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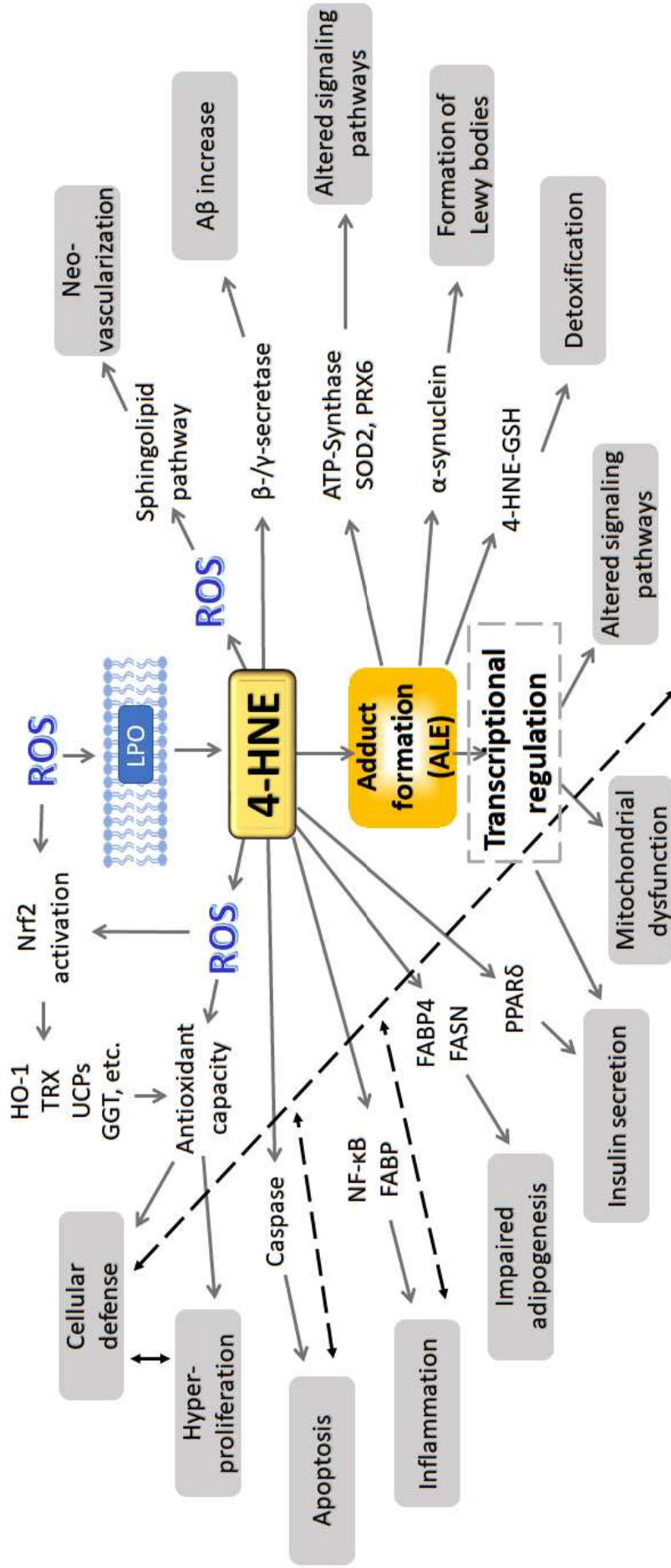
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# Neurodegeneration



## Psoriasis

## Metabolic syndrome

## Carcinogenesis – Cancer Regression

# The Relevance of Pathophysiological Alterations in Redox Signaling of 4-Hydroxynonenal for Pharmacological Therapies of Major Stress-Associated Diseases

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## Abbreviations

4-HNE, 4-hydroxy-2-nonenal; AD, Alzheimer's disease; AGEs, advanced glycoxidation end products; ALE, advanced lipoxidation end-products; AM, adrenomodulin; ANT, adenine nucleotide translocase; AP-1, activator protein 1; AR, aldose reductase; BAECs, bovine aortic endothelial cells; CRC, colorectal cancer; DHN, 1,4-dihydroxynonene; EMT, the epithelial-to-mesenchymal transition; FABP, fatty acid binding protein; FFPE, formalin-fixed paraffin-embedded; GCL, glutamate cysteine ligase; HIF, hypoxia-inducible factor; HMEC-1, human microvascular endothelial cells; HNSCC, head and neck squamous cell carcinoma; IEC-6, rat small intestine epithelial cells; IRS-1, insulin receptor substrate-1; JAK, janus kinase; MKN-45, human gastric adenocarcinoma cells; MMP, matrix metalloproteinase; MRP1, multidrug resistance-associated protein 1; mtROS, mitochondrial ROS; NAT, normal tissue adjacent to tumor; ND, neurodegenerative disease; NET, neutrophil extracellular traps; PD, Parkinson's disease; PR+BC, progesterone receptor positive breast cancer; RAMPs, receptor-activity-modifying proteins; RCS, reactive carbonyl species; SAEC, human small airway epithelial cells; SePP, selenoprotein P; SFN, sulforaphane; STEAP4, six-transmembrane epithelial antigen of prostate 4; TASA, tumor-adjacent specific activation; tDC, tumor-associated dendritic cells; THP-1, human monocytic cells; TNBC, triple-negative breast cancer; TNFR1, tumor necrosis factor receptor 1; VSMC, vascular smooth muscle cells; WB, western blot; XBP1, X-box binding protein 1;  $\Delta\psi_m$ , mitochondrial membrane potential

**Highlights**

- 4-HNE plays important roles in pathophysiology of major stress-associated diseases
- 4-HNE affects signaling pathways in concentration- and cell-type dependent manner
- Pathophysiological effects of 4-HNE mostly involve relevant protein modifications
- Current pharmacological designs are unaware of pathophysiological roles of 4-HNE
- Integrative biomedicine should tackle the pathophysiology of 4-HNE signaling

**Abstract**

Modern analytical methods combined with the modern concepts of redox signaling revealed 4-hydroxy-2-nonenal (4-HNE) as particular growth regulating factor involved in redox signaling under physiological and pathophysiological circumstances. In this review current knowledge of the relevance of 4-HNE as “the second messenger of reactive oxygen species” (ROS) in redox signaling of representative major stress-associated diseases is briefly summarized. The findings presented allow for 4-HNE to be considered not only as second messenger of ROS, but also as one of fundamental factors of the stress- and age-associated diseases. While standard, even modern concepts of molecular medicine and respective therapies in majority of these diseases target mostly the disease-specific symptoms. 4-HNE, especially its protein adducts, might appear to be the bioactive markers that would allow better monitoring of specific pathophysiological processes reflecting their complexity. Eventually that could help development of advanced integrative medicine approach for patients and the diseases they suffer from on the personalized basis implementing biomedical remedies that would optimize beneficial effects of ROS and 4-HNE to prevent the onset and progression of the illness, perhaps even providing the real cure.

**Keywords:** 4-hydroxynonenal, lipid peroxidation, oxidative stress, metabolic syndrome, diabetes mellitus, obesity, inflammation, cardiovascular diseases, atherosclerosis, neurodegeneration, Alzheimer’s disease, Parkinson’s disease, cancer, growth control, anti-cancer therapy, redox signaling, psoriasis

## Introduction

For many decades reactive aldehydes represented by the “substance reactive with thiobarbituric acid” (TBARS) were considered mostly as toxic by-products (or the end products) of oxidative degradation of lipids, notably of poly-unsaturated fatty acids (PUFAs). However, the last years brought many important changes increasing substantially our knowledge in the fields of oxidative stress research and lipid (per)oxidation. Modern analytical methods combined with the modern concepts of redox signaling revealed in particular 4-hydroxy-2-nonenal (4-HNE) as growth regulating factor involved in redox signaling under physiological and pathophysiological circumstances. Therefore, in this paper the authors shortly summarize current knowledge of the relevance of 4-HNE as “the second messenger of reactive oxygen species” (ROS) in redox signaling of representative major stress-associated diseases.

## 4-HNE, a bioactive marker of oxidative stress

While ROS, were firstly perceived as detrimental inducers of different diseases, such as cancer, nowadays, their importance in redox signaling and proper cellular functioning cannot be neglected as well. As recently suggested, different signals/stressors sensed by cells (both normal and malignant) urge them to coordinate their response to these signals, and ultimately direct their fate into decay or survival, even enhanced growth. Production and fine balance of ROS that will specifically target necessary signaling pathways seem to be a substantial mechanism involved. Thus produced ROS are not just orchestrating the fate of the cells that produced them but also other cells within the tissue [1]. In this context, ROS are acting as signaling molecules that modify specific, mainly cysteine, residues of a target protein to regulate cellular processes from proliferation to differentiation and apoptosis. The types of ROS and the vicinity of the target protein determines whether signal will be translated further. Therefore, their production and fine-tuning is intertwined with the metabolism and antioxidative machinery. Beside proteins, ROS can also affect other macromolecules such as lipids and DNA.

Oxidation of lipids results in a generation of reactive aldehydes, such as 4-HNE, in a process known as lipid peroxidation. 4-HNE is considered as a secondary messenger of ROS with a broad, highly concentration-dependent and cell/tissue-specific, regulatory functions. Its pleiotropic mode of action involves its three functional groups (carbonyl group, C=C double bond, and hydroxyl group) in reactions with different macromolecules such as proteins, DNA and lipids [2–4]. Once formed, 4-HNE is rapidly metabolized by forming adducts with glutathione (GSH) (direct or catalyzed by glutathione S-transferases (GST)); by oxidation to 4-hydroxy-2-nonenoic acid catalyzed by aldehyde dehydrogenases (ALDH); by reduction to 1,4-dihydroxynonene (DHN) catalyzed by alcohol dehydrogenase (ADH); by reduction of 4-HNE and GSH-4-HNE to GSH-DHN by several members of the aldo-keto reductase family such as aldose reductase (AR) [5,6]. Detoxification mechanisms vary among different cell/tissues. Therefore, levels of 4-HNE (and consequently its bioactivities) depend on the severity of oxidative stress, the structure of PUFAs, their content and distribution, especially of  $\omega$ -6 PUFA: linoleic and arachidonic acids, and the levels of the 4-HNE-metabolizing enzymes and GSH. Thus, in a concentration-dependent manner, 4-HNE can act as a cytotoxic, genotoxic and mutagenic agent or as a signaling molecule that is known to regulate proliferation, differentiation, apoptosis, senescence, autophagy and cell cycle arrest (reviewed in [3,4,7,8]).

The abilities of 4-HNE to act as a signaling molecule rely on its covalent binding to proteins and changing their activity. Hence, the reactions of this particularly reactive aldehyde are rather selective, preferring some amino acid residues over others with different reactivity among them, which is a modified signaling protein-dependent as well. Cysteine (Cys), histidine and lysine residues of proteins are the predominant targets of 4-HNE's binding to proteins and Michael adduction being preferred modification over Schiff base adduction [9,10]. Cys residues are the main targets for 4-HNE induced protein modifications, although it also forms adducts with other amino acids like Histidine and Lysine but with lower affinity [11]. Today, numerous targets for 4-HNE adduct formation have been identified [5].

#### **4-HNE can mediate redox signaling pathways**

Redox signaling is needed for the normal physiology of cells but is also involved in a variety of pathophysiological processes [12]. Mechanisms of redox signaling comprise of reversible oxidation and reduction of molecules involved in cellular signaling pathways or covalent modification of signaling proteins by ROS such as peroxides or reactive aldehydes represented by 4-HNE [13].

Redox signaling depends on the kinetics, concentration, and location of reactive species and target molecules. The diffusion distance of reactive species is of particular importance for ROS, such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), that have shorter lifetime than reactive aldehydes like 4-HNE [14]. This is particularly evident in the process of inflammation where a variety of ROS are produced during oxidative burst of granulocytes. Thus, depending on the amount and location of released ROS during respiratory burst of granulocytes, ROS may have opposite effects, such as tumor promotion or suppression [15,16], while 4-HNE may be of crucial importance for understanding such a complex pathophysiology of oxidative stress in cancer development. Namely, in the vicinity of the cellular or extracellular lipids containing PUFAs, granulocyte derived ROS can induce 4-HNE formation. In a manner of positive feedback loop, at higher concentrations 4-HNE can further modulate oxidative burst of granulocytes through modification of glyceraldehyde 3-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that regulate redox signaling suppressing ROS formation [17]. However, such effects were not observed for the lower 4-HNE levels [18]. Complexity of the opposing regulatory effects of 4-HNE depending on its amount and the availability of target macromolecules has been revealed in different pathophysiological processes. For example, the 4-HNE mediated neovascularization has concentration dependent effects on redox signaling in a way that low levels of 4-HNE ( $\leq 1 \mu\text{M}$ ) provoke neovascularization via ROS and activation of sphingolipid pathway, while higher concentration does not have such effects [19]. Similarly, the recent study by Dodson and colleagues has shown that at low concentration (5 – 10  $\mu\text{M}$ ) 4-HNE specifically targets and modifies proteins involved in the initiation of autophagy, such as mTOR and Beclin1 [20]. On the contrary, at higher concentration 4-HNE ( $\geq 15 \mu\text{M}$ ) suppresses autophagy while it targets mitochondrial adenosine triphosphate (ATP) synthase inducing mitochondrial dysfunction [20].

Furthermore, we have recently reported that 4-HNE at low concentrations ( $< 1.2 \mu\text{M}$ ) cooperates with singlet oxygen to inactivate membrane-associated catalase specifically in malignant cells, while at higher concentration 4-HNE is able to directly inactivate both membrane-associated and cytoplasmic catalase in a cascade of cytotoxic events that destroy malignant cells through



apoptosis or necrosis [21]. The 4-HNE mediated redox signaling in carcinogenesis will be discussed in detail later in this paper.

Redox signaling also has a detrimental role in reproductive biology. Ageing oocytes accumulate ROS and are thought to represent main contributors for oocyte health deterioration. Indeed, increased ROS levels promote 4-HNE generation in oocytes in a concentration dependent manner. Elevated 4-HNE further promotes cytosolic ROS production in oocytes and also covalently binds to tubulins affecting oocyte health and meiotic development [22]. Interestingly, in a similar manner 4-HNE affects budding of the single-cell organism of *Saccharomyces cerevisiae* [23], while in human fetal development it 4-HNE-protein adducts could also accumulate in placenta thus affecting the growth of fetus [24].

