)) alone could also induce the expression of Higd1a. Moreover, the liver expression of Higd1a was elevated in NAFLD patients compared with those with a normal liver (Figures 1(i) and 1(j)). These results indicated that the expression of Higd1a could be induced under high-fat exposure.

3.2. Higd1a Protects Cells from Stress under High-Fat Exposure. Given that previous studies demonstrated the protective effects of Higd1a on cells under hypoxic conditions (12), we then detected whether it could protect cells from stress under high-fat exposure. HepG2 and LO2 cells



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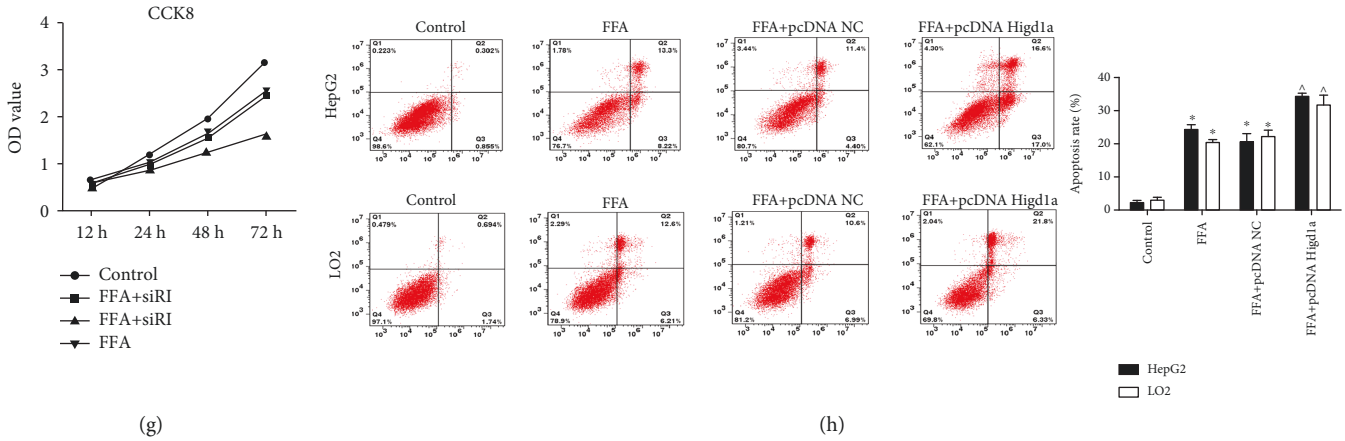


(g) (h)

FIGURE 2: Overexpression of Higd1a protects cells from apoptosis under high-fat exposure and vice versa. (a) HepG2 and LO2 cells were transfected with pcDNA NC or pcDNA Higd1a and treated with FFAs (0.4 mM OA + 0.2 mM palmitate) for 72 hours, and the overexpression of Higd1a was validated by qRT-PCR and western blot (b); cells in the FFA+pcDNA Higd1a group exhibited increased expression compared with those in the FFA+pcDNA NC group. (c) Cell proliferation in the abovementioned groups was measured by the CCK8 kit. (d) Cell apoptosis was assessed by flow cytometry using Annexin V/PI staining, and less apoptosis was induced in the FFA+pcDNA Higd1a group compared with the FFA+pcDNA NC group. (e) Cells were transfected with siRNA NC or siRNA Higd1a and then treated with FFAs for 72 hours; Higd1a knockdown was validated by qRT-PCR and western blot (f). (g) Cell proliferation in each group was measured by the CCK8 kit, and cell proliferation was more inhibited in the FFA+siRNA Higd1a group compared with the FFA+siRNA NC group. (h) Cell apoptosis in each group was measured by flow cytometry using Annexin V/PI staining, and more apoptosis was induced in the FFA+siRNA Higd1a group compared with the FFA+siRNA NC group ($^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the FFA+pcDNA NC group; $^{\wedge}P < 0.05$ compared with the FFA+siRNA NC group).

were transfected with pcDNA NC or pcDNA Higd1a and treated with FFAs for 72 hours. Higd1a overexpression was confirmed by qRT-PCR and western blot (Figures 2(a) and 2(b)). CCK8 assay and flow cytometry using Annexin V/PI staining revealed increased cell proliferation and decreased cell apoptosis in the FFA+pcDNA Higd1a group compared with the FFA+pcDNA NC group (apoptosis rate in HepG2/LO2 cells was 13.33 ± 1.53%/16.00 ± 1.63% in the FFA+pcDNA Higd1a group versus 24.33 ± 3.06%/25.67 ± 2.49% in the FFA+pcDNA NC group) (Figures 2(c) and 2(d)). Cells were then transfected with siRNA NC or siRNA Higd1a and treated with FFAs; Higd1a knockdown was validated by qRT-PCR and western blot (Figures 2(e) and 2(f)). As expected, cell proliferation was further inhibited in the FFA+siRNA Higd1a group, and more apoptosis was induced in the FFA+siRNA Higd1a group compared with the FFA+siRNA NC group (apoptosis rate in HepG2/LO2 cells was 33.67 ± 2.08%/31.67 ± 3.40% in the FFA+siRNA Higd1a group versus 20.67 ± 4.16%/21.67 ± 2.87% in the FFA+siRNA NC group) (Figures 2(g) and 2(h)). Given that Higd1a is localized to the mitochondrial inner membrane, we detected the impact of Higd1a on mitochondrial transmembrane potential (MMP). As expected, upregulating Higd1a before treatment with FFAs protected MMP, whereas downregulating Higd1a exerted opposite effects (Figures 3(a) and 3(b)). These results suggest that Higd1a plays positive roles in protecting cells from stress under high-fat exposure.

3.3. ROS Increases Higd1a Expression under High-Fat Exposure. The production of ROS is accelerated under lipotoxicity, and ROS induce a large series of proteins related to energy metabolism [18]. We then investigated whether elevated ROS could induce Higd1a expression. Flow cytometry using DCFH-DA staining revealed elevated intracellular ROS in the FFA group (cells treated with 0.4 mM OA + 0.2 mM palmitate) (Figure 4(a)). We then added the ROS inhibitor *N*-acetyl-L-cysteine (NAC) to the FFA group and assessed Higd1a expression. Interestingly, both Higd1a mRNA and protein levels were decreased (Figures 4(b) and 4(c)). In addition, Higd1a expression increased when cells were treated with hydrogen peroxide (H_2O_2), the established ROS inducer, and the expression decreased after cells were treated with NAC (Figures 4(d) and 4(e)). These results indicate that ROS could induce the expression of Higd1a under high-fat exposure.

3.4. ROS Promote the Expression of Higd1a by Upregulating HIF-1a under High-Fat Exposure. HIF-1a is a master transcriptional regulator of the cellular response to hypoxia and induces the transcription of genes involved in cell proliferation, cell survival, and cell metabolism [19]. Previous studies reported that Higd1a may be induced by HIF-1a under hypoxic conditions, but different points of views exist [12, 13]. Given that HIF-1a may also be induced by ROS, we then investigated whether HIF-1a played a role in ROS-mediated Higd1a expression. As expected, HIF-1a expression was increased in the FFA group and returned to approximately normal levels when NAC was added to the culture media (Figures 5(a) and 5(b)). Cells were then transfected with siRNA NC, siRNA HIF-1a, pcDNA NC, or pcDNA HIF-1a, separately, and treated with FFAs for 72 hours. Interestingly,