One of the primary sensors and oxidative stress regulators is nuclear factor erythroid 2-related factor 2 (Nrf2). Inactive Nrf2 is normally present in the cytoplasm sequestered by Kelch-like ECH-associated protein 1 (Keap1). After direct attack of ROS or in the presence of 4-HNE, Nrf2 is released from Keap1 repression, and rapidly translocates to nucleus [25,26]. These effects are attributed to 4-HNE modification of Keap1 Cys151 and Cys288 altering Keap1 structure and Nrf2 repression [27]. Nrf2 in the nucleus binds to antioxidant response elements triggering expression of antioxidative and phase II drug metabolizing/detoxifying genes, among which the redox signaling mediators are heme oxygenase-1 (HO-1), thioredoxin (TRX), thioredoxin reductase (TrxR), glutamate cysteine ligase (GCL) and  $\gamma$ -glutamyl transpeptidase (GGT). Both, HO-1 and TRX are antioxidant proteins while GCL and GGT are the key players in the GSH synthesis, homeostasis and metabolism [4,26,28–30]. Additionally, GGT can also be induced via 4-HNE activated p38 mitogen-activated protein kinase (p38MAPK) pathway [31]. Recent study demonstrated that uncoupling protein (UCP)-3 expression in cardiomyocytes, also under control of Nrf2 transcription factor, is triggered by 4-HNE altering cellular bioenergetics [32]. Beside modulation of p38MAPK pathway the role of 4-HNE in the regulation of other kinase signaling pathways has been well documented [33,34].

**Table 1. Some redox signaling pathways mediated by 4-HNE in a concentration- and cell-type specific manners.**

Cell type	4-HNE conc	Effect / Target	Mechanism	Reference
THP-1	20 $\mu$ M	Tissue factor	Decryption via mtROS formation and TRX inhibition	[35]
MKN-45 and PC-3	<1.2 $\mu$ M	Membrane-associated catalase	Inactivation via tumor-derived ROS and formation of $^1\text{O}_2$	[21]
	1.2 - 10 $\mu$ M		Direct inactivation	
HMEC-1	0.5 - 1 $\mu$ M	Angiogenesis	Neovascularization through redox-dependent sphingolipid pathway	[19]
	2.5 - 20 $\mu$ M		No effect	
Granulocytes (rat)	12.5 $\mu$ M	Oxidative burst	No effects	[18]
Granulocytes (human)	30 $\mu$ M		Inhibition	[17]

Isolated mitochondria	35 $\mu$ M	Mitochondrial uncoupling	Activate through UCPs and ANT	[36], [37]
SAEC	25 $\mu$ M	Mitochondrial dysfunction	Reduces $\Delta\psi_m$ , oxygen consumption and inhibits TRX while promotes mtROS	[38]
IEC-6	80 $\mu$ M	Apoptosis	Induces apoptosis via ROS generation, MKP-1 degradation and ERK1/2 activation	[39]
IPEC-1	40 $\mu$ M			
VSMC	1 - 30 $\mu$ M	Apoptosis	Induces apoptosis through mtROS	[40]
VSMC	1 $\mu$ M	MMP production	Induces MMP-2 via mtROS mediated AKT/NF $\kappa$ B signaling activation	[41]
Oocytes (mice)	5 - 20 $\mu$ M	Deterioration of oocyte health	Increases cytosolic ROS and binds to tubulins	[22]
Primary Neurons	5 - 10 $\mu$ M	Autophagy	Activation of neuron autophagy targeting proteins involved in autophagy initiation	[20]
	$\geq 15$ $\mu$ M		Inhibition of autophagic flux and induction of mitochondrial dysfunction targeting ATP synthase	
BAECs	1 $\mu$ M	Antioxidant defense	Modification of TRX1 affecting redox balance and promoting monocyte binding	[42]
Primary subcutaneous preadipocytes (human)	10 $\mu$ M	Adipogenesis	Induces ROS, catalase activity, and impairs adipogenesis	[43]

BAECs - Bovine aortic endothelial cells; HMEC-1 - Human microvascular endothelial cells; IEC-6 - rat small intestine epithelial cells; IPEC-1 - Intestinal porcine epithelial cell; MKN-45 - Human gastric adenocarcinoma cells; PC-3 - Human prostate adenocarcinoma cells; SAEC - Human small airway epithelial Cells; THP-1 - Human monocytic cells; VSMC - Vascular smooth muscle cells.

### Mitochondrial redox signaling modulated by 4-HNE

Mitochondria have the central role in cellular bioenergetics and are also one of the main sites of cellular ROS production [44]. Mitochondrial ROS (mtROS), in the form of superoxide ( $O_2^{\bullet-}$ ) are formed during oxidation of electron transport chain metabolic intermediates. Beside,  $\alpha$ -ketoglutarate dehydrogenase complex, pyruvate dehydrogenase complex, branched-chain keto acid dehydrogenase, 2-oxoadipate dehydrogenase and *sn*-glycerol-3-phosphate dehydrogenase also contribute to significant amounts of mtROS in specific tissues [45–47]. The levels of mtROS can also to some extent be affected by other factors such as protein S-glutathionylation [48] and the level of mitochondrial membrane potential ( $\Delta\psi_m$ ) [49,50]. Another main source of cellular ROS production are NADPH oxidases (NOXs). The NOX-derived cytosolic ROS, including  $H_2O_2$  or mtROS, can activate redox-sensitive kinases and promote further ROS generation either in the cytosol or mitochondria [51,52]. The NOX4, on the other hand, is localized in the inner membrane of mitochondria where it directly contributes to onset of mtROS [53]. The  $O_2^{\bullet-}$  and  $H_2O_2$  are the main mtROS, and their overall concentration is modulated by delicate antioxidant defense system. Majority of  $O_2^{\bullet-}$  is rapidly removed by superoxide dismutase (SOD) enzymes yielding  $H_2O_2$  [54],



that can further be degraded via either GSH and Trx-2 systems or catalase [46,55,56]. Excess mtROS can damage mitochondrial macromolecules and impair normal mitochondrial function [55]. Mitochondrial phospholipids that contain PUFAs, among which is cardiolipin, are one of the main mtROS targets resulting in formation of 4-HNE [57]. Bioactive 4-HNE can modulate a number of mitochondrial signaling pathways either directly or indirectly through covalent modification with macromolecules, such as proteins altering their structure and function [11]. Experiments on isolated mitochondria, reported that 4-HNE induces mitochondrial uncoupling through UCPs and the adenine nucleotide translocase (ANT) mitigating excessive mtROS production [36,37]. However, high  $\Delta\psi_m$  is required before 4-HNE can trigger the mild uncoupling [58]. Interestingly, 4-HNE was shown to reduce, in a concentration-dependent manner,  $\Delta\psi_m$  of small airway epithelial cells (SAEC) mitochondria [38]. The same study also demonstrated that 4-HNE reduces mitochondrial oxygen consumption and TRX, while it promotes mtROS. In addition 4-HNE induced mtROS formation and inhibited TRX system affecting phosphatidylserine externalization leading to induction of tissue factor decryption from cryptic to prothrombotic [35]. Especially important role of 4-HNE on mtROS generation of vascular smooth muscle cells (VSMC) has been well documented [40,41]. Namely, depending on the levels of 4-HNE, the 4-HNE-induced mtROS generation can mediate apoptosis of VSMC [38] or affect serine/threonine kinase (AKT)/nuclear factor  $\kappa$ B (NF $\kappa$ B) signaling pathways enhancing matrix metalloproteinase (MMP)-2 production [35]. In an excellent review, a range of 4-HNE induced vascular cells redox signaling effects have been summarized, as well as 4-HNE roles in the pathogenesis of vascular diseases [59].

### Metabolic syndrome and 4-HNE signaling

Pancreatic  $\beta$ -cells are particularly vulnerable to excessive ROS as they express lower levels of antioxidant defense systems (e.g. SOD and catalase) compared to other cells [60]. Nevertheless, although key proteins necessary for normal cellular functions are sensitive to excessive ROS, redox signaling is crucial for normal insulin secretion and  $\beta$ -cell function. This has been described in details in a comprehensive review [61].

Nutrient overload impairs ETC of mitochondria resulting in an overproduction of  $O_2^{\bullet-}$  and mitochondrial dysfunction [61]. Studies on cardiac cells suggested that harmful effects of high glucose - induced mtROS could be diminished by overexpression of mitochondrial peroxiredoxin-3 [62]. Additionally, high glucose alters NADPH oxidase and lipoxygenases (LOXs) promoting intracellular ROS [63]. Glucose autooxidation and glycation of proteins also contribute to nutrient overload inducing oxidative stress [64]. Up to certain extent  $O_2^{\bullet-}$  and 4-HNE may induce mild uncoupling thus protecting  $\beta$ -cells from oxidative damage. Activated UCPs are also suggested to play role in insulin secretion in the state of nutrient overload [65]. A  $\beta$ -chain of F-ATP synthase, major cellular ATP producer, as well as FAD-containing subunit of succinate dehydrogenase are other mitochondrial targets of 4-HNE [66,67].

It was also shown that high glucose activates phospholipase A2, leading to hydrolysis of fatty acids in the membrane phospholipids of  $\beta$ -cells. Arachidonic and linoleic fatty acids are then released to the intracellular space where there are (per)oxidized to 4-HNE [68]. Nutrient overload also activates 12-LOX in pancreatic islets, thus catalyzing oxidation of PUFA-s and 4-HNE generation [69,70]. Insulin resistance and microvascular dysfunction are some of the key features in metabolic syndrome and 4-HNE was shown to play a role in both processes. In the former, 4-HNE

compromises function of transient receptor potential vanilloid subtype-1 through Cys621, contributing to microvascular dysfunction in metabolic syndrome [71], which is a complex multifactorial process.

Under very frequent alterations of systemic metabolic preconditions, such as obesity or insulin resistance, there is a strong inflammatory response within adipose tissue with interleukin (IL)-6 being the major inflammatory mediator. Locally secreted IL-6 was reported to affect adipogenesis at least to some extent in progression of obesity-associated metabolic syndrome [72]. Inflammatory process further contributes to increased 4-HNE in metabolic syndrome via action of cyclooxygenases (COXs) [73]. The  $\beta$ -cells can tolerate up to 25  $\mu$ M 4-HNE and retain normal glucose-stimulated insulin secretion [74], however at higher concentrations 4-HNE causes  $\beta$ -cell dysfunction [72,75]. The 4-HNE further enhances the activity of nuclear receptor peroxisome proliferator-activated receptor (PPAR)- $\beta/\delta$  [76–78] triggering the detoxification process through amplification of insulin secretion [67]. These data suggest an important adaptive response of  $\beta$ -cells to nutrient overload with 4-HNE activated PPAR $\delta$  as a key player [73].

Adipocytes from insulin resistant individuals tend to accumulate more intracellular 4-HNE during in vitro differentiation process which could be attributed to lower levels of antioxidant defense systems [43]. Acute and chronic exposure to physiological 4-HNE decreases lipid content, promotes adipogenic and lipolytic gene expression and alters adipogenic proteins and adipokines in 3T3-L1 adipocytes [79]. In adipose tissues 4-HNE induces activation of the protein kinase A and inhibition of AMP-activated protein kinase pathways contributing to obesity-related lipolytic activation [80].

Excessive 4-HNE promotes fatty acid synthesis and inhibits beta-oxidation of fatty acids leading to fat accumulation [78]. Furthermore, 4-HNE alters glucose and fatty acid uptake, elevates intracellular ROS and protein oxidation levels in adipocytes [81]. Proteomic studies revealed Cys117 of adipocyte fatty acid binding protein as 4-HNE target, impairing its affinity for fatty acids [82]. Our recent studies on primary human subcutaneous preadipocytes derived from either insulin sensitive or insulin resistant individuals, provided new insights in regulatory role of 4-HNE. Acute and chronic preadipocyte exposure to physiological 4-HNE inhibited preadipocyte growth, impaired adipogenesis and promoted insulin resistance [77]. The 4-HNE downregulated key pro-adipogenic genes SREBF1 and FASN, while it had opposite effect on antiadipogenic genes like fatty acid binding protein (FABP)-4. Moreover, exposure to 4-HNE reduced phosphorylation of Ser636 of insulin receptor substrate-1 (IRS-1) in differentiating human preadipocytes. In 3T3 L1 adipocytes 4-HNE was found to adduct to IRS-1/-2 promoting its degradation and a marked decrease [83].

Modulatory role of 4-HNE in adipose tissue metabolic dysfunction associated with insulin resistance was later confirmed for the omental tissue derived preadipocytes as well [84]. We found that 4-HNE has adverse effects in vitro in case of adipocytes obtained from insulin-sensitive or from insulin-resistant patients, which might be related further to the therapeutic effectiveness of metformin, the natural-substance based medicament which acts through regulation of cyclic adenosine monophosphate and protein kinase activities. Complementary to that, recent study suggested that carnitine acid may improve 4-HNE-induced insulin resistance and inverse the inhibition of IRS-1 phosphorylation [85].

In conclusion we propose 4-HNE to be considered as one of major etiopathogenic factors of the metabolic syndrome, because it might play important role in the cellular energy control and in systemic metabolic alterations building the vicious circle of obesity, diabetes mellitus and

cardiovascular diseases as stress-associated diseases of the modern society. In favor of this assumption is also observed accumulation of 4-HNE originating from the oxidized low-density lipoprotein (oxLDL) in atherosclerotic blood vessels, as well as within and around blood vessels in the adipose tissue of patients with metabolic syndrome [84].

### **Redox signaling pathways involving 4-HNE in cardiovascular disorders and related therapies**

Atherosclerosis is a disease of medium and large size arteries caused by progressive intimal accumulation of oxLDL and proliferation of arterial, i.e. VSMC. There are numerous risk factors of atherosclerosis including metabolic syndrome, hypertension, hypercholesterolemia, diabetes mellitus, cigarette smoking, obesity, stress, aging as well as gender and genetic susceptibility. The LDL-oxidation generated lipid peroxidation products, in particular 4-HNE, modify proteins and other biomolecules of arterial wall inducing chronic inflammatory reaction. In thus generated advanced lesions endothelium breaks down and platelets aggregation with fibrin occurs in an inflammatory process associated with neovascularization of atherosclerotic lesions. The expansion of such lesions, together with thrombosis produce occlusion of the arteries and subsequent major atherosclerotic complications including ischemic heart disease, myocardial infarction, stroke, infarction of the other organs and gangrene of the extremities.

Accordingly, the fundamental process of atherogenesis is peroxidation of PUFAs generating 4-HNE and related protein-binding aldehydes within the lipid moiety of oxLDL as well as within the blood vessel wall. The 4-HNE, either produced by non-enzymatic peroxidation of PUFAs or by enzymatic reactions triggered by 15-LOX acts as major second messenger of oxidative stress generating reversible and irreversible advanced lipoxidation end-products (ALE) [86–88]. In normal arterial intima there are no immunohistochemically detectable ALEs, but in the central lipid core of atherosclerotic plaques, aldehyde adducts are detected by anti-HNE-lysine, anti-HNE-histidine [89–93] and anti HOCl-modified-LDL antibodies [94,95]. HNE-histidine adducts are also expressed in VSMC of human aorta in age-related manner, but not in damaged elastic fibers in tunica media [96].