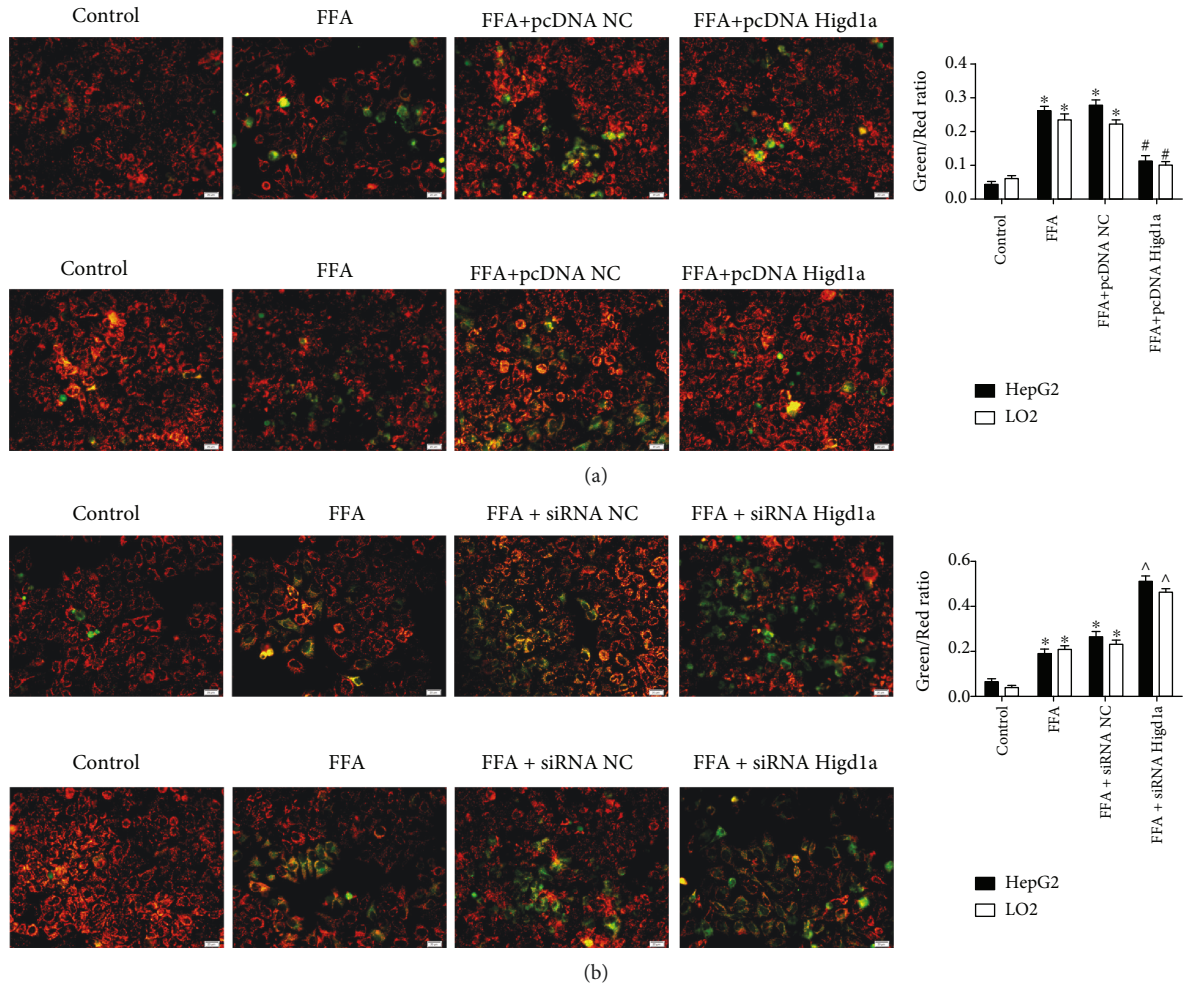


Figure 3: Overexpression of Higd1a protects mitochondrial transmembrane potential under high-fat exposure and vice versa. (a) Mitochondrial transmembrane potential (MMP) in the control group, FFA group, FFA+pcDNA NC group, and FFA+pcDNA Higd1a group was measured by fluorescence microscopy (Olympus, Japan) (JC-1 dye was used to evaluate MMP; red fluorescence indicated cells with normal MMP, and green fluorescence indicated cells with impaired MMP), and the impairment of MMP was less severe in the FFA +pcDNA Higd1a group compared with the FFA+pcDNA NC group. (b) MMP in the control group, FFA group, FFA+siRNA NC group, and FFA+siRNA Higd1a group was measured by fluorescence microscopy, and the impairment of MMP was more severe in the FFA +siRNA Higd1a group compared with the FFA+siRNA NC group ($^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the FFA+pcDNA NC group; $^{\wedge}P < 0.05$ compared with the FFA+siRNA NC group).

Higd1a expression in the FFA+siRNA HIF-1a group was considerably reduced compared with that in the FFA+siRNA NC group (Figures 5(c) and 5(d)). In contrast, Higd1a expression was considerably increased in the FFA+pcDNA HIF-1a group compared with the FFA+pcDNA NC group (Figures 5(e) and 5(f)). Moreover, cells transfected with siRNA HIF-1a exhibited reduced Higd1a expression compared with cells transfected with siRNA NC when treated with H_2O_2 (Figures 5(g) and 5(h)). These results suggest that ROS promote Higd1a expression partially through upregulating HIF-1a.

3.5. ROS Promote Higd1a Expression by Upregulating PGC-1a under High-Fat Exposure. PGC-1a is a powerful transcriptional coactivator that regulates cell metabolism and mitochondrial biogenesis. Sen et al. [20] reported that PGC-1a acts as a key modulator of P53 and promotes cell survival upon metabolic stress. PGC-1a is also induced by ROS under oxidative stress [21], so we investigated whether PGC-1a regulates the expression of Higd1a under high-fat exposure. First, we found that PGC-1a expression was significantly increased in the FFA group, whereas minimal changes in expression were noted in the FFA plus NAC group (Figures 6(a) and 6(b)). We then transfected cells with siRNA NC, siRNA PGC-1a, pcDNA NC, or pcDNA PGC-1a, separately, and treated cells with FFAs. We found that Higd1a mRNA and protein expression were decreased if PGC-1a was knocked down before cells were treated with FFAs (Figures 6(c) and 6(d)), whereas expression was increased if PGC-1a was overexpressed (Figures 6(e) and 6(f)). In addition, knocking down PGC-1a before cells were treated with H_2O_2 reduced Higd1a expression (Figures 6(g) and 6(h)). These results indicated that PGC-1a is involved in ROS-induced upregulation of Higd1a.

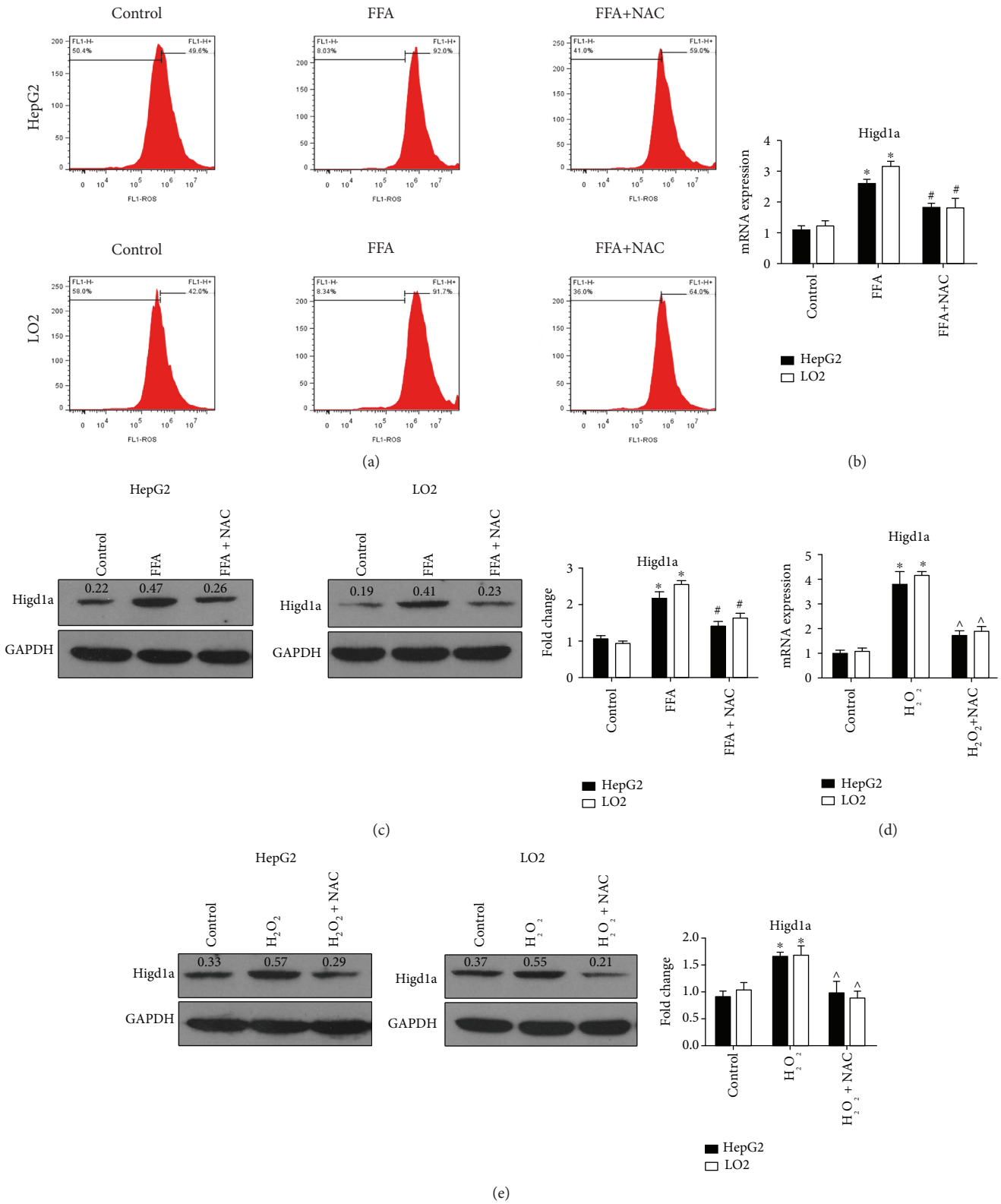
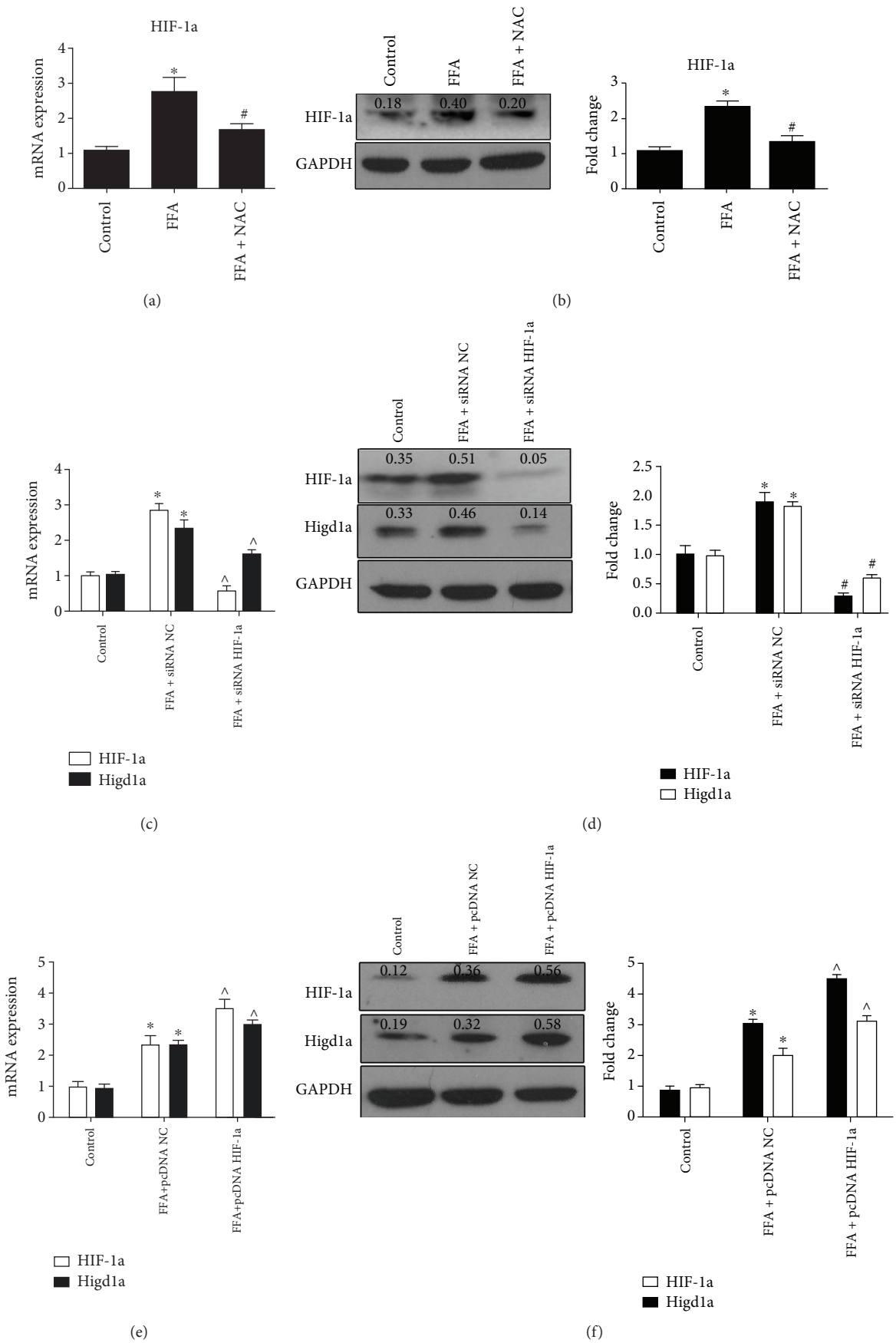


Figure 4: ROS promote Higd1a expression under high-fat exposure. (a) Flow cytometry using DCFH-DA identified intracellular ROS production in the control group, FFA group, and FFA+NAC group, and intracellular ROS was significantly elevated in the FFA group. (b) qRT-PCR and western blot (c) detected Higd1a expression in the abovementioned groups, and Higd1a expression was elevated in the FFA group but decreased in the FFA+NAC group. (d) qRT-PCR and western blot (e) detected the expression of Higd1a in the control group, H_2O_2 group, and the H_2O_2+NAC group, and Higd1a expression was increased in the H_2O_2 group but decreased in the H_2O_2+NAC group ($^*P < 0.05$ compared with the control group; $^\#P < 0.05$ compared with the FFA group; $^\wedge P < 0.05$ compared with the H_2O_2 group).



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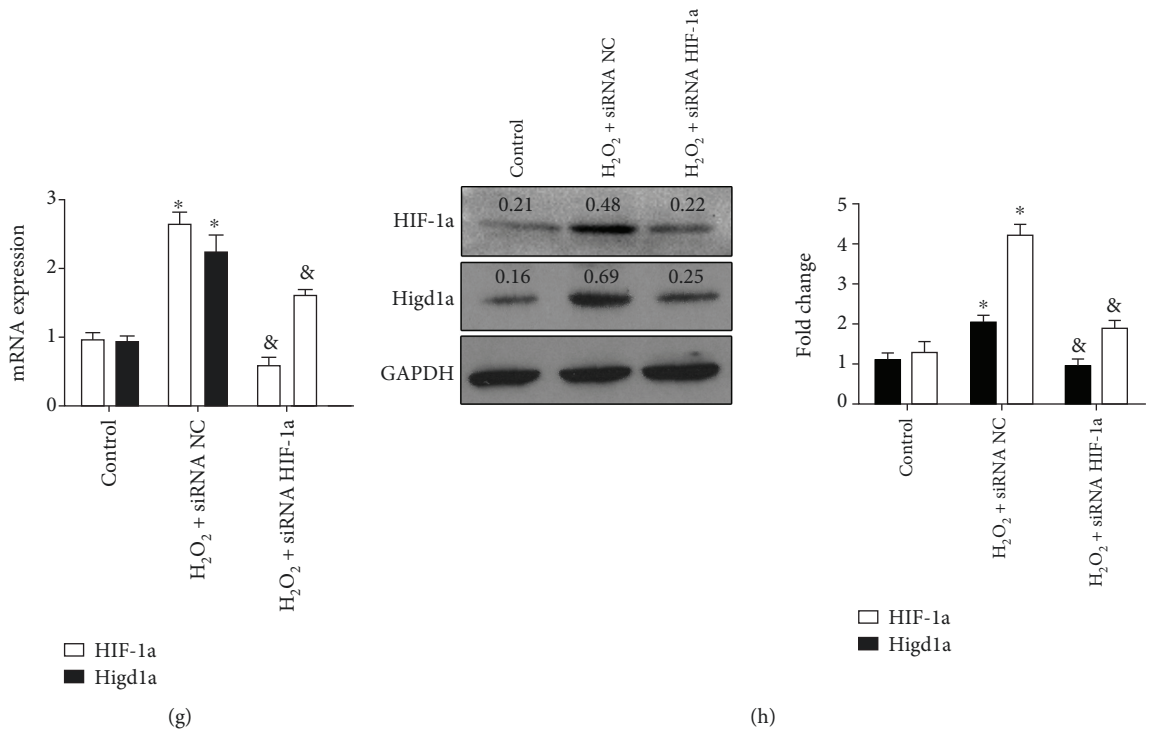


Figure 5: ROS promote Higd1a expression by upregulating HIF-1a. (a) HIF-1a expression in the control group, FFA group, and FFA+NAC group was measured by qRT-PCR and western blot (b), and HIF-1a expression was elevated in the FFA group but decreased in the FFA+NAC group. (c) Cells were transfected with siRNA NC or siRNA HIF-1a and then treated with FFAs; qRT-PCR and western blot (d) were used to detect Higd1a expression in each group, and Higd1a expression was decreased in the FFA+siRNA HIF-1a group compared with the FFA +siRNA NC group. (e) Cells were transfected with pcDNA NC or pcDNA HIF-1a and then treated with FFAs, and qRT-PCR and western blot (f) demonstrated that Higd1a expression was increased in the FFA+pcDNA HIF-1a group compared with the FFA+pcDNA NC group. (g) Cells were transfected with siRNA NC or siRNA HIF-1a and then treated with H_2O_2, and qRT-PCR and western blot (h) revealed that HIF-1a and Higd1a expressions were increased in the H_2O_2+siRNA NC group but decreased in the H_2O_2+siRNA HIF-1a group ($^*P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the FFA group; $^{\wedge}P < 0.05$ compared with the FFA+siRNA NC (pcDNA NC) group; $^{\&}P < 0.05$ compared with the H_2O_2+siRNA HIF-1a group).

3.6. PGC-1a and HIF-1a Exert a Synergistic Effect on Inducing Higd1a Expression. Given that both PGC-1a and HIF-1a exhibited regulatory effects on Higd1a, we then investigated the synergistic role of these two genes in regulating Higd1a. Cells were transfected with siRNA NC, siRNA PGC-1a, siRNA HIF-1a, or siRNA PGC-1a plus HIF-1a, separately. FFAs were added to the culture medium after transfection, and Higd1a protein expression was detected by western blot. As expected, the group cotransfected with siRNA PGC-1a and HIF-1a exhibited reduced Higd1a expression compared with other groups (Figure 7(a)). Next, we transfected cells with pcDNA NC, pcDNA PGC-1a, pcDNA HIF-1a, or pcDNA PGC-1a plus pcDNA HIF-1a and treated cells with FFAs. Western blot revealed increased Higd1a expression in the FFA+pcDNA PGC-1a+pcDNA HIF-1a group compared with other groups (Figure 7(b)). These results suggest that PGC-1a and HIF-1a exert a synergistic effect in inducing Higd1a expression.