As the plaque forms, the intima comprises growing VSMC and chronic inflammation based on activated and oxidized lipid-intoxicated macrophages (“foamy cells”), lymphocytes and connective tissue. The change of arterial tissue structure in plaque results in hypoxia due to insufficient oxygen and nutrient diffusion from blood and increased metabolism of inflammatory cells, activates the hypoxia-inducible factor (HIF)/vascular endothelial growth factor (VEGF) pathway and subsequent neoangiogenesis within a plaque [97–101].

Such a complex inflammatory process showed the evidence for a significant role of Toll-like receptor 4 (TLR4) in the onset of innate immunity atherosclerotic inflammation, while 27-hydroxycholesterol (27-OH) and 4-HNE enhance the production of interleukins IL-8, IL-1 $\beta$  and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and up-regulate metalloproteinase MMP-9 production by macrophages, through activation of TLR4/NF $\kappa$ B-dependent pathway, with a subsequent plaque instability and even its rupture [102].

Moreover, 27-OH and 4-HNE can promote up-regulation of COX-2 and membrane-bound prostaglandin E synthases with consequently increased production of prostaglandin E<sub>2</sub> and inducible

nitric oxide synthase (iNOS), leading to the release of nitric oxide, as observed in vitro for human pro-monocytic U937 cells [103].

Therefore, atherosclerosis is generally understood to be mainly the consequence of a complicated, chronic inflammatory process associated with different stages of plaque development. As mentioned before, neovascularization of the atherosclerotic plaque is an important pathophysiological process of atherosclerosis, similarly like neovascularization is one of major features of any persistent inflammatory processes. Colocalization of 4-HNE-adducts with CD31 in endothelial cells in neovascularization suggests relationship between low concentration of 4-HNE which stimulate formation of the tubes by human microvascular endothelial cells, through a redox-dependent sphingomyelinase and sphingosine kinase-1 (nSMase2/SK1) pathway. That was also confirmed by the carbonyl scavengers hydralazine and bisvanillyl-hydralazone, which inhibit the nSMase2/SK1 pathway activation and tube formation. Interestingly, high concentrations of 4-HNE are not angiogenic, probably because at highly supraphysiological concentrations 4-HNE exerts rather cytotoxic than regulatory or stimulating effects [19].

Adrenomodulin (AM) is a member of the calcitonin superfamily that acts via calcitonin receptor-like receptor and the three receptor-activity-modifying proteins (RAMPs). Expression of NADPH subunit, p67phox, was found to increase in the injured arteries of heterozygous knockout mice (RAMP2<sup>+/-</sup>) and drug-inducible endothelial cell-specific RAMP2<sup>-/-</sup> (DI-E-RAMP2<sup>-/-</sup>) mice. In addition, 4-HNE, RAMP2<sup>+/-</sup> and DI-E-RAMP2<sup>-/-</sup> are increased predominantly in neointima of injured arteries. This suggests that endothelial RAMP2 may be a target for regulation of oxidative stress and that AM might exert protective effects against vascular injuries [104].

Lipid peroxidation and ALEs play important roles in cardiovascular diseases, so they are used as biomarkers of illness and as potential therapeutic targets. The lipid sources in the cardiovascular diseases mostly originate from vascular tissue, including myocardium, as well as from epicardial adipose tissue and bloodstream (circulating lipoproteins and lipids). However, due to its important physiological role for so-called reverse cholesterol transport from the peripheral tissue to liver high density lipoprotein has mostly beneficial effects, while LDL is important for transport of the lipids to the cells, thus being the risk factor for the onset of the cellular lipid peroxidation. The endothelial injury causes the infiltration of oxLDL-cholesterol into the intima where its progressive oxidation leads to formation of oxysterols and core-aldehydes in the intimal plaque.

Statins are one of the most important medicaments used to reduce danger of cardiovascular diseases. In addition to their lipid-lowering effect, statins exhibit non-lipid lowering, so-called “pleiotropic” effects based partly to their antioxidant properties. Essentially statins inhibit hydroxyl-methyl-glutaral coenzyme A reductase (HMG-CoA), which acts by synthesizing L-mevalonat from HMG-CoA. After multi-step process of conversion mevalonate finally serves as precursor for synthesis cholesterol. Mevalonate synthesis pathway occurs mostly in hepatocytes, but it is also active in the cells of cardiovascular system. Statins inhibit prenylation of small GTPases in cells of the cardiovascular system, where they also display a pleiotropic effect independent of their lipid lowering effect. Namely, in healthy subjects treated with hydrophilic statin rosuvastin, reduced levels of C-reactive protein independently of any effect on LDL levels were observed, while in human endothelial cells of the coronary arteries statin treatments were found to be efficient against the oxLDL-induced formation of lipid membrane rafts and reduction in superoxide anion generation [105].

Statins suppress NOXs activities in the vascular wall and reduce expression of NOX1 with subsequent translocation of Rac1 and reduced generation of superoxide anion. On the other hand, statins could downregulate p47 phox and p22 phox protein levels by increasing PPAR $\alpha$  activity [106] in parallel enhancing vascular eNOS expression, on transcriptional and posttranscriptional levels [107].

Accumulation of oxLDL in a core of atherosclerotic plaque is accompanied not only by productions of ALEs but also of advanced glycoxidation end products (AGEs) and the adducts of oxidized phosphatidylcholine with ApoB-100, the major protein of LDL, which modulate the lipoprotein uptake by inflammatory (macrophage) cells [108]. Prevention and inhibition of AGE formation in oxidative stress-based diseases like atherosclerosis are part of several therapeutic strategies targeting different levels of AGE formation and catabolism [109]. Inhibition of the AGE formation could be possible also indirectly by some antioxidants or metal ion chelators or directly quenching reactive carbonyl species (RCS) [109].

Antioxidants are xenobiotics such as natural flavonoids and antioxidant micronutrients ( $\beta$ -carotenes, retinol, vitamin E, and vitamin C). Hydrophilic vitamin C is effective in trapping oxygen, nitrogen and sulfhydryl radicals also acting through reduction of the lipid hydroperoxides, complementary to the hydrophobic vitamin E, which acts as scavenger of lipid peroxy radicals. Hence, vitamin C can recycle thus generated tocopherol radical back into the bioactive vitamin E, so do GSH and lipoic acid [109]. Pyridoxamine (PM), a natural form of vitamin B6, is carbonic quencher that may prevent production of ALE and AGE [110], while it also prevents modification of lysine residues on the RNase-treated with oxLDL in the model of copper-catalyzed lipid (LDL) peroxidation [111]. PM acts also by directly trapping MDA modified serum albumin, thus under physiological conditions reducing generation of lipofuscin-like fluorescence [112]. In addition, modification of lysine residues on serum albumin by 4-oxo-nonenal can be decreased after treatment with PM [113], but it is not effective in case of albumin modification by 4-HNE, which is mostly binding to the histidine residues of the albumin.

Pyrido indole derivatives, such as stobadine, have ability to scavenge oxygen radicals, quench singlet oxygen, repair oxidized base and to maintain oxidation of SH group by one electron donation. By conjugation of MDA and 4-HNE, stobadine was found to act against lipid peroxidation and consequential protein modification, so it has been postulated to act as cardioprotectant that may even correct hypertriglyceridemia and hypercholesterolemia in diabetic rats [114,115].

ALEs can also be inhibited by metal ion chelators or agents that possess ability to chelate metal ion and inhibit lipoxidation reaction in cardiovascular disease. Many drugs are used in treatment of cardiovascular disease such as angiotensin-converting enzyme inhibitors, blockers of angiotensin receptor, inhibitors of aldose reductase, hydralazine, and other ALEs and AGEs inhibitors including carnosine, pyridoxamine and metformin [109,116]. The RCS quenchers reduce ALE forming covalent adducts with electrophilic carbonyl derivatives. Hence, GSH acts as endogenous antioxidant that makes covalent adducts with electrophilic compounds eventually generating mercapturic acid. The quenching activity of GSH could be also catalyzed by GST that are up regulated during detoxification of 4-HNE and related reactive aldehydes [117]. Similarly, N-acetyl-cysteine (NAC), a precursor of GSH, is often used in animal models to reduce reperfusion injuries in acute myocardial infarction [118] also reducing MDA production and myocardial fibrosis in compensated left ventricular hypertrophy during heart failure [115,119]. Moreover, NAC inhibits oxLDL-induced endoplasmic reticulum stress in human endothelial cells [120] and in LDL-receptor knockout mice



attenuates atherosclerosis and reduces LDL oxidation itself, consequently being able to stabilize atherosclerotic plaques in ApoE deficient mice [121].

Carnosine is a dipeptide of  $\beta$ -alanine and histidine with RCS scavenging properties showing also strong metal ion chelation in vivo and in vitro [122]. Carnosine directly binds to  $\alpha,\beta$ -unsaturated aldehydes such as 4-HNE [123,124]. Carnosinase-resistant analog, carnisolon, displayed higher scavenging potency and selectivity than carnosine in reduction of 4-HNE adducts in atherosclerotic plaque in ApoE-null mice model, as it protected VSMCs in vitro from the 4HNE-induced injury [125]. Carnosinol in a dose dependent manner attenuated also formation of 4-HNE adducts in skeletal muscle and liver while modifying also dyslipidemia, insulin resistance, steatohepatitis and inflammation [126].

Vasodilator and antihypertensive drugs such hydralazine can also have excellent 4-HNE scavenging activity. Hydralazine traps 4-HNE and reduces formation of 4-HNE adducts on the platelet-derived growth factor receptor- $\beta$  in VSMCs treated in vitro with oxLDL, and in vivo in aortal atherosclerotic plaque of hypercholesterolemic rabbits [127].

While intracellular degradation of ALEs has been mainly reported to be a proteasome-dependent process [128], some studies reported degradation based on lysosomal degradation or proteases' activity [129]. Carnosine can directly react with ALEs leading to formation of carbonyl-carnosine adducts, which make crosslink with unmodified proteins such proteasome components thus enhancing degradation of ALEs, also modulating NO, known to act like activator of proteasome activity [122,130].

Finally, it should be mentioned that inactivation of oxidized phospholipids with modified form of natural IgM E06 antibodies could reduce inflammation associated with atherosclerosis [130]. Therefore, expanding knowledge on signaling in oxidative stress in cardiovascular diseases proposed several available therapeutic strategies for integrative biomedicine treatments of cardiovascular diseases in the future.

Of course, the same may be stated also for the other diseases, in particular chronic stress- and age-associated diseases, the most of which are degenerative. Among these, neurodegenerative diseases deserve particular attention.

#### **4-HNE involvement in redox signaling pathways of neurodegenerative diseases and respective therapies**

Neurodegeneration, stroke and brain tumors are closely tied to oxidative stress due to high levels of ROS found in these severe diseases of the central nervous system (CNS) [131]. However, answer to the question whether ROS were preceding these brain diseases or are mere a pathological consequence remains still open. By looking at ROS only as deleterious metabolic by-products which need to be detoxified, one would easily find explanation for elevated ROS levels in brain diseases due to its susceptibility to oxidative stress. Often, this susceptibility is corroborate with major brain biochemical characteristics (e.g. high PUFA and iron content, high oxygen consumption, low antioxidant defense, etc.), but almost never with redox signaling [132]. However, ROS play physiological roles in CNS development through controlling proliferation and stemness of neuronal progenitor cells [133], regulation of neuronal polarization, connectivity and plasticity [134,135], and axonal pathfinding and regeneration [136].



Besides ROS, numerous physiological processes of the brain necessitate metal ions like calcium, potassium, sodium and zinc, but also redox metals – iron and copper, which are constitutive parts of numerous brain proteins as well as signaling molecules [137]. Under homeostatic conditions, ROS and redox metals in the fine interplay assure proper functioning of the brain. When this balance is disrupted, oxidative stress occurs leading to ferroptosis as cell becomes overwhelmed with toxic oxidative metabolites. Ferroptosis, as a new form of cell death dependent on iron and ROS with intense membrane lipid peroxidation as a main characteristic [138], is being recognized as a leading process in the progression of neurodegenerative disorders [139]. Lipid peroxides that are accumulating during ferroptosis, especially 4-HNE, modify primarily proteins thus altering signaling pathways involved in proper cellular functioning and contributing to pathogenesis of neurodegeneration [140–142].

### **Overview of two most common neurodegenerative diseases**

Neurodegeneration is usually defined as the ‘large group of neurological disorders with heterogeneous clinical and pathological expressions affecting specific subsets of neurons in specific functional anatomic systems that arise for unknown reasons and progress in a relentless manner’ [143]. The most common neurodegenerative disease (ND) is Alzheimer’s disease (AD), followed with Parkinson’s disease (PD), multiple sclerosis, amyotrophic lateral sclerosis, Huntington’s disease and others. All of the mentioned ND share common characteristics including neuroinflammation, oxidative stress, impaired mitochondrial function, deposition of aggregated proteins and high levels of metals [144]. Due to different mechanism underlying each ND, approved therapies are mechanistically specific and extensive, so the attention of this review will be on the two most common neurodegenerative diseases – AD (Figure 1.) and PD (Figure 2.).

### **Alzheimer’s disease**

Pathological hallmarks of AD include synapse and neuronal loss, formation of extracellular senile plaques with amyloid  $\beta$  as major component, and intracellular neurofibrillary tangles constituted of hyper-phosphorylated tau proteins [145]. Abundance of oxidatively modified DNA, proteins and lipids in AD emphasizes the role of oxidative stress as an early event in AD development [146]. Recent research of Youssef and colleagues revealed increased accumulation of  $H_2O_2$  in early stage of AD which activates Nrf2 [147]. Consequently, Nrf2 activates transcription of autophagy protein NDP52, which binds to phosphorylated tau protein and leads to its autophagic clearance [148]. Amyloid  $\beta$  through interaction with copper can also produce  $H_2O_2$ , thus acting as a prooxidant, while its soluble oligomers bind to N-methyl-D-aspartate (NMDA) receptor causing increased calcium influx, which impairs mitochondrial functions and again leads to increased ROS levels [149]. Furthermore, post mortem analysis of AD brains revealed high levels of zinc, copper and iron in senile plaques implying potential role of metals in AD pathogenesis (reviewed in [150]). Namely, amyloid  $\beta$  protein precursor has two metal binding domains – the structural zinc-binding domain, and the functional copper-binding domain. Thus, zinc and copper are in focus of researcher evaluating the AD pathogenesis. Zinc binding to amyloid  $\beta$ , was found to induce precipitation of insoluble amyloid  $\beta$  aggregates, which further increases amyloid  $\beta$  neurotoxicity [151,152]. Besides, this could lead to impaired neuronal signaling due to reduced zinc levels at synaptic cleft [153].