3.7. Knocking Down Higd1a Aggravated Liver Injury in NAFLD Mice. We established an in vitro model to demonstrate that increased Higd1a expression exerted a positive role in protecting cells from oxidative stress, and we next explored whether Higd1a had similar in vivo effects. Mice were fed a standard diet (SD) or a high-fat diet (HFD) for 6 weeks. Mice fed a HFD were then subjected to caudal vein injection adenoviral virus expressing a shRNA against Higd1a or a control shRNA at a titer of $4 * 10^9$. After 7 additional days, all mice were sacrificed and analyzed. As expected, Higd1a expression in the liver was increased in mice fed a HFD compared with mice fed a SD and reduced in mice injected with adenovirus expressing Higd1a shRNA (Figures 8(a) and 8(b)). Liver tissues were then stained with HE for histological examination. Mice fed a HFD developed a fatty liver, and hepatic steatosis was more severe in mice injected with adenovirus expressing Higd1a shRNA (Figure 8(c)). These results enhanced the positive role of Higd1a in protecting cells from oxidative stress.

4. Discussion

Based on our research, this is the first study to demonstrate that Higd1a is elevated under high-fat exposure, potentially protecting cells from oxidative stress. We first demonstrated that ROS promote the expression of Higd1a by inducing HIF-1a and PGC-1a.

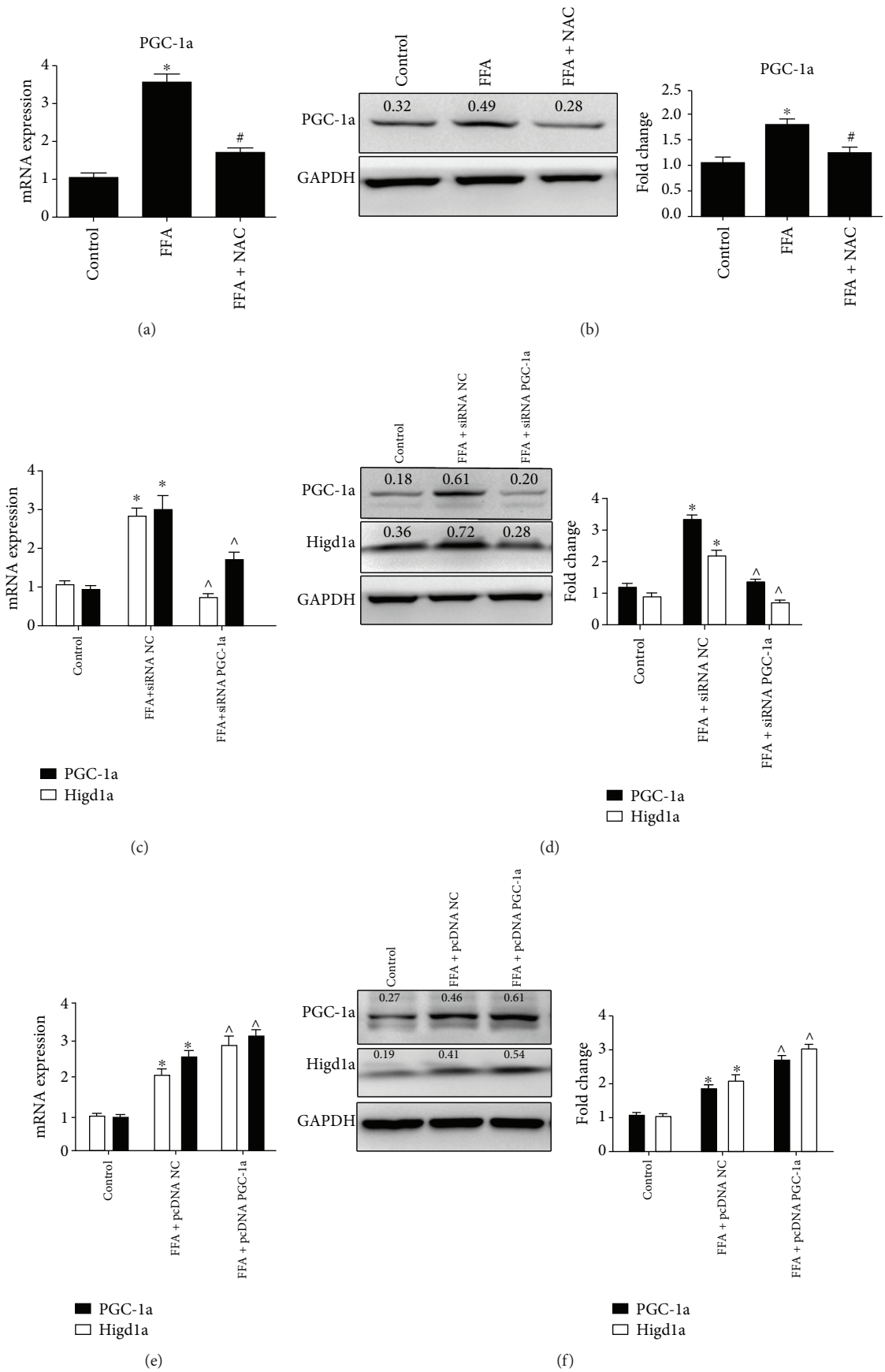
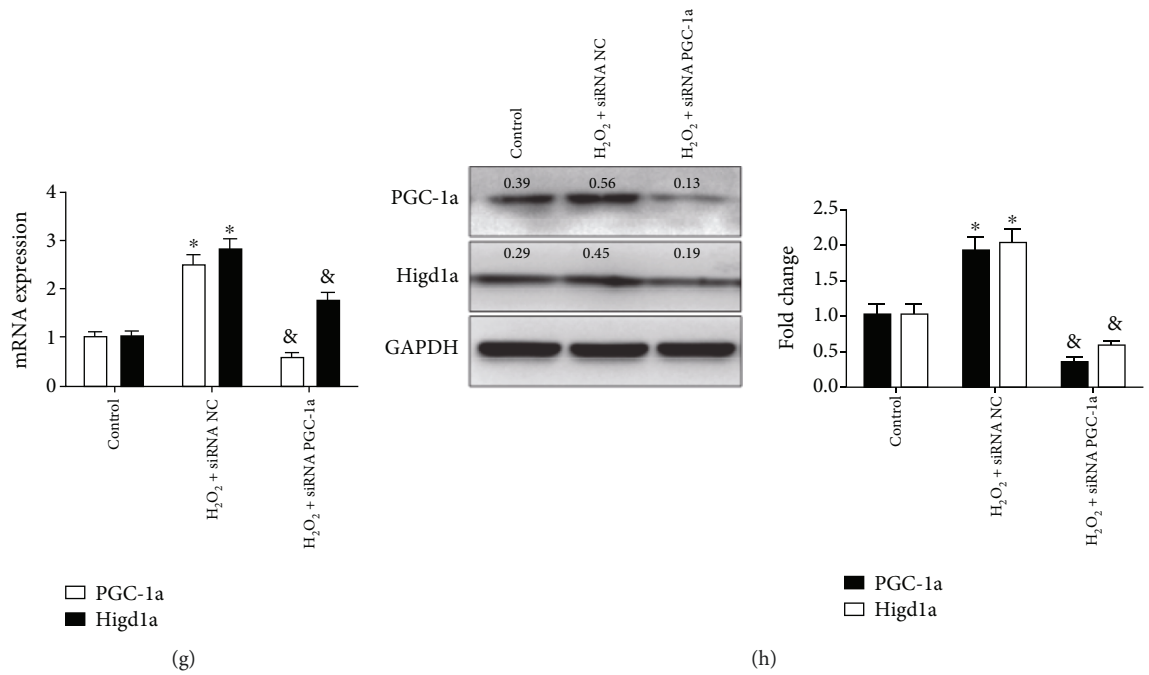


Figure 6: Continued.



(g) (h)

Figure 6: ROS promote Higd1a expression by upregulating PGC-1a. (a) PGC-1a expression in the control group, FFA group, and FFA+NAC group was measured by qRT-PCR and western blot (b), and levels were elevated in the FFA group but decreased in the FFA+NAC group. (c) Cells were transfected with siRNA NC or siRNA PGC-1a and then treated with FFAs; qRT-PCR and western blot (d) were used to detect Higd1a expression in each group, and Higd1a expression was decreased in the FFA+siRNA PGC-1a group compared with the FFA +siRNA NC group. (e) Cells were transfected with pcDNA NC or pcDNA PGC-1a and then treated with FFAs, and qRT-PCR and western blot (f) demonstrated that Higd1a expression was increased in the FFA+pcDNA PGC-1a group compared with the FFA+pcDNA NC group. (g) Cells were transfected with siRNA NC or siRNA PGC-1a and then treated with H_2O_2, and qRT-PCR and western blot (h) demonstrated that PGC-1a and Higd1a expressions were increased in the H_2O_2+siRNA NC group but decreased in the H_2O_2+siRNA PGC-1a group ($^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the FFA group; $^{\wedge}P < 0.05$ compared with the FFA +siRNA NC (pcDNA NC) group; $^{\&}P < 0.05$ compared with the H_2O_2+siRNA PGC-1a group).

Hypoxia-inducible gene domain family member 1A (Higd1a) is a HIF-1a targeted mitochondrial protein that is typically induced by hypoxia. Higd1a binds to mitochondrial γ-secretase complex, decreases γ-secretase activity, and thus reduces ROS production and mitochondrial dysfunction [14]. Higd1a also interacts with the mitochondrial electron transport chain and decreases oxygen consumption and AMPK activity to promote cell survival [11]. Moreover, Higd1a binds to the cytochrome c oxidase complex and increases its activity, thus protecting cells from an energy crisis [15]. In this study, Higd1a expression was elevated under high-fat exposure. In addition, knocking down Higd1a impaired MMP and induced cell apoptosis and vice versa. These results indicate that Higd1a plays a positive role in protecting cells from oxidative stress under high-fat exposure, and Higd1a may represent a potential target in treating NAFLD. Higd1a is localized to the mitochondria, and a prior report suggests that Higd1a is translocated from the mitochondria to the nucleus upon metabolic stress [22]. Similar to AIF, GAPDH, or FKP51, Higd1a may also exhibit novel nuclear roles that could fine tune cellular fates [23–25]. Although we did not explore the exact mechanism by which Higd1a protects cells, we hypothesize that some key proteins related to cell death or survival may be regulated by Higd1a, and this hypothesis will be further assessed in future research.

Whether HIF-1a promotes Higd1a expression is controversial. An et al. [13] reported that HIF-1a could bind to the promoter of Higd1a and induce Higd1a expression. However, Ameri and Maltepe [12] reported that Higd1a is not induced by HIF-1a in hypoxic cells but is instead triggered by additional metabolic stressors. If the promoter of Higd1a is methylated, its expression is inhibited. In this study, we discovered that HIF-1a expression is elevated in the fatty acid group. Knocking down HIF-1a reduced the expression of Higd1a and vice versa. These results indicate that HIF-1a induces Higd1a expression under high-fat exposure.

PGC-1a is a transcriptional coactivator that regulates genes involved in energy metabolism and exhibits powerful transcriptional activity when linked to a heterologous DNA-binding domain or docked to a transcription factor [26]. Under stress, PGC-1a activates gluconeogenesis, fatty acid β-oxidation, ketogenesis, and bile acid homeostasis by coactivating key hepatic transcription factors, such as HNF4a, FOXO1, GR, and FXR [27–31]. Loss of PGC-1a leads to significant functional deficits in oxidative metabolism and exacerbates steatohepatitis. This is the first study to identify that together with HIF-1a, PGC-1a also promotes Higd1a expression under high-fat exposure, and these results could help us to better understand Higd1a regulation.

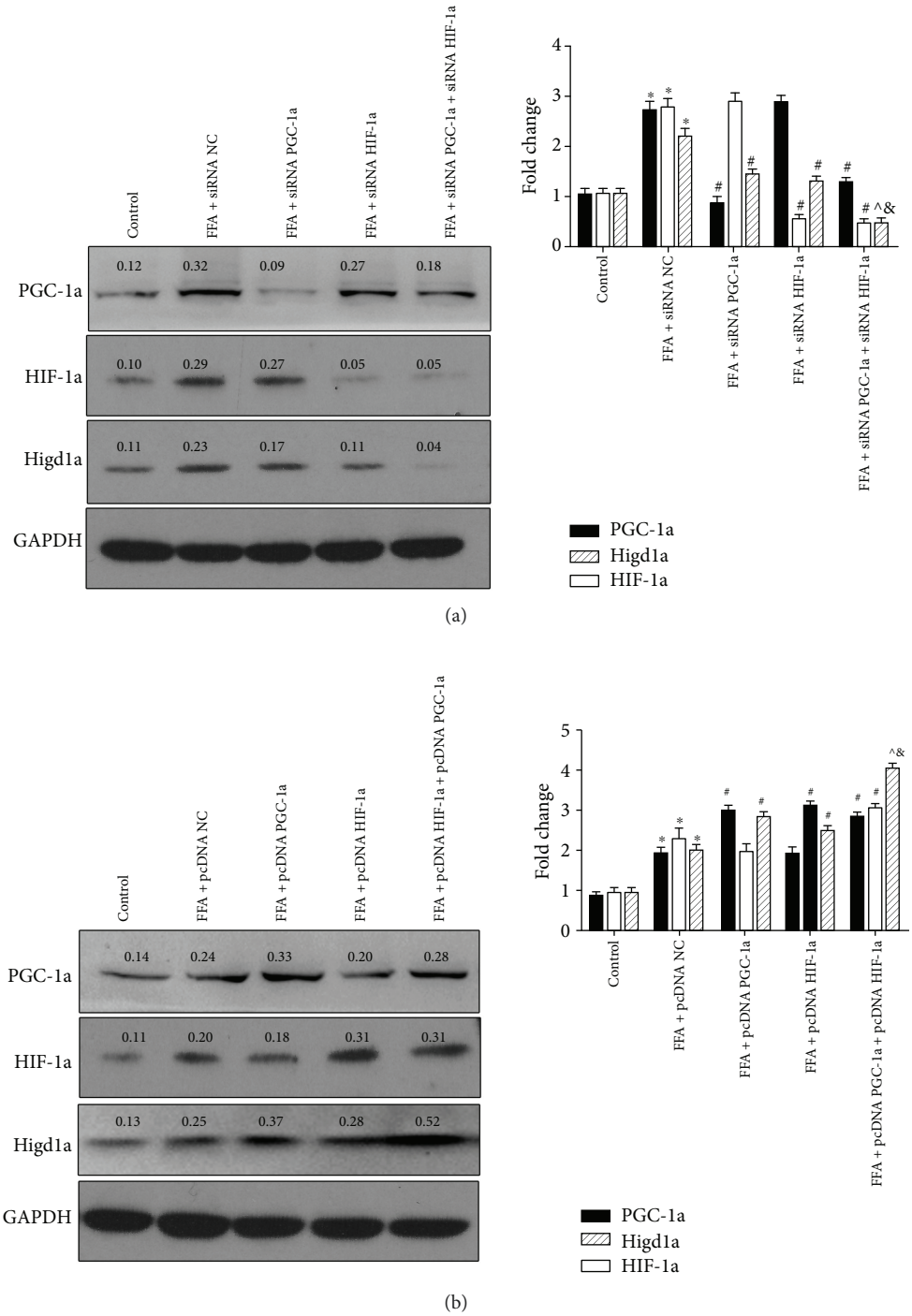


Figure 7: PGC-1a and HIF-1a exert synergistic effects on inducing Higd1a expression. (a) LO2 cells were transfected with siRNA NC, siRNA PGC-1a, siRNA HIF-1a, or siRNA PGC-1a+siRNA HIF-1a and then treated with FFAs; the protein expression of PGC-1a, HIF-1a, and Higd1a was measured by western blot, and Higd1a expression was decreased in the FFA+siRNA PGC-1a+siRNA HIF-1a group compared with other groups. (b) LO2 cells were transfected with pcDNA NC, pcDNA PGC-1a, pcDNA HIF-1a, or pcDNA PGC-1a+pcDNA HIF-1a and then treated with FFAs, and western blot revealed that Higd1a expression was increased in the FFA+pcDNA PGC-1a+pcDNA HIF-1a group compared with other groups ($^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with FFA+siRNA NC group or FFA +pcDNA NC group; $^{\wedge}P < 0.05$ compared with the FFA+siRNA PGC-1a group or FFA+pcDNA PGC-1a group; $^{\&}P < 0.05$ compared with the FFA+siRNA HIF-1a group or FFA+pcDNA HIF-1a group).

When cells are exposed to high levels of fat, β-oxidation is accelerated. In addition, more electrons may leak from the respiratory chain, and more ROS will be generated [32]. Our study first demonstrated that elevated ROS could promote the expression of Higd1a potentially by upregulating HIF-1a and PGC-1a. Although our research is