Increased copper levels in amyloid  $\beta$  plaques are tightly correlated with decreased intracellular copper levels and concomitantly with reduction of SOD [154], while increasing intracellular copper inhibits amyloid  $\beta$  accumulation and tau phosphorylation [155]. Known as metal most often acting as pro-oxidant in ROS generation, iron is also connected with AD pathogenesis. Several studies implicated iron in amyloid  $\beta$  production through furin activity. Namely, furin, an enzyme responsible for proper functioning of secretases, is lowered under high iron levels, thus contributing to production of amyloid  $\beta$  [156,157]. Metal dyshomeostasis that occurs during oxidative stress, enhances mitochondrial dysfunctionality thus closing viscous circle of continuous ROS production and increasing lipid peroxidation. Lipid peroxidation is an early event in AD pathogenesis confirmed by 4-HNE presence in the AD brains throughout the course of the disease. The 4-HNE-modified proteins in AD encompass proteins involved in energy metabolism, mitochondrial dysfunction, cytoskeletal integrity, antioxidant defense, excitotoxicity and neuronal communication (extensively reviewed in [158]).

### AD therapies

The two main types of AD therapies include cholinesterase inhibitors (donepezil, galantamine or rivastigmine) to treat mild to moderate AD and NMDA receptor antagonist (memantine) for treating moderate to severe AD [159]. None of these medications truly cure AD, rather helps to relieve major symptoms.

Cholinesterase inhibitors are first therapy due to presynaptic reduction of acetylcholine found in AD pathology. Donepezil is the most widely prescribed drug for AD treatment that inhibits hydrolysis of acetylcholine thus increasing its concentration in extrasynaptic space, leading to modest improvements of cognitive and behavioral functions in AD patient [159]. Besides, recent research revealed antioxidative properties of donepezil in AD patients. Namely, donepezil induces reduction of acetylcholinesterase level, protein carbonylation and protein oxidation, while increasing antioxidative defense in AD patients [160]. Interconnection of donepezil and 4-HNE was described by Jeong and coworkers on rat brain hippocampal neurons after pilocarpine induced seizure, where long term donepezil treatment resulted in reduced oxidative injury measured by decreased 4-HNE immunostaining and also by decreased neuronal death [161].

Memantine is uncompetitive, voltage-dependent NMDA receptor antagonist that inhibits excessive calcium influx as a consequence of NMDA receptor overstimulation. In moderate to severe AD patients, memantine used alone or in combination with donepezil maintained superior cognitive performance and function [162]. Concerning the fact that amyloid  $\beta$  oligomers are inducers of NMDA receptor, memantine seems to be appropriate therapy. By blocking NMDA receptor, memantine suppresses overaccumulation of intracellular calcium ions which would consequently lead to increased ROS, thus acting as antioxidant [149]. Besides, memantine would block activation of NMDA receptor due to excessive glutamate accumulation originated as a result of 4-HNE-modification and thereby inactivation of glutamate transporter EAAT2 [163].

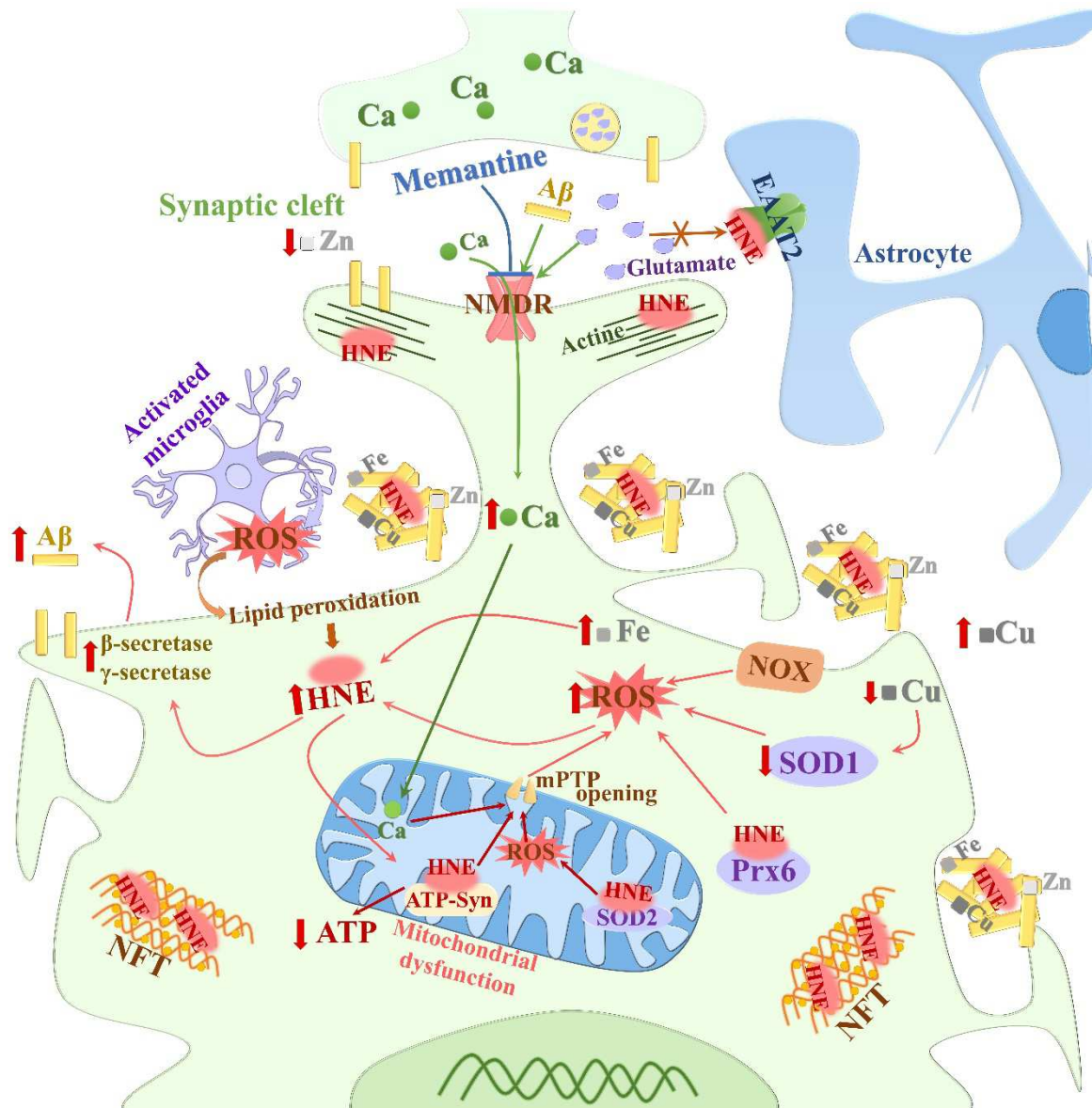


Figure 1. AD neuronal degeneration overview. Redox environment of the brain, together with astrocytes and microglia contributes to neuronal degeneration in AD development and onset. Amyloid  $\beta$  oligomers bind to NMDR thus allowing increased calcium influx. High levels of intracellular calcium leads to mPTP opening due to mitochondrial dysfunction and increased ROS levels. In iron rich environment, ROS triggers lipid peroxidation resulting in 4-HNE increase. Additionally, activated microglia contributes to lipid peroxidation by secreting inflammatory cytokines and producing high ROS levels. 4-HNE accumulates in NFT and senile plaques, but also modifies main cellular proteins including: ATP-Syn leading to ATP decrease; SOD2 and Prx6 leading to further ROS increase; Actine resulting in impaired cytoskeletal trafficking; EAAT2 thus blocking glutamate transport and resulting in glutamate increase in synaptic cleft. 4-HNE also potentiates  $\beta$ - and  $\gamma$ - secretase activity leading to amyloid  $\beta$  increase, which together with increased glutamate levels, keep NMDR active thus closing vicious circle. Accumulation of metals in senile plaques results in a decreased signal transmission, due to lowered zinc levels at synaptic cleft and decreased SOD1 activity due to decreased intracellular copper levels. Combined together, these processes lead to neuron degeneration and death. Memantine, as an approved AD drug, blocks NMDR, thus partially inhibiting and delaying neuronal degeneration. (A $\beta$  – amyloid  $\beta$ ; ATP-Syn – ATP synthase; mPTP – mitochondrial potential transition pore; NFT – neurofibrillary tangles;

NMDR - N-methyl-D-aspartate receptor; EAAT2 - glial glutamate transporter; Prx6 - peroxiredoxin; Ca - calcium; Zn - zinc; Cu - copper; Fe - iron).

### **Parkinson's disease (PD)**

Parkinson's disease is a neurodegenerative disease characterized by premature selective loss of dopaminergic neurons and accumulation of Lewy bodies composed of misfolded  $\alpha$ -synuclein [164]. Dopamine is an inhibitory neurotransmitter that in healthy brain regulates excitability of striatal neurons responsible for controlling balanced body movements. Since in PD brain dopaminergic neurons degenerate, dopamine levels are decreased thus resulting in diminished inhibition of neuronal activities. Dopamine metabolism includes several enzymes acting in a sequence, starting with oxidative deamination by monoaminoxidase (MAO), continuing with catechol-O-methyl transferase and ALDH to homovanilic acid as a main degradation product [165]. Besides, dopamine is susceptible to autooxidation in the oxygen presence yielding quinones. All of these reactions as byproducts have ROS which can further increase in the presence of redox metals. Dopaminergic toxicity could thus be triggered by dopamine itself through mitochondrial inhibition, ROS and oxidatively modified biomolecules, making oxidative stress a major hallmark of PD [166]. Reduced levels of GSH and decreased activity of the mitochondrial respiratory chain complex I found in post mortem analysis of PD brain support these facts [167,168]. The presence of 4-HNE in Lewy bodies of PD patients indicate its possible involvement with  $\alpha$ -synuclein accumulation in PD pathogenesis [169,170]. In vitro studies revealed higher susceptibility of 4-HNE-modified  $\alpha$ -synuclein aggregates to form oligomers and insoluble fibrils than  $\alpha$ -synuclein alone [171]. Moreover, 4-HNE-modified  $\alpha$ -synuclein showed higher toxicity on dopaminergic neurons in vitro than other cell types due to induction of intracellular ROS formation [172]. On the other hand, 4-HNE can contribute PD pathogenesis through impairing dopamine receptor function by acting directly on sulfhydryl groups at the binding site of the receptor [173].



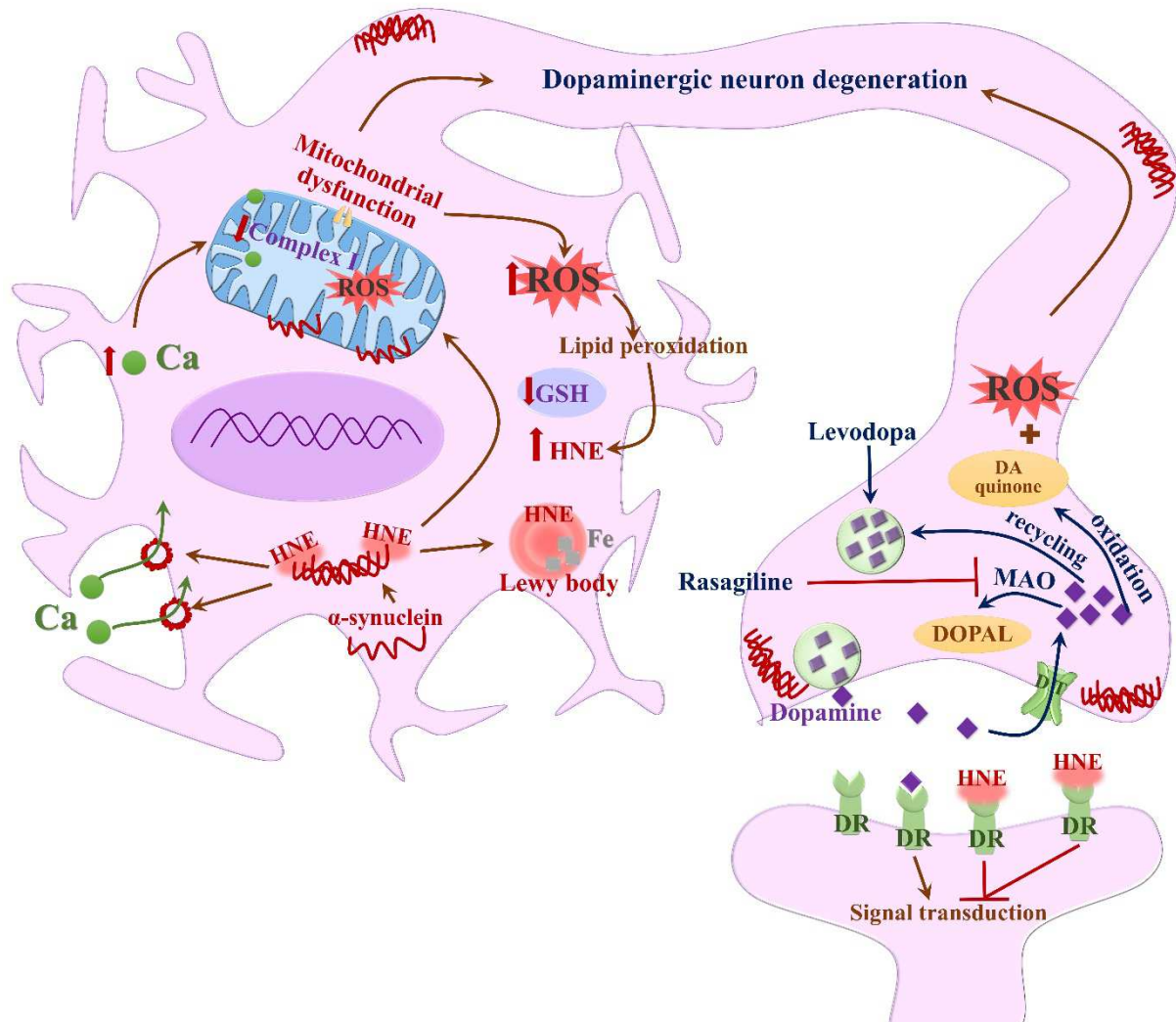


Figure 2. PD neuronal degeneration overview. Oxidative stress, mitochondrial damage and  $\alpha$ -synuclein misfolding and aggregation are processes included in degeneration of dopaminergic neurons and the onset of PD. Misfolded  $\alpha$ -synuclein binds to cell membrane creating pores through which calcium can freely enter the cell and accumulate at toxic levels. Calcium together with  $\alpha$ -synuclein causes mitochondrial damage and dysfunction through complex I inhibition that leads to increased ROS production and consequently lipid peroxidation and 4-HNE formation. The 4-HNE binds to  $\alpha$ -synuclein thus making it more cytotoxic to cell, while  $\alpha$ -synuclein oligomers aggregate and form Lewy bodies, which also accumulate 4-HNE and iron. In the environment rich in ROS and depleted of antioxidants (primarily GSH), neuronal damage progresses. The  $\alpha$ -synuclein can also block dopamine release from vesicles, thus directly interfering with neurotransmission. On the other hand, 4-HNE can bind to sulfhydryl groups of dopamine receptor that result in blocking of dopamine binding and signal transduction. In such states, dopamine reuptake occur by presynaptic neuron where it can be degraded by MAO to DOPAL, recycled or oxidized to DA-quinone. The last process is the most damaging as it produces ROS as byproducts. To ensure neurotransmission dependent on dopamine, PD patients are receiving Levodopa as dopamine precursor and Rasagiline as MAO inhibitor in order to increase dopamine availability and assure signal transduction. (DA – dopamine; DOPAL – 3,4-dihydroxyphenylacetaldehyde; DT – dopamine transporter; DR – dopamine receptor; Ca – calcium; Fe – iron).