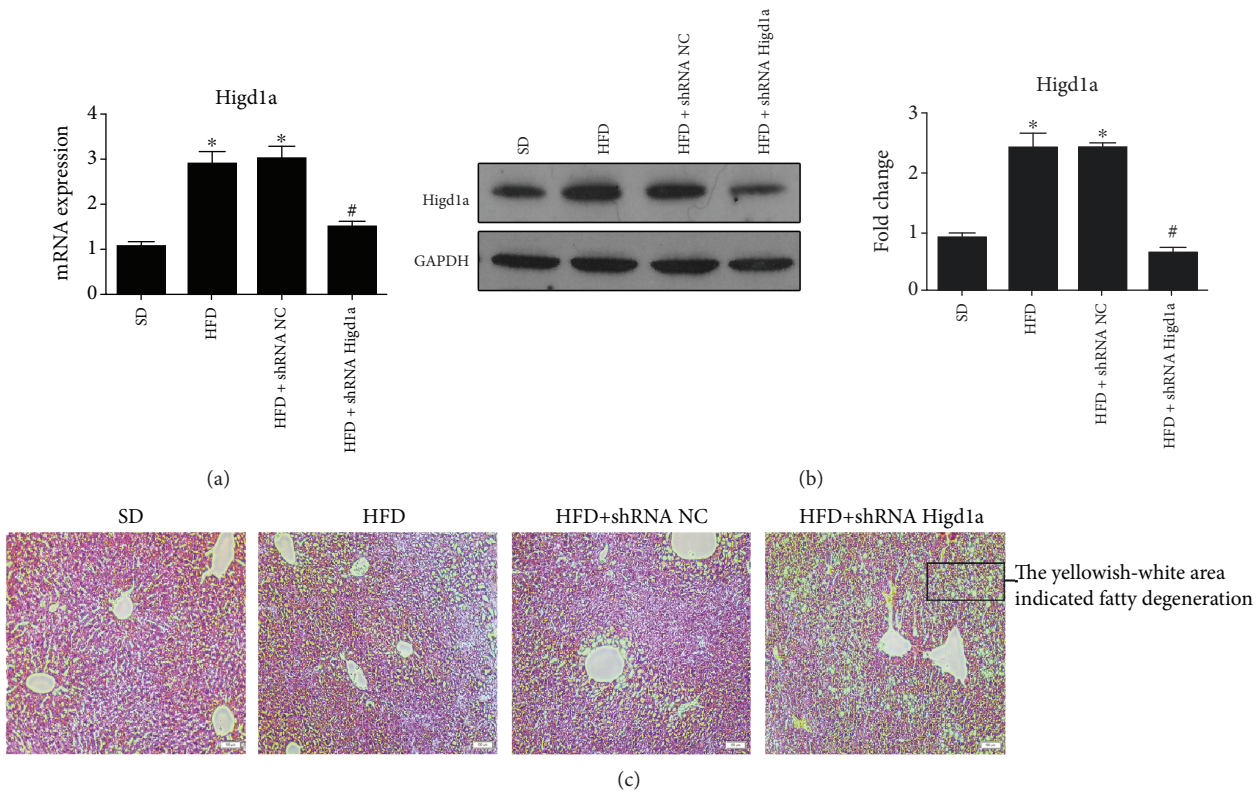


Figure 8: Knocking down Higd1a aggravated liver injury in NAFLD mice. Mice were fed a standard diet (SD group) or high-fat diet (HFD group). Mice fed a HFD were subjected to caudal vein injection with adenoviral virus expressing Higd1a shRNA (HFD+shRNA Higd1a group) or controlled shRNA (HFD+shRNA NC group), and qRT-PCR (a) and western blot (b) were used to measure Higd1a mRNA and protein expression in each group; liver tissues in each group were stained with HE for histological examination, and more severe liver injury was noted in the HFD+shRNA Higd1a group compared with other groups (c) ($^{*}P < 0.05$ compared with the SD group; $^{\#}P < 0.05$ compared with the HFD+shRNA NC group).

preliminary, these results could promote more studies in this field.

In conclusion, our study found that increased Higd1a expression could protect cells from oxidative stress, and ROS induce Higd1a expression by upregulating HIF-1a and PGC-1a expressions upon high-fat exposure.

Data Availability

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

Conflicts of Interest

The authors confirm that there are no conflicts of interest.

Authors' Contributions

TL, WJX, and YZ conceived and designed the experiments. TL, WJX, SJ, YG, and QHY performed the experiments. QCZ and SJ analyzed the data. TL, WJX, and YZ wrote the paper. Tong Li and Wen-jing Xian contributed equally to this study.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: Oil Red staining identified lipid droplets in the FFA (0.4 mM OA + 0.2 mM palmitate) group. Supplementary Table 1: sequences of siRNA. Supplementary Table 2: primer sequences for qRT-PCR used in the study. *(Supplementary Materials)*

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Research Article

DHA Oral Supplementation Modulates Serum Epoxydocosapentaenoic Acid (EDP) Levels in Breast Cancer Patients

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Introduction. The omega-3 polyunsaturated fatty acids, as docosahexaenoic acid (DHA), are considered mediators regulating the resolution of inflammation during cancer and may be associated with better outcomes. Epoxydocosapentaenoic acids (EDPs), metabolites of the DHA, are hypothesized to be responsible for some beneficial effects. In the present study, we aimed to assess the circulating 19,20-EDP levels in breast cancer (BC) patients and in healthy controls before and after DHA oral supplementation and the potential differences in the DHA conversion in 19,20-EDPs between patients with different BC presentations. *Methods*. BC patients and healthy controls were supplemented with DHA (algal oil) for 10 days (2 g/day). Blood samples were collected at baseline (T0) and after supplementation (T1) to assess EDP (19,20-EDP) serum levels by liquid chromatography spectrometry. *Results*. 33 BC patients and 10 controls were studied. EDP values at T0 were not different between patients and controls. At T1, we found an increase in 19,20-EDP levels in BC patients ($P < 0.00001$) and in controls ($P < 0.001$), whereas no differences in 19,20-EDPs were present between the two groups; when considering the type of BC presentation, patients with BRCA1/2 mutation showed lower 19,20-EDPs levels with respect to BC patients without the mutation ($P = 0.03$). According to immunohistochemical subtype, luminal A-like BC patients showed at T1 higher 19,20-EDP levels compared to nonluminal A ($P = 0.02$). *Conclusions*. DHA oral supplementation was associated with increased 19,20-EDP serum levels in BC patients, independent of the type of BC presentation, and in controls. Patients carrier of BRCA1/2 mutation seem to possess lower ability of DHA epoxidation, whereas luminal A-like BC patients showed higher EDP conversion. This behavior should be tested in a larger population.

1. Introduction

Polyunsaturated fatty acids (PUFAs) of the omega-3 series are essential nutrients whose dietary intake, with food and/or supplements, is associated with well-documented health benefits [1, 2]. Omega-3 PUFAs, primarily found in dietary fish oils and in plants, are substrates able to modulate and limit inflammatory responses, likely those that underlie mechanisms of chronic diseases such as cancer [3], including breast cancer (BC) [4].

The omega-3 PUFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid, may be considered modulators of

the mechanisms regulating the onset, prolongation, and resolution of inflammation and, therefore, are believed to be protective against inflammatory response [1].

Metabolites of DHA, named as epoxydocosapentaenoic acids (EDPs), deriving from the conversion of DHA by cytochrome P450 epoxygenases, are suggested to be responsible for some of the beneficial effects attributed to omega-3 PUFAs and omega-3-rich foods, such as fish oil, and to mediate some of the health-promoting effects of DHA [5].

Evidences showed that omega-3 fatty acid metabolites and mainly EDPs mediate several beneficial effects in chronic conditions, including chronic pain and angiotensin II-dependent hypertension, by anti-inflammatory properties, and in kidney disease [6–10].

Experimental studies have documented that specific EDPs - 16,17-EDP and, in particular, 19,20-EDPs - are potent mediators in suppressing inflammation and inhibiting angiogenesis, endothelial cell migration, endothelial cell proliferation, and the growth and metastasis of human breast and prostate cancer [6, 11, 12].

Consumption of omega-3 fatty acid-rich diets raises the circulating and tissue levels of 19,20-EDPs in animals and in humans, which is the most prominent change in the profile of PUFA metabolites determined by dietary omega-3 fatty acids [13–15]. Therefore, it is reasonable to think that the metabolism of DHA to 19,20-EDPs may be responsible, at least in part, for some of the beneficial effects referred to dietary omega-3 fatty acids.

As previously shown [5, 16], 19,20-EDP is the most abundant EDP regioisomer detected *in vivo* with potential positive effects in cancer. In addition, data suggest that in BC patients carrying BRCA1 and BRCA2 mutations, a lipid and metabolite dysregulation is present [17].

In this perspective, we aimed to assess the circulating 19,20-EDPs levels in BC patients and in healthy controls and the potential differences in the DHA conversion in 19,20-EDPs between sporadic BC patients and BC patients with a family history of breast malignancy, either positive or not for BRCA1/BRCA2 gene mutation.

2. Subjects and Methods

This was an interventional, spontaneous, single-center, controlled study performed on patients from the Department of Surgical Sciences, Sapienza—University of Rome, Italy. After approval of the local Ethics Committee and after obtaining written informed consent from each participant, women with diagnosis of BC and healthy women without personal and/or family history of BC, participating to a study conducted to observe changes in DHA levels and omega-3 index before and after DHA oral supplementation [18], were considered. All procedures were in accordance with the ethical standards of the Helsinki Declaration issued in 1975 and later amendments. Exclusion criteria were self-reported consumption of omega-3 PUFA supplements, omega-3 PUFA-supplemented foods in the previous 6 months, and other neoplastic diseases, as previously described [18]. We previously demonstrated that a sample size of 33 BC patients and 10 controls was able to show significant changes in DHA circulating levels and omega-3 index [18]. Therefore, the same study size (and the same population) was used for the aims of the present investigation.

2.1. Breast Cancer Patients and Healthy Controls. We enrolled breast cancer patients at their first diagnosis, before starting any anticancer treatment. We recorded participants' demographic and anthropometric characteristics (age, weight, height, body mass index, and body weight change over the prior 6 months), the presence/absence of comorbidities (i.e., hypercholesterolemia, hypertriglyceridemia, and diabetes), and serum metabolic and nutritional biomarkers. Histological diagnosis, tumor staging, and a detailed medical history were collected. According to international classification, which is based on the familiar and past medical history, the participants were divided into sporadic (S) group, including BC patients without family history of breast malignancy; familiar (F) group, including BC patients with BC familial history, but negative for BRCA1 or BRCA2 gene mutation; and mutated (M) group, including BC patients with documented BRCA1 or BRCA2 gene mutation [18]. We also considered healthy age- and body mass index-matched women, serving as the control group (C) [18]. The questionnaire on participants' self-reported dietary habits was administered focusing on the consumption of seafood and its frequency [19], allowing to identify "low or good seafood consumer" [18].