## PD therapies

As in the case of AD, drug treatments in PD serve to relieve symptoms and postpone disease progression. Two major drug types are in use alone or in combination – dopaminergic drugs in order to restore dopamine levels (levodopa and carbidopa) and MAO-B inhibitors to maintain dopamine levels (selegiline, rasagiline) [174,175]. While outcomes of levodopa treatments are still contradictory [176], rasagiline was demonstrated to have neuroprotective properties that comprise of: mitochondrial protection through prevention of membrane potential decline; regulation of cell survival Bcl-2 family proteins; inhibition of MAO-B at transcriptional level and suppression of oxidative stress through increase of the antioxidant enzymes [177].

Neurodegenerative disorders are still major mystery in spite of huge progress in revealing mechanisms of their development and onset. One can be sure that oxidative stress and lipid peroxidation play an important role in the pathogenesis of the neurodegeneration. However, due to limitations in measurements at the site of the disease (within the brain) in the real time, it is difficult to undeceive causes from consequences of the disease. The most valuable information from post mortem brains should also be taken with caution, as personalized treatment protocols are mentioned very rarely for every patient. In addition, with our knowledge on antioxidative properties and redox implications of the approved drugs for ND treatment, question remains what is the real set of oxidative stress events in ND and respective therapies? Redox signaling implications in ND lead to presumption of antioxidant supplementation or iron chelation as promising adjuvant therapies. However, these strategies are still under trials and for now did not result in unambiguous benefits for patients.

Finally, it should be said that iron, like many other pro-oxidants, has multiple beneficial effects, yet acting as a sword with the double age, from such points of view resembling also antioxidants. Therefore, essential principles of modern integrative and personalized medicine should be implemented to better understand, monitor, prevent and treat neurodegenerative diseases as stress- and age-associated diseases.

## 4-HNE in redox signaling pathways of inflammatory diseases: The example of psoriasis

As in case of ND, multifunctional pro-oxidants represented by iron can play fundamental role also in the skin diseases, often manifested by systemic stress response [178]. However, while translation models of neurodegenerative diseases and related brain disorders rely on the induction of intracerebral oxidative stress and lipid peroxidation, often resembling autoimmune processes, as in case of encephalitis, the specific complexity of these diseases in humans is based on the lack of functional systemic immune defense within the CNS. On the other hand, most of the major, especially chronic, human diseases comprise inflammatory and even auto-immune processes as essential component of their pathophysiology.

Psoriasis - a chronic immune-mediated inflammatory disease, with not completely described etiology, is associated with hyperproliferation of skin keratinocytes, sometimes complicated by arthritis [179]. This disease affects 2-8% of the Western (USA and Europe) population [180]. The development and severity of the disease depends on genetic preconditions, various infections,



exposure to xenobiotics, incorrect body mass index, stress, cigarette smoking, air pollution and other factors associated with inflammatory reactions [181–184].

### **Redox imbalance in psoriasis**

Under inflammatory conditions, activated phagocytic cells generate large amounts of ROS and reactive nitrogen species, while inflammation is also accompanied by hypoxia, with VEGF and angiogenesis upregulation that further promotes generation of ROS, as was observed also for psoriasis [185,186]. On the other hand, the increased generation of pro-oxidants is accompanied by a lower antioxidant capacity, manifested by a decrease of vitamins C and E, as well as the lowered levels/activities of GSH/TRX and GPX/TrxR, which are accompanied by high activity of SOD in plasma and in circulating immune cells [187–189]. Consequently, redox imbalance is observed in the peripheral blood of psoriatic patients, so thus generated ROS can oxidize membrane phospholipids containing PUFAs leading to the increase of lipid peroxidation products resulting from both oxidative fragmentation (4-HNE and MDA) and from cyclization (isoprostanes), the elevated levels of which were found in plasma, blood cells and synovial tissues of patients with psoriasis [189–191]. Therefore, many studies consistently indicate significant changes in the lipid profile of people with diagnosed psoriasis [189,191–193]. These changes concern not only the levels of individual compounds (mainly phospholipids, triglycerides, lipoproteins and cholesterol), but also the modification of their structure, including oxidative modifications and products of their metabolism [189,191,194]. Hence, it can be assumed that the development of psoriasis is associated with changes in the lipid metabolism, including peroxidation resulting from the disturbed interaction between the redox balance and inflammatory factors.

Both ROS and 4-HNE can diffuse from the site of origin and on the way react with nucleophilic compounds, including lipids, proteins and DNA thus causing cellular damage important for the onset of inflammation, also modify cell signaling [195]. In particular the 4-HNE-protein adducts have been extensively examined as a biomarkers of oxidative protein modifications so their increased levels have been reported in various pathological states, including psoriasis and its arthritic form [189,191,196,197]. The immunological consequences of these interactions imply primarily the 4-HNE-albumin adducts, to which autoantibodies have greater affinity than to the plain albumin, as was observed in patients with inflammatory diseases [198]. Because 4-HNE modifies also mitochondrial proteins participating in process of angiogenesis [199], this particular reactive aldehyde induces angiogenesis both through activation of the TLRs as well as by up-regulation of VEGF expression [200]. In addition, both inflammation and angiogenesis are regulated by transcription factors regulating redox balance. Therefore, modifications of oxidative stress can change the stability of blood vessels and the inflammatory response, as presented by the Figure 3.

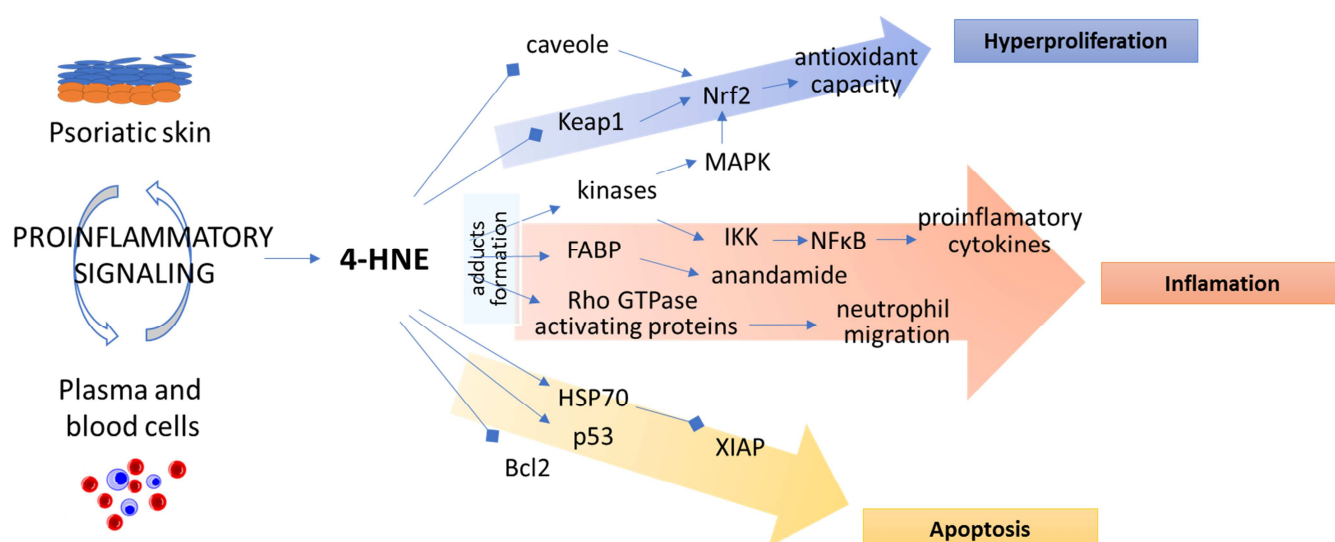


Figure 3. The involvement of 4-HNE as the signaling molecule in the blood and skin cells of psoriatic patients.

Considering the fact that psoriasis vulgaris is associated with skin lesions, it is important to mention that 4-HNE also modulates proteins important in healthy functioning skin, such as fatty acid binding protein (including epithelial FABP), the level of which is strongly up-regulated in plasma of psoriatic patients [201], consequently enhancing the uptake, transport and cellular metabolism of fatty acid derivatives. As a result, increased expression of FABP leads to higher level of circulating anandamide, which is known as an anti-inflammatory messenger [202]. It was found that 4-HNE binding to FABP at Cys-120 modifies FABP to more stable form than unmodified ones [203], what suggests that 4-HNE additionally supports proinflammatory signaling (Figure 3).

Moreover 4-HNE forms adducts with proteins involved in the catalytic activities of psoriatic patients that were not observed in the plasma, lymphocytes or keratinocytes from healthy individuals [197,204]. Among such proteins are: kinase 7 activated by 4-HNE, as well as Rho GTPase-activating protein 12, whose adducts with 4-HNE may stimulate neutrophil migration and disruption of the intercellular connections between the skin cells [197]. Moreover, the 4-HNE-protein adducts were found to act as factors promoting inflammatory interactions between lymphocytes and keratinocytes in case of psoriasis development [204].

#### 4-HNE in modulation of transcription factors in psoriasis

The effectiveness of 4-HNE-cell signaling is mainly due to its capacity to regulate mitogen activated protein kinase (MAPK) pathways and the activity of redox-sensitive transcription factors such as Nrf2, activator protein 1 (AP-1), and NFκB.

One of the main roles of 4-HNE in pathogenesis of psoriasis regarding stimulation of signal transduction is the aldehyde's effect on the activity of kinases participating in MAPK, Janus kinase/signal transducer and activator of transcription (JAK/STAT), and IκB kinase (IKK)/IκB/NFκB signaling pathways [205,206], the expression of which is highly increased in inflamed tissues, including psoriatic skin cells [207,208]. 4-HNE significantly increases the activity of these signal transduction cascades, what triggers cell response to transcription factors, cytokines, and other

intercellular signal molecules, leading to cell proliferation, differentiation, and even apoptosis [195] (Figure 3).

At the beginning of the inflammation, activation of immune cells can initiate downstream signalling pathways, through TLRs which activate transcription factors, such as NF $\kappa$ B and AP-1, that in turn induce pro-inflammatory gene expression, exert antimicrobial functions and recruit additional immune cells [209,210] and enhance the levels of mitochondrial ROS and 4-HNE, while decreasing the matrix metalloproteinases [200]. The nuclear transcription factor NF $\kappa$ B is the main regulator of the inflammatory response that drives the activation of genes associated with the transcription of inflammatory mediators, such as ILs, TNF- $\alpha$  and prostaglandins, as well as inflammatory enzymes, like inducible nitric oxide synthase and COXs [211]. NF $\kappa$ B is constitutively activated in autoimmune diseases such as psoriasis [212]. In addition, ROS, as well as 4-HNE, can activate NF $\kappa$ B directly or indirectly through other stimuli such as TNF- $\alpha$  [211,213]. The activity of NF $\kappa$ B components (RelA/p65, c-Rel, RelB, p50, and p52) is inhibited in the cytosol by I $\kappa$ B, however, the phosphorylation of I $\kappa$ B by IKK leads to I $\kappa$ B blocking and NF $\kappa$ B activation. Therefore, 4-HNE-inducing stimulation of IKK additionally stimulates NF $\kappa$ B transcriptional activity and the onset of inflammation [34] (Figure 3). Because of that, there are many promising attempts to inhibit NF $\kappa$ B/I $\kappa$ B in psoriatic skin cells using natural antioxidants, whose action is likely based on inhibition of the 4-HNE-IKK interaction [214,215].

4-HNE was also found as an effective Nrf2/Keap1 pathway activator [32,195]. Nrf2 is widely distributed in skin cells and, as a transcription factor, is responsible for cytoprotective and antioxidant proteins expression [216]. Nrf2 level and activity are controlled mainly by its cytosolic inhibitor Keap1, however, in psoriatic patients Nrf2 is up-regulated resulting in keratinocyte hyperproliferation and accelerated differentiation [217]. That might be connected with increased level of 4-HNE that, by binding to Keap1, modifies its structure and disrupts its interaction with Nrf2 resulting in prevention its ubiquitination and degradation [218]. Moreover, through the MAPK activation 4-HNE leads to Nrf2 phosphorylation, which favors its nuclear translocation and transcriptional activity [219]. On the other hand, 4-HNE stimulates Nrf2 in keratinocytes by breaking “caveoles”, i.e. the membrane structures with the ability to bind proteins, what further stimulates the proliferation of these skin cells [220], leading to formation of the psoriatic plaques.

### **The involvement of 4-HNE in decay of psoriatic cells**

4-HNE acting as a signaling molecule at elevated levels can also lead to processes that directly or indirectly induce the stress-induced apoptosis [221] (Figure 3). It is well known that the main function in oxidative stress-induced apoptosis is performed by the transcription factor/tumor suppressor p53, the increased expression of which was found in keratinocytes isolated from the skin of psoriatic patients [222]. However, binding of 4-HNE to p53 may additionally stimulate its transcriptional activity thereby leading to cell apoptosis [223] consequently accelerating the death of epidermal cells and aggravating the symptoms of psoriasis.

In addition, 4-HNE may affect apoptosis by modifying the structure and activity of heat shock proteins (HSPs), the levels of which increase in patients with psoriasis [224]. The 4-HNE modified HSP70 was found to reduce the expression of apoptosis-inhibiting X-linked inhibitor of apoptosis protein, which induces apoptosis [225].

The pro-apoptotic bioactivities of 4-HNE are based not only on up-regulation of pro-apoptotic factors but also on down-regulation of anti-apoptotic proteins, including B-Cell Lymphoma-2 (Bcl-2) [226], which level was found to be significantly lowered in psoriatic patients [227]. Furthermore, the above mentioned 4-HNE-induced activity of intracellular kinases leads to phosphorylation of Bcl-2 at Thr56, Ser70 causing blockage of the antiapoptotic properties of this protein [228].

Another type of cells death observed in psoriatic patients is NETosis (neutrophil extracellular traps, NETs) [229]. Namely, it is well known that NETs created by neutrophils are involved in the development of psoriasis [230]. NETs are networks composed of extracellular fibers and DNA and are the first line of defense against infection that occurs by absorbing microbes, participating in the secretion of antimicrobials and killing extracellular pathogens, with minimal damage to host cells [231]. In the peripheral blood and skin of psoriatic patients formation of NETs is increased and correlates with disease severity [229]. It was demonstrated that oxidized phospholipids components are the most potent NETs inducers, while 4-HNE was also effective, yet less potent [232]. However, it seems that 4-HNE can also inhibit constitutive neutrophil apoptosis by reducing activation of initiator caspases as well as inactivation of caspase-3 by modification of its critical cysteine residue [233]. In addition, 4-HNE is known to inhibit as in negative-feedback manner neutrophil functions, including glycolysis, phagocytosis and the oxidative burst, although being generated by these inflammatory processes [17].