2.2. DHA Oral Supplementation. We considered women enrolled in a study consisting in assuming 2 g of DHA per day in the form of strawberry-flavored algal oil syrup (from Schizochytrium sp. microalgae) containing omega-3 fatty acids >46% of the total fatty acids (DHA in the triglyceride form) (Richoil® syrup, provided free of charge by DMF Dietetic Metabolic Food, Limbiate, Italy) for 10 consecutive days [18]. Moreover, during those days the participants were prescribed to maintain a standard normo-balanced diet and the usual physical activity level. The participants had a reference telephone number to contact for discussing any difficulties during the supplementation period and to ensure compliance.

2.3. Blood Sample Collection and Epoxydocosapentaenoic Acid Assay. Blood samples were obtained on fasting from all the participants at baseline (T0) and after the 10 days of oral DHA supplementation (T1). Aliquots of serum were stored at -80°C until analysis.

Epoxydocosapentaenoic acid serum levels were assessed by a liquid chromatography–tandem mass spectrometry method. Twenty microliters of serum samples were added to sixty microliters of cold methanol 100%. After extensive vortex (60 sec), samples were maintained at 4°C for ten minutes and then centrifuged at 13,000 revolutions per minutes (rpm) for 15 minutes. Fifty μL of supernatant was directly transferred to an autosampler vial for the injection into the chromatographic system. Ultra-performance liquid chromatography (UPLC) analysis was performed with a Sciex Liquid Chromatography System series ExionLC (AB Sciex, Ontario, Canada). Chromatographic separation

was performed using a reverse-phase column (100 × 2.1 mm, Kinetex Biphenyl, 2.6 μm, 100 Å pore size; Phenomenex, Torrance, USA). The column was maintained at 60°C. The mobile phases were UPLC-grade water (eluent A) and methanol (eluent B); the elution was performed at a flow rate of 600 μL/min, using an elution gradient as follows: firstly 0.5 min with 5% eluent B and 1 min of linear gradient to 95% eluent B, followed by an additional period of 0.5 min in isocratic conditions and finally 1 min of 5% eluent B. The injection volume was 5 μL, and the total run time analysis was 3 minutes. Mass spectrometry was performed on a 5500 triple quadrupole system (QTRAP Sciex, Ontario, Canada) equipped with an atmospheric pressure chemical ionization source (AB Sciex). The detector was set in negative mode. The Q1 and Q3 quadrupoles were tuned for the unit mass resolution. The instrument was set in the multiple-reaction monitoring mode. Mass spectrometer parameters were optimized to maximize sensitivity for each transition (Table 1). Data were acquired and processed with Analyst 1.7.0.

2.4. Statistical Analyses. Patients' characteristics were described using mean ± standard deviation (SD) for continuous normally distributed variables, including EDP levels, and separately by participant group, and percent for dichotomous variables. EDP values were nonnormally distributed and therefore transformed to the natural log (Ln) and one-way analysis of variance (ANOVA) and Student's *t*-test were used to evaluate differences among groups. R version 3.5.1 was used as statistical software.

3. Results

3.1. Participants' Characteristics. Baseline characteristics of the participants are reported in Table 2. In summary, a total of 33 BC patients and 10 healthy women were studied at baseline, well tolerated the daily doses of oral DHA, and completed the supplementation for 10 days, as previously described [18]. The participants were distributed as follows: 10 BC patients in the S group, 12 BC patients in the F group, 11 BC patients in the M group, and 10 women in the C group. Mean age was 47.3 ± 8.9 years for BC patients and 48.3 ± 5.66 years for the C group.

3.2. Epoxydocosapentaenoic Acid Levels at Baseline. We initially tested the potential presence of EDPs in the supplemented algal oil syrup, and none of them were detected.

At T0, no significant differences were observed in 19,20-EDP levels between BC patients and controls ($P = 0.43$), neither between each group of BC patients (S, F, and M groups). No association was found between 19,20-EDP levels and seafood dietary habits.

3.3. Epoxydocosapentaenoic Acid Levels after DHA Supplementation. After DHA supplementation (T1), 19,20-EDP levels significantly increased in BC patients ($P < 0.00001$) (Figure 1(a)) and in controls ($P < 0.001$) (Figure 1(b)). This significant change was confirmed in all the BC subgroups ($P \leq 0.001$). Moreover, we did not find differences in 19,20-EDP levels between BC patients (S, F, and M groups) and controls (Table 2).

Table 1: Mass spectrometry parameters.

Precursor Ion *(m/z)*	Fragments *(m/z)*	DP	EP	CE	CXP
	299.1	-95	-5.5	-13.3	-9.2
343.3	285.1	-95	-5.5	-14.8	-10.7
	241.3	-95	-5.5	-15.1	-11.0

m/z: mass/charge ratio; DP: declustering potential; EP: entrance potential; CE, collision energy; CXP, collision cell exit potential.

Considering that we previously found that the M group presented a different DHA incorporation after oral supplementation compared to the other BC patients [18], we specifically analyzed 19,20-EDP levels in BRCA 1/2 mutation carriers vs the other BC patients without the mutation ($n = 22$), observing that 19,20-EDP levels (ng/dl) were lower in the M group (4.84 ± 0.82 vs 5.61 ± 1.10 $P = 0.03$) (Figure 2).

As for baseline, no association was seen between 19,20-EDP levels and seafood dietary habits.

3.4. Epoxydocosapentaenoic Acid Levels according to Immunohistochemical BC Subtype. Stratifying BC patients according to immunohistochemical subtype, the majority of BC patients ($n = 17$, 52%) presented as luminal A, 6 as luminal B, 6 as triple negative, and 4 patients as Her2-positive. At T0, no significant differences were observed in 19,20-EDP levels between the different immunohistotypes.

At T1, we found that 19,20-EDP levels (ng/mL) in luminal A patients were significantly higher with respect to patients with other immunohistotypes (5.75 ± 1.06 vs 4.93 ± 0.93; $P = 0.02$) (Figure 3).

4. Discussion

Our data show that DHA oral supplementation in BC patients was able to change not only the circulating DHA levels and omega-3 index [18] but also 19,20-EDP serum levels, which have been correlated with cancer growth and progression [14]. In particular, by a 10-consecutive day DHA algal oil (2 g/day) oral supplementation, we found a significant increase in 19,20-EDP serum levels in BC patients and in healthy women (control group). Moreover, in each of the 3 BC groups (S group, F group, and M group), we observed a significant increase in EDP serum levels. However, no significant changes from baseline in 19,20-EDP levels were documented between BC groups and controls.

Our results, obtained using an easy-to-perform and well-tolerated supplementation, show the importance of modifying a specific metabolic profile (lipid metabolites) that might have an impact on the prognosis, and possibly on the therapeutic response, in BC patients. This may have potential implications in specific BC presentations, such as patients carrying BRCA 1/2 mutation, where the therapeutic options may be limited or with possible lower efficacy. In fact, our data showed that, after DHA oral supplementation, 19,20-EDP levels were lower among the M group with respect to the other BC patients. However, due to the small number of patients in the M group, these results should be

Table 2: Participants' characteristics.

All participants $N = 43$	S group $N = 10$	F group $N = 12$	M group $N = 11$	C group $N = 10$
Age, years	49.4 ± 9.8	47.92 ± 8.46	44.82 ± 9.59	48.3 ± 5.66
Body weight, kg	62.8 ± 12.43	69.42 ± 11.87	58.91 ± 9.44	60.6 ± 7.4
BMI, weight (kg)/height^2 (m)	23.68 ± 4.08	25.61 ± 4.52	22.32 ± 2.65	23.68 ± 2.93
Cholesterol, mg/dL	203.2 ± 31.36	210.09 ± 22.33	206.75 ± 35.57	217.7 ± 31.77
Glycemia, mg/dL	96.2 ± 8.85	92 ± 8.10	94.33 ± 12.26	88.72 ± 9.93
Comorbidities[#] (y/no)	3/7	3/9	2/9	1/9
Ln EDPs (ng/mL) at T0	3.44 ± 0.74	3.64 ± 1.22	3.16 ± 0.72	3.16 ± 0.79
Ln EDPs (ng/mL) at T1	5.84 ± 1.05	5.41 ± 1.15	4.84 ± 0.82	5.03 ± 0.99

[*]Data are shown as mean ± SD. P values are not significant for the patients' characteristics shown between groups. Abbreviations include: S: sporadic group; F: familiar group; M: mutated group; C: control group; BMI: body mass index; EDPs: epoxydocosapentaenoic acids. [#]Dyslipidemia and type 2 diabetes.