### Psoriasis treatments

Not fully understood etiology of psoriasis limits current therapies to only alleviate symptoms by extinguishing inflammation or inhibiting skin cell proliferation. Because the development of psoriasis is associated with inflammation and oxidative stress, the primary severity of oxidative stress and lipid peroxidation causes the NF $\kappa$ B activation with cytokines and chemokines causing inflammation development, which in turn results in increased oxidative stress and its consequences. However, when inflammation is the primary event, it leads to oxidative stress and increased lipid peroxidation, which intensifies inflammation [234]. Therefore, in order to increase the effectiveness of pharmacotherapy of psoriasis, for many years compounds have been sought for simultaneously targeting oxidative stress and inflammation aiming to block their vicious circle.

The usual pharmacotherapy of inflammation includes several classes of drugs, such as corticosteroids, nonsteroidal anti-inflammatory drugs and natural products, the most of which may reduce the level of oxidative stress by decreasing the onset of ROS/lipid peroxidation and/or by increasing the antioxidant capacity of the affected skin cells, while some can also directly reduce the biological activity of NF $\kappa$ B and/or directly modify the level of TNF- $\alpha$  [235,236]. Antioxidants such as SOD and NAC have been shown to inhibit oxidative stress and 4-HNE levels in synoviocytes, acting as ROS scavengers, while NAC-induced NF $\kappa$ B reduction was associated with reduced proinflammatory cytokine release and angiogenesis [190].

The therapeutic potential of other medical remedies, such as dimethyl fumarate, is associated with the induction of several antioxidant pathways increasing the levels of GSH, NAD(P)H quinone oxidoreductase 1, Nrf2, as well as acting anti-inflammatory by lowering the levels of cytokines and adhesive molecules [237] (Figure 4).

However, the action of many drugs used to treat psoriasis shows an unexpected effect on the lipid peroxidation process (including 4-HNE generation). Commonly used cyclosporin that acts by

inhibition of membrane transport proteins has been found to act also as factor enhancing 4-HNE level in plasma [238]. Similarly, even topically used dithranol (anthralin) by its prooxidative effects causes formation of superoxide anion and  $H_2O_2$ , which stimulate proinflammatory cytokines following NF $\kappa$ B activation [239].

Similar situation occurs also when using physical therapeutic agent, ultraviolet radiation (UV) (UVA and UVB light), aiming to increase the effectiveness of pharmacotherapy of psoriasis vulgaris, which, in turn, may be destructive for the skin, even leading to cancer [240]. Both of these types of irradiation produce different biological effects including ROS and 4-HNE enhanced generation [241]. On the other hand, UVA and UVB irradiation have been shown to decrease presentation of antigens, resulting in diminished T-cell responses and inflammatory suppression [242] (Figure 4).

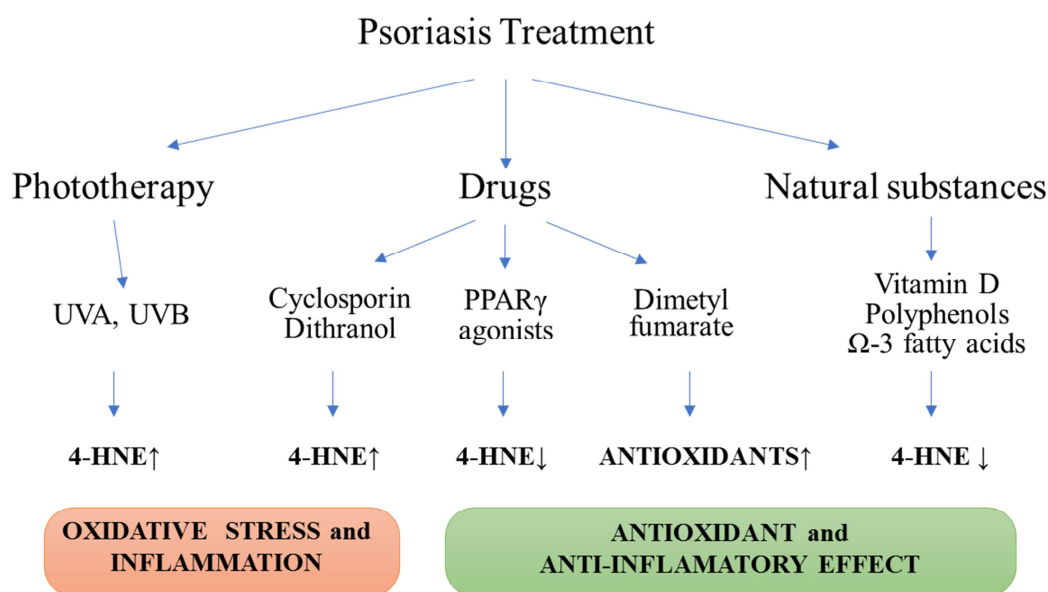


Figure 4. The examples of psoriasis treatment and their impact on 4-HNE level.

The other drugs based on anti-inflammatory signaling are PPAR $\gamma$  modulators. These drugs use their immunomodulatory and antiproliferative effects [243]. The PPAR $\gamma$  expression has been found to be strongly reduced in psoriatic plaques, which may suggest that PPAR $\gamma$  activation may contribute to the prevention of psoriatic lesions [244]. On the other hand, activation of PPAR $\gamma$  in mouse models of skin hyperproliferative diseases with thiazolidinediones, exerts antiproliferative effects and suppresses the inflammatory signal [245]. A similar effect was observed by an *in vitro* study using cultures of normal human keratinocytes and lymphocytes in which the PPAR $\gamma$  agonist (GED-0507-34L) suppressed the inflammatory process by inhibiting NF $\kappa$ B, and consequently reduced expression of pro-inflammatory cytokines such as IL-6, IL-8, IL-12, IL-21, IL-23, TNF- $\alpha$  and COX-2 [246], thereby reducing generation of 4-HNE (Figure 4).

Unfortunately, anti-psoriatic therapies with synthetic drugs are often characterized by side effects, while biological drugs treatments are expensive. To minimize these problems, herbal preparations, nutraceuticals or dietary supplements are increasingly used as alternative or complementary therapy. Currently, approximately 80% of people around the world use preventive



and therapeutic natural products because of their relative safety, effectiveness and low cost, if matching the integrative biomedicine approach.

The most frequently studied and used to treat psoriasis natural antioxidants are polyphenols such as flavonoids, lignans, flavoglucanols, quinones, stilbenes, phenylpropanoids and diarylheptanoids, which can all modulate the inflammatory pathways. Among them, quercetin, apigenin, luteolin, and baicalein showed anti-inflammatory activity in vitro and in animal models [247,248]. One of such polyphenols that interacts with multiple pathophysiological targets that can safely and inexpensively reduce inflammatory processes is curcumin, which significantly reduces 4-HNE levels as well 4-HNE mediated oxidative modifications of a protein substrates even by 80% [249,250]. Moreover, the polyphenols-rich extract from green alga *Codium fragile* applied topically on skin in therapy against psoriasis decreased 4-HNE levels and protein oxidation protecting skin against pro-inflammatory and oxidative damages induced by UVB [251].

Antioxidants usually prove as beneficial in the treatment of psoriasis because cell signaling pathways such as MAPK or JAK/STAT are sensitive to redox reactions and have been shown to be involved in the progression of psoriasis [205]. As an example of topical or orally used natural substances are vitamin D analogues (calcipotriol, tacalcitol, calcitriol) [252]. Vitamin D, as a fatty soluble antioxidant molecule, is known as a lipid protector and is used in therapies for a number of diseases [253,254]. It has been shown that vitamin D protects against oxidative stress [255] and prevents lipid peroxidation [256], effectively lowering the 4-HNE level in cells under both physiological and oxidative conditions [257].

Other natural compounds used as oral or topical remedies for the treatment of psoriasis include omega-3 fatty acids such as eicosapentaenoic acid, due to their anti-inflammatory properties, which are particularly manifested by reduced levels of TNF- $\alpha$  and 4-HNE-protein adducts (even by 30%) in the blood, especially if used by long-term administration [258–260]. Yet, recent research results show that dietary supplementation of eicosapentaenoic acid only affects lipid metabolism, reducing the level of eicosanoids that are a potential source of itching [261].

An important element in therapy with natural preparations, including antioxidants, is that, when accompanied by other antioxidant compounds, they show synergy of action. Therefore, it is believed that the most effective form of therapy is the use of a mixture of natural antioxidants, which are usually plant extracts. Since different components generally modify different metabolic pathways, this usually leads to different pharmacological effects. However, regardless of the type of therapy used an important role is also played by the balanced diet. Namely, it was found, among others, that regular consumption of fresh fruit, vegetables and spices rich in different phytochemicals can reduce oxidative stress and inflammation and may relieve symptoms of psoriasis [262–264].

Therefore, we may conclude that as long as the exact and detailed etiopathogenesis of psoriasis is not revealed, we could rely mostly on nutritional approach and anti-inflammatory medicaments aiming to prevent or control at least some aspects of psoriasis, like any other chronic inflammatory disorder. However, 4-HNE, acting as a signaling molecule regulating proliferation, differentiation and apoptosis might help us to make major progress in these fields, in particular if we keep in mind that complex, often opposite effects of 4-HNE are not only concentration-dependent, but depend also on its ability to modify various enzymes, cytokines and their receptors even regulating the activities of the antioxidants (enzymatic and non-enzymatic, especially GSH). That might be crucial not only for metabolic, degenerative and inflammatory aspects of the stress-associated diseases, but in particular for the onset of the most fearsome of them all – the cancer.



### **The involvement of 4-HNE in carcinogenesis and anti-cancer therapies**

Although a lot of efforts have been done, we are still eager to gain knowledge that could contribute to the fight against cancer not just supporting prevention but also eradication of the advanced malignancies. The cancer therapy elusiveness could be attributed to heterogeneity of malignant neoplasms, their ability to adapt and protect themselves as well as to the fact that they are mostly “silent killers”, not showing symptoms until it is late. Every day researches are trying to find new distinguished pathways, molecules involved in tumor initiation and progression that could be specifically targeted and modulated to stop the disease development and hopefully, ultimately cure the disease. Myriad of interplaying signaling pathways influencing genetic, epigenetic, metabolic alterations, as well as different environmental cues, are involved in cancer initiation and progression [265–267]. However, new insights also brought to the attention the “Dr. Jekyll and Mr. Hyde” behavior of many diverse contributors/suppressors in tumorigenesis, highlighting the complexity of tumors.

ROS are an example of such behavior (reviewed in [266,268–271]). Excessive ROS induce oxidation of lipids yielding 4-HNE, a known bioactive marker of oxidative stress [88], and a pleiotropic molecule that can, in a concentration-dependent manner, affect diverse cellular processes from proliferation to differentiation and apoptosis in normal and cancer cells [3,87]. The development of antibodies against 4-HNE-protein adducts [272,273], and thus established immunochemical methods such as immunohistochemistry [274], immuno electron microscopy [275] and ELISA [276,277], along with the development of high performance liquid chromatography (HPLC) methods, proteomics and other mass spectrometry (MS) methods [278], enabled studying the involvement of 4-HNE in cancer and other (patho)physiological, especially inflammatory processes [11]. It should be mentioned that immunochemical methods detecting the 4-HNE-protein adducts appears to be among the most popular and reliable methods for clinical detection of lipid peroxidation in human diseases, especially in cancer [279].

Therefore, we have highlighted data regarding occurrence of 4-HNE in cancer patients’ tissue or serum/plasma samples in Table 2.

**Table 2.** Short overview on literature revealing the occurrence of 4-HNE in malignant tumors using diverse samples (tumor tissue slides, homogenates and serum samples of cancer patients and healthy controls) and methods (IHC, HPLC, ELISA, WB)

Tumor	4-HNE occurrence in tumor	Samples	Method	Ref
Lung cancer (primary tumors and secondary metastasis)	<p>Primary and secondary malignant cells showed similar 4-HNE intensity: predominantly ++(2-moderately positive) in cytoplasm and mostly pronounced in the necrotic regions.</p> <p>Diverse 4-HNE pattern of surrounding tumor tissue was observed between primary and secondary tumor: higher intensity of surrounding tissue of secondary metastasis (between ++(2-moderate) and +++(3-very positive)) vs. weakly positive +(1) observed for primary tumor.</p> <p>Negative correlation was observed between 4-HNE intensity of the surrounding tissue and tumor volume of secondary metastasis, while primary tumors were lacking this correlation.</p>	FFPE sections of primary lung tumors (n=19) and secondary lung tumors (n=17)	IHC	[280]
Lung cancer	<p>Higher 4-HNE concentration was observed in lung cancer patients, especially in women.</p> <p>Stronger link between redox status and lipid metabolism alterations in non-smoking lung cancer patients, in comparison to current or former smokers.</p>	Serum samples of lung cancer patients (n=92) and healthy controls (n=82)	ELISA	[281]
Non-small cell lung cancer (NSCLC)	<p>Higher levels of 4-HNE (measured by GC/MSMS) and 4-HNE-protein adducts (measured by ELISA) in both tumors (SCC and AC) in comparison to adjacent normal lung tissue.</p> <p>AC group had higher levels of 4-HNE-protein adducts than SCC.</p> <p>IHC revealed abundance of cytoplasmic 4-HNE-protein adducts for both, SCC and AC, mostly in necrotic regions of tumors. Adjacent stromal cells were mostly positive for cytoplasmic 4-HNE-protein adducts.</p>	<p>Tissue homogenates, plasma and FFPE sections of squamous cell carcinoma (SCC) n= 38 (female/men n=12/26)</p> <p>adenocarcinoma (AC) n=34 (female/men n=16/18)</p>	<p>GC/MS</p> <p>MS</p> <p>ELISA</p> <p>IHC</p>	[241]
Astrocytic and ependymal tumors of the brain	<p>4-HNE-protein adducts were found in all tumors.</p> <p>The incidence of 4-HNE-immunopositive tumor cells is correlated with increasing grades of malignancy.</p> <p>Endothelium of almost all tumor vessels was positive for 4-HNE, while higher positivity of the walls of the vessels was observed in DA and anaplastic AA than in PA and GBM.</p> <p>Number of 4-HNE-positive microvessels was associated with the grade of malignancy in both astrocytic and ependymal tumors.</p>	<p>FFPE sections of glial tumors (n=160):</p> <ul style="list-style-type: none"> <li>- Astrocytic tumors (n=120): pilocytic astrocytomas (PA; n=30), diffuse astrocytomas (DA; n=30), anaplastic astrocytomas (AA; n=30), glioblastomas multiforme (GBM; n=30)</li> <li>- Ependymal tumors (n=40): ependymomas (E; n=30), anaplastic ependymomas (AE; n=10)</li> </ul>	IHC	[282]
Glioblastomas	<p>4-HNE-protein adducts were found in all tumors with the immunopositivity ranging from moderate (22/30) to strong (8/30) and the intensity ranging from moderate (20/30) to strong (9/30).</p> <p>2/3 of blood vessels walls and all mesenchymal stroma were 4-HNE positive in which intensity ranged from weak to moderate. Necrotic regions were mostly negative. In control group, only sporadically reactive astrocytes were moderately positive with low stroma intensity.</p> <p>4-HNE expression was proportional with the expression of CD133.</p>	FFPE sections of glioblastomas (GBM; n=30) and control samples (without pathological changes, tumor, and other inflammatory and reactive changes; n=10)	IHC	[283]
Astrocytic tumors	<p>4-HNE positivity was proportional with malignancy of astrocytomas (GB&gt;AA&gt;DA).</p> <p>In DA, weak 4-HNE positivity was predominantly present in perivascular tumor cells while malignant variants (AA and GB) had moderate to strong and diffusely distributed 4-HNE positivity in all tumors.</p>	FFPE sections of astrocytomas (n=45): diffuse astrocytomas (DA; n=15), anaplastic astrocytomas (AA; n=15), glioblastomas (GB; n=15)	IHC	[284]

Colorectal cancer	<p>In more than 50% of samples the positivity of cytoplasmic 4-HNE in cancer cells was high while in other was low. Nuclear 4-HNE positivity was observed in 10% of cancer and stromal cells.</p> <p>Epithelial (in particular nuclear) and cytoplasmic stromal 4-HNE positivity are correlate with the poor outcome.</p> <p>Association of cytoplasmic stromal 4-HNE positivity with survival is independent of tumor stage and co-morbidity.</p>	FFPE sections of colorectal cancers (CRC; n=62) in elderly population (patients aged 85 years and older)	IHC	[285]
Colorectal cancer	<p>Levels of 4-HNE in primary CRC were higher than in normal colon tissue.</p> <p>The level of 4-HNE is correlated with the clinical stage of CRC.</p>	Tissue homogenates from primary colorectal cancer (CRC; grades II, III, IV; n=81) and controls (unchanged colon regions distant from the cancer )	HPLC	[286]
Colorectal cancer	<p>4-HNE was increased in cancer tissue in comparison to the controls.</p> <p>Enzymatic antioxidants (SOD, GPX and glutathione reductase) were increased except catalase, which was decreased along with non-enzymatic antioxidants (vitamin E and C) in cancer tissue.</p>	Tissue homogenates from colorectal cancer (CRC; n=55) and controls (microscopically unchanged colon tissue far from the cancer)	HPLC	[287]
Colon adeno-carcinoma	<p>Levels of 4-HNE-protein adducts were decreased in all cancer tissue samples except in two extremely advanced cases (T4/G3) where they were within normal range.</p> <p>Correspondingly, TGF-<math>\beta</math>1 protein was decreased or negligible compared with the corresponding normal tissue surrounding the tumor, again in all except those two very advanced cases (T4/G3) where it was in normal range.</p>	Tissue homogenates from colon adenocarcinoma patients (n=15; women/men n=8/7; TNM: T2/T3/T4 n=5/8/2; grade:G2/G3, n=11/4) and controls (microscopically unchanged tissue distant from the cancer)	Fluorescence Measurement	[288]
Colorectal carcinoma	<p>4-HNE-protein adducts were detected in cytoplasm of carcinoma cells and the intensity was stronger (in 29/37) than in adjacent normal tissue and adenoma cells. Recurrent cases showed strong intensity.</p> <p>Higher intensity of carcinoma cells in the invasive front in comparison to the tumor center was observed in a few cases.</p> <p>4-HNE positivity was also observed in the cytosol of infiltrating inflammatory cells.</p> <p>Western blot analyses of colorectal adenocarcinoma and nontumorous normal mucosa of the same patient, revealed that 4-HNE modifies many of the major proteins even in normal cells. However, several proteins were increased (39, 42, and 52 kD) in all adenocarcinoma samples.</p>	FFPE sections of colorectal adenocarcinoma (n=37), adenoma (n=6), and controls (nontumorous normal tissue)	IHC WB	[289]
Colorectal carcinoma	<p>The cytoplasm of cancer cells was diffusely stained for 4-HNE-protein adducts while in normal tissue it was weakly stained. The overall scores determined by multiplying the percentage score by intensity score revealed 5-fold higher levels of 4-HNE-protein adducts in cancer tissue in comparison to normal counterparts.</p> <p>WB analysis of 4-HNE-protein adducts revealed 3 strong bands, between 35 and 75 kDa which intensities were stronger in tumor tissue than in normal tissue.</p> <p>Content of GSH and levels of GPX-1, GPX-3 and SePP were decreased in tumor tissue while level of GPX-2 was increased. Levels of SePP were lower at stage II and IV in comparison to stage II.</p>	FFPE sections and homogenates of colorectal carcinoma (n=41; women/men, n=25/16) of different staging and controls (nontumorous tissue)	IHC WB	[290]

Renal tumors	<p>Positivity for 4-HNE-protein adducts was observed in all 6 morphologic types of renal tumors, although intensity varied among tumor types:</p> <ul style="list-style-type: none"> <li>- clear-cell type RCC: low amount of 4-HNE-protein adducts in nuclei and focal areas adjacent to cell membrane and within cell cytoplasm</li> <li>- granular-cell type RCC: moderate 4-HNE staining predominantly in cytoplasm</li> <li>- Mixed-cell type RCC: light labeling in clear cells and moderate in granular cells</li> <li>- Papillary carcinoma: trace levels of 4-HNE in both nuclei and cytoplasm</li> <li>- Transitional cell carcinoma of the renal pelvis: low levels of 4-HNE-protein adducts with moderate to heavy labeling of adjacent uninvolved transitional epithelium in nuclei and cytoplasm</li> <li>- Wilms' tumor: trace nuclear staining of 4-HNE-protein adducts</li> <li>- Renal oncocytoma: moderate levels of 4-HNE-protein adducts in cytoplasm and nuclei</li> </ul> <p>Immunogold electron microscopic analysis of clear-cell variant (n=1), granular-cell variant (n=1), papillary carcinoma (n=1) and healthy kidney (n=1) revealed nuclear and mitochondrial immunolabeling with minimal cytoplasmic and cell surface staining.</p>	<p>FFPE sections of renal tumors (n=38):</p> <ul style="list-style-type: none"> <li>- Malignant: renal cell carcinoma [RCC; n=18: clear cell type (n=13), granular cell type (n=2), mixed type (n=3)]; papillary carcinomas (n=4); transitional carcinoma of the renal pelvis (n=10); Wilms' tumor (n=4)</li> <li>- Benign renal oncocytomas (n=2)</li> </ul>	IHC  Immuno-gold method	[291]
Renal-cell carcinoma	<p>RCC cells were positively stained for 4-HNE-protein adducts showing fine to coarse staining pattern in their cytoplasm.</p> <p>This pattern was not observed in nontumorous tissue where proximal tubules showed faint diffuse staining.</p> <p>Staining intensity did not correlate with the clinical or pathological features.</p>	Cryosections of renal cell carcinoma (RCC; n=15)	IHC	[292]
Esophageal squamous cell carcinoma	<p>Higher expression of 4-HNE in ESCC tissue in comparison to non-malignant esophageal epithelial tissues was associated with the clinical stage.</p> <p>Patients with 4-HNE-positive cancer had poorer clinical outcome.</p> <p>The strongest 4-HNE positivity observed in severe dysplastic tissue accompanied with severe inflammation.</p>	FFPE sections of esophageal squamous cell carcinoma (ESCC) (n=57), esophageal carcinoma in situ (n=11) and, benign esophageal epithelial tissue samples (n=23)	IHC	[293]
Breast cancer	<p>4-HNE-protein adducts were primarily observed in cytoplasm and the 4-HNE positivity was mainly weak to moderate and increasing from UDH (45.8%) and ADH (52.4%) to DCIS (63.2%) and invasive breast cancers (73.0%). The only strong positivity of 4-HNE was observed in invasive breast cancers.</p> <p>4-HNE increases during breast carcinogenesis.</p> <p>Association of 4-HNE with the traditional prognostic factors of breast cancer in the invasive breast cancer cohort was not observed.</p>	FFPE sections of breast tumors (n=219): usual ductal hyperplasia (UDH; n=24); atypical ductal hyperplasia (ADH; n=21); ductal carcinoma <i>in situ</i> (DCIS; n=22); invasive carcinoma (n=115)	IHC	[294]
Breast cancer brain and lung metastasis	<p>High 4-HNE immunostaining in 93% (42/45) of the lung metastases and only in 16% (9/55) of brain metastases.</p> <p>High NRF2 staining in 78% (32/41) of the lung metastases and in 30% (14/48) of the brain metastases.</p> <p>Strong association between 4-HNE and NRF2 protein level was observed in the majority of the patients.</p>	Tissue microarrays of brain metastases (BrM, n=55) and lung metastases (LM, n=45) from breast cancer patients	IHC	[295]
Thyroid neoplasia	4-HNE-protein levels were higher in cytoplasm of all tumor tissues (FTA, FTC, and PTC) in comparison to matched normal tissue.	Constructed tissue microarray (TMA) slides of well-differentiated thyroid	IHC	[296]

	<p>Yet, percentage and intensity of nuclear 4-HNE positivity was higher in normal thyroid tissue.</p> <p>Levels of both cytoplasmic and nuclear 8-oxo-dG were higher in all tumor tissues.</p>	neoplasia (n=133; women/men, n=104/29): follicular thyroid adenoma (FTA, n=71), follicular thyroid carcinoma (FTC, n=17), papillary thyroid carcinoma (PTC, n=45), and matched normal tissue		
Hepatocellular carcinoma	<p>Levels of 4-HNE-protein adducts gradually decrease in tumor tissue in comparison with adjacent normal tissues during HCC progression:</p> <ul style="list-style-type: none"> <li>- 0A grade: lower levels in tumor tissue in 4/8</li> <li>- B grade: lower levels in tumor tissue in 3/5</li> <li>- C grade: lower levels in tumor tissue in 10/10</li> <li>- PVT (C grade): lower levels in tumor tissue in 9/9</li> </ul> <p>Levels of cardiolipin (CL; source of 4-HNE), their oxidation product were decreased in tumor and PVT tissues comparing to tumor adjacent tissue (concomitant with 4-HNE levels). PUFA content in CL was decreased, favoring saturated or MUFA.</p>	FFPE sections of hepatocellular carcinoma (HCC; n=46): 0 and A grade (n=8), B grade (n=4), C grade (n=19): HCC (n=10) and portal vein tumor thrombus (PVT, n=9)	IHC	[297]
Cutaneous malignant melanoma	<p>Presence of 4-HNE revealed different patterns:</p> <ul style="list-style-type: none"> <li>- absent in all samples of simple nevi while present in all dysplastic nevi (3/5 samples with more than 50% of positive cells) with moderate to strong intensity</li> <li>- LMM – both, lentigo maligna and invasive MM areas showed strong to moderate intensity of 4-HNE in all samples with high positivity in 2/3 samples</li> <li>- SSMM – moderate to strong positivity in 13/15 samples with a slight decrease of intensity towards tumor depth</li> <li>- NMM – 60% negative while the positive showed weak positivity and intensity</li> <li>- AML – all samples were diversely positivity exhibiting moderate to strong intensity</li> <li>- Amelanotic MM – similar to NMM; 50% weak to moderate positivity with weak to moderate intensity</li> <li>- Metastases – 60% negative samples while positive showed weak to moderate positivity with ranging intensity from weak to moderate and strong.</li> </ul>	<p>FFPE sections (n=55) of:</p> <ul style="list-style-type: none"> <li>- control (n=10): simple nevus (n=5); dysplastic nevus (n=5);</li> <li>- primary malignant melanoma (MM, n=35): superficial spreading MM (SSMM, n=15); nodular MM (NMM, n=10), lentigo MM (LMM, n=3), acral lentiginous melanoma (ALM, n=3), amelanotic MM (n=4)</li> <li>- metastases (n=10)</li> </ul>	IHC	[298]
Prostate cancer	<p>A pilot study to compare presence of 4-HNE with already revealed strong correlation of acrolein presence with the progression of prostate carcinoma.</p> <p>4-HNE and acrolein showed different patterns:</p> <ul style="list-style-type: none"> <li>- strong positivity of acrolein in cytoplasm and nuclei of prostatic cancer cells and in tumor stroma</li> <li>- presence of 4-HNE was observed only in tumor stroma, and not in tumor cells of prostatic carcinoma.</li> </ul>	pilot analysis	IHC	[274]

Abbreviations: IHC-Immunohistochemistry; FFPE-Formalin-Fixed Paraffin-Embedded; ELISA- Enzyme-Linked Immunosorbent Assay; HPLC- High Performance Liquid Chromatography; WB-Western Blot;

As can be seen from the data collected, the appearance of 4-HNE is not unique across different tumors and highly depends upon the tissue of origin, tumor grade/stage, and even a patient. For example, the increase in 4-HNE expression was correlated with the progression of brain tumors [282–284], esophageal squamous cell carcinoma [293], and breast cancers [294]. Opposite to that, in hepatocellular carcinoma, 4-HNE expression gradually decreased with the tumor grade showing stronger expression in adjacent tissue than in tumor [297]. Similarly, stronger expression of 4-HNE in adjacent tissue, was observed for metastatic lung cancers [280], supporting the assumption that non-malignant cells in the vicinity of cancer generate 4-HNE to defend themselves against invading cancer [21,280,297], although some studies revealed higher 4-HNE levels in tumor tissue samples than in matching controls [241,281]. Such a discrepancy might be on one hand explained by the fact that every cancer is as individual disease as is the patient suffering from it, while on the other hand, cancer growth is associated with oxidative stress, yet in case of cancer decay due to necrosis, abundant 4-HNE is being generated within malignant tissue by destroyed cancer cells, as well as by stromal and inflammatory cells [15,16,241,297]. Studies involving colorectal cancer are showing conflicting data (for these and other examples please see Table 2.). Discrepancies in data of 4-HNE presence in tumors of the same origin could be attributed to insufficient information about the tumor type/grade/stage (diverse presence of 4-HNE in different types/grades/stages of the same tumor), age/sex diversity of patients (e.g. female patients with lung cancer had higher levels of 4-HNE than male patients [281]), and to sample size as well as to which samples (tissue or serum/plasma) were used and what were controls. As on the colon cancer example, it is very rarely the case that hardly available normal, control colon tissue is analyzed serving as control specimens, instead either non-malignant tissue near cancer colon tissue, which is not really normal as already mentioned, or inflamed colon tissue (as in Chron's disease) is analyzed.