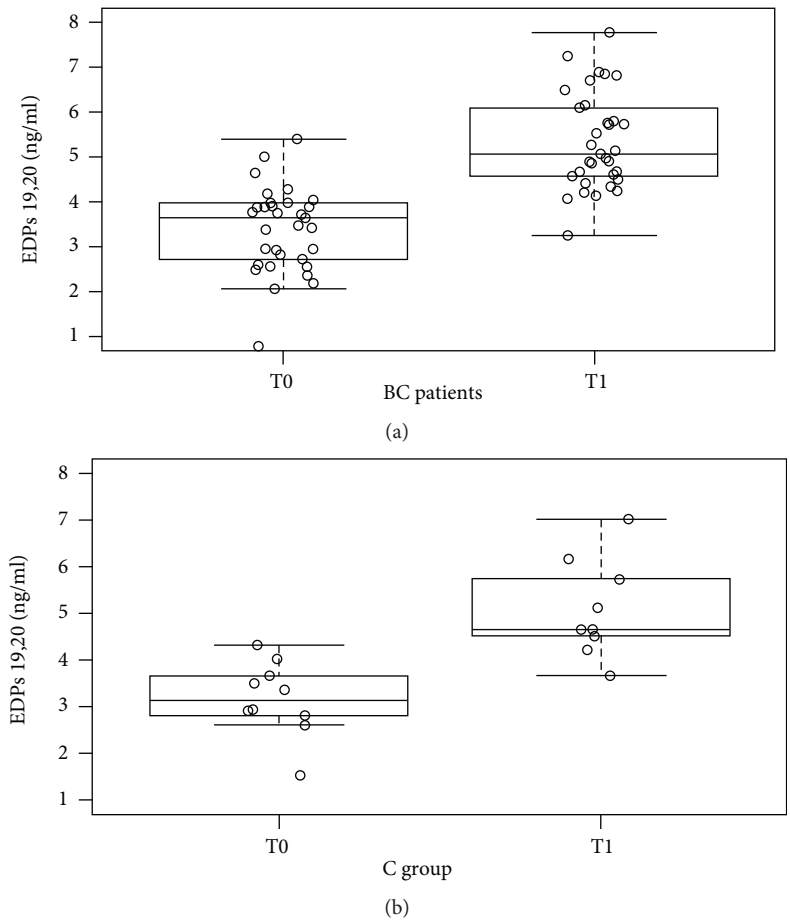

Figure 1: (a) Whisker plot of 19,20-EDP levels (ng/mL) in BC patients at baseline (T0) and after supplementation (T1). 19,20-EDP levels were higher at T1 with respect to T0 ($P < 0.00001$). Abbreviations: EDPs: epoxydocosapentaenoic acids; BC: breast cancer. (b) Whisker plot of 19,20-EDP levels (ng/mL) in C group at baseline (T0) and after supplementation (T1). 19,20-EDP levels were higher at T1 with respect to T0 ($P < 0.001$). Abbreviations: EDPs: epoxydocosapentaenoic acids; C group: control group.

further confirmed given the high heterogeneity of patients with BC, whose metabolic characteristics, which might be related to the disease progression and likely to a different therapeutic response, may profoundly differ depending on the type of BC presentation and/or immunohistotype. In this respect, we also found that patients presenting with luminal A subtype showed, after supplementation, higher 19,20-EDP levels compared to patients presenting with a nonluminal A subtype.

This result appears interesting considering that luminal A is the most favorable BC subtype [20], and no data are available in the literature on the possible relation

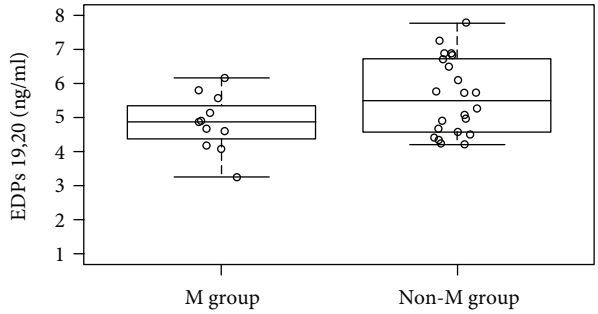

Figure 2: Whisker plot of 19,20-EDP levels (ng/mL) after supplementation (T1) in BC patients BRCA 1/2 mutation carrier (M group) and BC patients without the mutation (non-M group). 19,20-EDP levels were lower in the M group with respect to the non-M group (4.84 ± 0.82 vs 5.61 ± 1.10, $P = 0.03$). Abbreviations: EDPs: epoxydocosapentaenoic acids; BC: breast cancer.

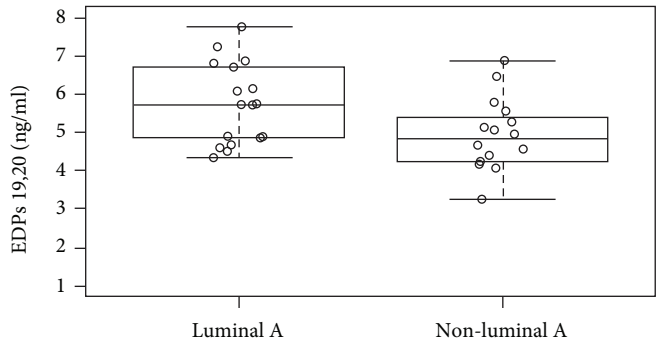

Figure 3: Whisker plot of 19,20-EDP levels (ng/ml) after supplementation (T1) in BC patients stratified by luminal A BC subtype or non-luminal A BC subtype. 19,20-EDP levels were higher in luminal A subtype with respect to non-luminal A subtype (5.75 ± 1.06 vs 4.93 ± 0.93, $P = 0.02$). Abbreviations: EDPs: epoxydocosapentaenoic acids; BC: breast cancer; T1: after 10 days of DHA oral supplementation.

between epoxides and prognosis in this clinical setting. However, serum 19,20-EDP levels of both luminal A and non-luminal A BC subtype did not differ from controls, although the sample size for this analysis is very small limiting its interpretation.

Metabolic derangements are common during BC [1]. Data obtained among patients presenting as BRCA mutation carriers indicate that the prevalence of metabolic disorders, in particular insulin resistance, is high [21]. Low insulin sensitivity and high diabetes rate have been observed in a 15-year follow-up in a group of BRCA mutation carriers who developed BC [21]. Considering that obesity and greater BMI values are modifiable risk factors for diabetes in BC patients with BRCA mutation, attention to novel metabolic and nutritional interventions, including omega-3 PUFA supplementation, should be implemented [4].

Previous clinical studies showed an increased formation of EDPs in humans upon DHA supplementation, indicating that our results are directly correlated with the effects of DHA oral intake [16, 22, 23]. In particular, Fischer et al. showed that EPA/DHA supplementation for 8 weeks doubled the levels of all regioisomeric DHA-derived metabolites, including 19,20-EDPs [24]. This behavior was confirmed by the results of our study.

Supplementation of DHA increases the levels of EDPs in most organs [5], and compared with other PUFA metabolites, EDPs are at least one thousand times more potent in terms of anti-inflammatory modulation [25]. These results further argue that EDP levels upon DHA supplementation cause potential multiple beneficial effects [21, 25].

Chronic inflammation is known as a potent contributor of BC growth and progression, playing a major role in the neoplastic process. Although hormonal modifications seem to play a driving role in breast carcinogenesis, the increased cytokine production may be a clinically relevant feature in BC [4]. However, how this condition has an impact on treatments' efficacy has to be better elucidated.

Several studies evaluated the effects of the supplementation of omega-3 PUFAs, including DHA, in resolving the inflammatory process [1, 2]. More importantly, this therapeutic strategy had a positive effect on patients' prognosis [4, 26]. Zhang et al. demonstrated that 19,20-EDP inhibits angiogenesis *in vitro* and *in vivo* and primary tumor growth [11]. To determine whether the anticancer effect was obtained from 19,20-EDP or from its metabolite derived by soluble epoxide hydrolase, the 19,20-dihydroxydocosapentaenoic acid (19,20-DiHDPA), the authors tested the effect of 19,20-DiHDPA on Met-1 tumor growth. The continuous infusion of 19,20-DiHDPA in mice did not have any effect on tumor growth, confirming that the anticancer effect was not from this diol metabolite [11]. Additional evidences indicate that inhibition of soluble epoxide hydrolase prevents vascular damages mediated by 19,20-EDPs [11]. However, in our clinical setting, information on how much

DHA supplementation is necessary to reach an effective EDP concentration is not available.

Although our study involved a homogeneous population of BC patients from a single breast cancer unit, it presents several limitations. Our cohort of participants is small, and the BC patients involved may not be representative of larger BC patients' population, possibly presenting a high individual intervariability. The limited sample size of each subgroup of participants might have reduced the possibility of identifying association between patients' characteristics and EDP levels.

Moreover, we believe that a relevant aspect that should be further evaluated is the association between EDP levels and prognosis in BC patients.

5. Conclusions

The prevention and treatment of BC represent a relevant public health issue, and research on the relationship between BC, diet, metabolic intervention, and lifestyle should be implemented.

The human data that we obtained should be confirmed in a larger population and, more importantly, evaluated in a longitudinal fashion related to patients' outcomes.

Data Availability

The data, including laboratory analyses, used to support the findings of this study are restricted by our Local Ethics committee [Azienda Policlinico Umberto I, Rome, Italy] in order to protect patient privacy. Data are available from Prof. Maurizio Muscaritoli (senior author, email: maurizio.muscaritoli@uniroma1.it) for researchers who meet the criteria for access to confidential data.

Conflicts of Interest

Authors have no potential conflicts of interest.

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