A recent comprehensive transcriptomic analysis of normal tissue adjacent to tumor (NAT; n=428), healthy tissue (n=1558) and tumor tissue (n=4550) of different origin (liver, colon, lung, thyroid, prostate, breast, bladder, uterus) has shown that NAT is distinct from both healthy and tumor tissue and although each tissue is unique, some differences are shared across tumor types including TNF- $\alpha$  and TGF- $\beta$  signaling pathways, hypoxia, and the epithelial-to-mesenchymal transition (EMT) [299]. Authors further suggest that this TASA (tumor-adjacent specific activation) signature is orchestrated by tumor itself as a possible mechanism of its progression, although the opposite interpretation is as likely, as was observed for the model of rodent liver cancer (LEC rats) [300,301].

Therefore, future research should consider this unique feature of NAT and whether NAT could be used as “normal” control when analyzing differences between tumor and normal tissue. In addition, to fully understand the interactions between tumor and NAT and how they affect tumor progression, additional analyses are needed that will reveal what happens on lipidomic and proteomic level, what is the influence of oxidative stress, and consequently of 4-HNE considering that it can modify proteins and thus affect diverse pathways and ultimately cell fate, as well as to explore diversity of antioxidative protection.

Hence, higher 4-HNE presence in adjacent tumor tissue could have dual explanation. Concomitantly with the previously mentioned tumor initiation of TASA signature to spread, Blendea et al. suggested these higher 4-HNE levels contribute to metastatic spread of cutaneous malignant melanoma [298]. Another explanation could consider it to be a protective mechanism of normal cells against tumor as suggested for lung cancer metastasis due to observed negative correlation between 4-HNE intensity of the surrounding tissue and tumor volume of secondary metastasis [280]. Higher



susceptibility of osteosarcoma cell lines as well as leukemic cells in comparison to normal cells of mesenchymal origin to pro-apoptotic action of 4-HNE [302–304] and a novel mechanism of selective induction of apoptosis in cancer cells, which is linked with the 4-HNE-induced inactivation of cancer-specific membrane-associated catalase and the subsequent increase of ROS [21], are corroborating later explanation. Both explanations might be plausible if considering the diversity of 4-HNE presence in tumors of different origins. As suggested, causes of this observed diversity could be alterations in lipid composition of the cell membranes, favoring saturated and monounsaturated fatty acids (MUFA) over polyunsaturated fatty acids (PUFA) (especially arachidonic and linoleic acids from which 4-HNE is formed); different pattern of detoxifying enzymes and antioxidant proteins (higher in cancer cells enabling better elimination of 4-HNE); presence of inflammation which might increase levels of 4-HNE that diffuse from inflammatory cells within surrounding tissue to tumor cells [8,305].

#### **4-HNE Involvement in Carcinogenesis**

4-HNE is involved in almost all hallmarks of cancer (reviewed in [2,306]) including insensitivity to growth inhibition, escaping apoptotic signals, unlimited replication potential, self-sufficiency in growth signals, escape from immune system, neoangiogenesis and metastasis formation. Diverse signaling pathways are affected by 4-HNE such as Nrf2/Keap1, NF $\kappa$ B, phosphatidylinositol 3-kinase (PI3K)/AKT, and others. Thus 4-HNE can be considered as a pro-carcinogenic activator initiating tumor development or its progression. Some of the examples are listed below.

##### *Pro-carcinogenic role of 4-HNE in tumor initiation*

The 4-HNE's preferential formation of DNA adducts (4-HNE-dGuo) and the G→T transversion at codon 249 of the *P53* gene has been considered as mutational hotspot in hepatocellular carcinoma and lung cancers, especially to those related to cigarette-smoke [307] thus implicating 4-HNE's pro-carcinogenic role in cancer initiation. Concomitantly, Long-Evans Cinnamon (LEC) rat model of hepatitis and liver carcinogenesis has revealed accumulation of 4-HNE-protein adducts in early stages of the diseases indicating its involvement in cancer initiation [301].

There are several examples of 4-HNE implication in the development of colorectal cancer. First, through the indirect effect of commensal bacteria and innate immunity on normal epithelia. Macrophages polarized by *Enterococcus faecalis* produce 4-HNE that drive chromosomal instability and transformation of normal colon epithelial cells. These phenotypically changed cells were shown to form poorly differentiated and invasive tumors in immunodeficient mice [308]. Another example is 4-HNE-induced expression of COX-2 leading to loss of APC and subsequent decrease in degradation of  $\beta$ -catenin and its translocation to the nucleus where it interacts with T-cell factor 4 for transcription of oncogenes [309]. Inflammation is additional risk factor leading to the development of colon cancer. Recent proteomic analysis identified higher levels of ferrireductase: six-transmembrane epithelial antigen of prostate 4 (STEAP4) in mouse model of colitis and patients with inflammatory bowel disease. Increase in STEAP4 leads to dysregulation of mitochondrial iron homeostasis which in turn elevates ROS and 4-HNE causing tissue injury and impact colon

carcinogenesis. Authors suggest involvement of the hypoxia/HIF-2 $\alpha$ /STEAP4/mitochondrial iron/mitochondrial ROS axis in promotion of colitis and colon cancer [310].

#### *4-HNE involvement in tumor progression*

Already in the early nineties we described the concentration- and serum-dependent effects of 4-HNE acting as cell growth stimulating factor in vitro and in vivo [311,312]. The fact that growth regulating effects of 4-HNE and of humoral (i.e. present in serum) growth factors (cytokines) are mutually dependent [313], made the basis for studies on 4-HNE as signaling molecule bifunctionally regulating c-fos expression affecting the EGF and PDGF signaling eventually causing either enhanced or suppressed growth of the HeLa cells [314,315], depending on the amount of protein adducts formed by 4-HNE [316]. The continuous studies in this direction are summarized in several review papers [11][274][306].

Complementary to that, it should be underlined that immunity plays important role in suppression of tumor spread. Dendritic cells are required to generate robust and sustained T-cell dependent anticancer immunity. Frequently, tumors impair normal dendritic cell functions to evade immune control. Constitutive activation of the endoplasmic reticulum stress response factor XBP1 in tumor-associated DCs (tDCs) was shown to direct ovarian cancer (OvCa) progression by suppressing anti-tumor immunity. 4-HNE-induced XBP1 activation triggers triglyceride biosynthetic program in tDCs leading to severe lipid accumulation and consequent inhibition of tDCs ability to support anti-tumor T-cells [317]. On the other hand, the murine model of spontaneous regression of W256 cancer revealed the importance of the innate immunity carried by granulocytes/neutrophils, which is relying on the lipid peroxidation generating acrolein, as well as MDA and 4-HNE that are regulating extracellular granulocyte cell-signaling in defense against cancer [14–18].

Furthermore, breast cancer growth and angiogenesis was shown to be promoted by 4-HNE-induced upregulation of HIF-1 $\alpha$  and increased expression of VEGF in a SIRT3-dependant manner [318], which might also explain to certain extent association of 4-HNE with the pathologic blood vessels proportional to the level of malignancy of human astrocytic tumors [282–284].

#### **Anti-cancer therapies and 4-HNE**

Optimally efficient anti-cancer therapy should selectively kill cancer cells while sparing normal cells. To date, the main selective feature of cancer cells exploited in therapy treatments includes observed higher ROS levels accumulated as a consequence of metabolic alterations due to high energy demands needed for cancer to expand. Therefore, the majority of the conventional anti-cancer therapy, aside of surgery, still relies on chemo- and radiation therapy exploiting these higher ROS levels and reinforcing them to levels that will lead cancer cells to death. [1]. Unfortunately, this feature is not sufficiently selective so unwanted side-effects also occur. Herein we will mention just some of the chemotherapeutics (doxorubicin (DOX), cisplatin, paclitaxel, tamoxifen, sorafenib) mediating their anti-cancer activity through generation of ROS and 4-HNE, and their role in toxicity against normal cells. Several examples of pharmacological mitigation of side-effects will be mentioned as well.

Chemotherapy-induced cognitive impairment referred to as “chemobrain” is observed in patients upon DOX treatment. The proposed mechanism of “chemobrain” involves enrichment of 4-

HNE-protein adducts that oxidizes apolipoprotein A-I leading to its inability to interact with ATP-binding membrane cassette transporter A1 thus increasing TNF- $\alpha$  in the periphery. TNF- $\alpha$  further crosses blood-brain barrier by endocytosis of tumor necrosis factor receptor and activates the formation of local TNF- $\alpha$  by microglia leading to mitochondrial dysfunction and apoptosis in neurons and consequently to cognitive decline. Co-administration of the antioxidant drug, 2-mercaptoethane sulfonate sodium decreased protein oxidation and resulting elevation of TNF- $\alpha$  in plasma [319]. Recently a new term “tumor brain” was suggested. It implies that cognitive deficits could be observed even prior cancer diagnosis and treatment. TumorGraft<sup>TM</sup> models of triple-negative breast cancer (TNBC) or progesterone receptor positive breast cancer (PR+BC) xenografts revealed implication of oxidative stress, and alteration of specific pathways (MAPK, PI3K/AKT) needed for the proper function of hippocampal neurons. While the decreased expression of AKT1, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), neuronal PAS domain protein 4, and brain-derived neurotrophic factor was observed in both TNBC and PR+BC tumor-bearing animals, increased levels of 4-HNE were seen just in TNBC tumor-bearing mice whereas the levels were decreased in PR+BC tumor-bearing mice [320].

Another example of negative side-effect of DOX is cardiotoxicity. 4-HNE plays important role in DOX-induced cardiotoxicity, while even non-toxic doses of DOX could result in the accumulation of the 4-HNE-protein adducts by cardiomyocytes, which show a great range of individuality under such circumstances [321]. Increased levels of 4-HNE upon DOX treatment, inactivate NADH oxidoreductase activity of the mitochondrial apoptosis inducing factor (AIFm2) causing adduction of His 174 and translocation of AIFm2 from mitochondria thus facilitating apoptosis in heart tissue of mice and humans [322]. DOX-induced release of extracellular vesicles from cardiomyocytes comprising of high 4-HNE-modified protein content was suggested to be an early and sensitive biomarker of cardiac injury [323]. In addition, 4-HNE can modify several mitochondrial proteins involved in Krebs cycle and EMT thus altering their function and consequently energy metabolism. Treatment with Mn(III) meso-tetrakis(N-n-butoxyethylpyridinium-2-yl)porphyrin, an SOD mimic, can prevent this DOX-induced cardiac damage [324].

Fidarestat, an aldose reductase inhibitor, was found to increase sensitivity of colon cancer cells to DOX by decreasing the multi-drug resistance-1, MRP1, and ATP binding cassette subfamily G member 2 drug transporters and thus efflux of the drug from cancer cells. Additionally, fidarestat reduces DOX-induced cardiac toxicity by decreasing formation of 4-HNE-protein adducts, suggesting its possible usefulness as adjuvant therapy aiming to protect non-malignant cells [325].

Sulforaphane (SFN), an isothiocyanate found in cruciferous vegetables such as broccoli, is known for its dual role: selective cytotoxicity of cancer cells and chemoprotection of normal cells. While its cytotoxic activity is attributed to generation of 4-HNE and targeting specific pathways which enable selective growth advantage of cancer cells, its chemoprotective effect is due to upregulation of defense mechanisms, such as activation of Nrf2 pathway [326,327]. Studies in a rat orthotopic breast cancer model revealed attenuated cardiotoxicity and potentiated efficacy of DOX when used concomitantly with SFN suggesting that lower dosage of DOX can be used in concomitant treatments [328].

A mechanism of action of tamoxifen includes generation of 4-HNE which through activation of protein phosphatase 2A downregulates survival proteins such as AKT thus leading to apoptosis of breast cancer cells. However, cancer cells can adapt to tamoxifen treatment by activating Nrf2 pathway thus increasing expression of antioxidant genes (NAD(P)H:quinone oxidoreductase 1

(NQO1), HO-1, SOD1) and the multidrug resistant transporters (e.g. MRP1 that exports 4-HNE from cancer cells). Indeed, high expression levels of Nrf2, NQO1, MRP1, and ATP Binding Cassette Subfamily C Member 3 indicate poor prognosis for those breast cancer patients [329].

Mangafodipir, another SOD mimic, was suggested as a protective mechanism against cisplatin- or paclitaxel-induced ovarian damage (by decreasing levels of 4-HNE) while not affecting antitumor effects of anticancer drugs in tumor xenografts. Therefore, the administration of mangafodipir during cisplatin and/or paclitaxel therapy might be promising pharmacological strategy for protection of the normal cells [330].

Sorafenib is a standard cytostatic for treatment of kidney and liver malignancies. In renal cancer, c-Met-Nrf2-HO-1 axis was shown to be important in sorafenib chemoresistance. Combined treatment with pharmacological inhibitors of c-Met and HO-1 reduced the growth of renal tumors xenografts through activation of 4-HNE, apoptosis and DNA damage [331]. Cardiolipin a specific hepatic, mitochondrial phospholipid rich in linoleic acid is suggested as an important source of 4-HNE. Supplementation of liver cancer cells with tetralinoleoyl cardiolipin enhanced the Sorafenib-induced apoptosis in vitro. Hence, ketogenic diet (rich in PUFAs such as linoleic and arachidonic acid) could serve as an effective adjuvant therapy in treatment of hepatocellular carcinoma and other tumors by a mechanism linking mitochondrial 4-HNE formation with induction of apoptosis [297].

Sulfasalazine, used for treating rheumatoid arthritis, was also shown to act as a specific inhibitor of xCT-dependent cystine transport, which is important for generation of GSH, induction of ferroptosis and implicated in chemoresistance. In sulfasalazine resistant head and neck squamous cell carcinoma (HNSCC) and gastric tumors, which are known to highly express aldehyde dehydrogenase (ALDH)3A1, a co-treatment with dyclonine (oral anesthetic; inhibitor of ALDH) increases the accumulation of 4-HNE and thus cancer cell death suggesting it as a sensitizer to xCT-targeted cancer therapy [332].

### **Conclusions:**

Taken together the findings summarized in this review paper allow us to conclude that 4-HNE might be considered not only as second messenger of ROS, but also as one of fundamental factors of the stress- and age-associated diseases. While standard, even modern concepts of molecular medicine and respective therapies in majority of these diseases target mostly the disease-specific symptoms, 4-HNE, especially its protein adducts, might appear to be the bioactive markers that would allow better monitoring of specific pathophysiological processes reflecting their complexity. Eventually that could help development of advanced integrative medicine approach for patients and the diseases they suffer from on the personalized basis implementing biomedical remedies that would optimize beneficial effects of ROS and 4-HNE to prevent the onset and progression of the illness, perhaps even providing the real cure.

### **Dedication:**

Dedicated to Prof. Peter Eckl, former president of the International 4-HNE-Club, great researcher and dear friend, who suddenly passed away. Thank you for everything Peter, we will miss you.

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other researchers revealing importance of 4-HNE as “second messenger of reactive oxygen species” in health and in stress- and age-associated diseases.

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