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REVIEW ARTICLE

Pathological aspects of lipid peroxidation

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Abstract
Lipid peroxidation (LPO) product accumulation in human tissues is a major cause of tissular and cellular dysfunction that plays a major role in ageing and most age-related and oxidative stress-related diseases. The current evidence for the implication of LPO in pathological processes is discussed in this review. New data and literature review are provided evaluating the role of LPO in the pathophysiology of ageing and classically oxidative stress-linked diseases, such as neurodegenerative diseases, diabetes and atherosclerosis (the main cause of cardiovascular complications). Striking evidences implicating LPO in foetal vascular dysfunction occurring in pre-eclampsia, in renal and liver diseases, as well as their role as cause and consequence to cancer development are addressed.

Keywords: *Oxidative stress, oxidative homeostasis, reactive oxygen species, lipid peroxidation, 4-Hydroxynonenal, neurodegenerative diseases, Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, liver disease, fatty liver, atherosclerosis, redox equilibrium, cell signalling, oxidized phospholipids, oxysterol, diabetes, diabetes complications, 4-hydroxydodecadienal, 4-hydroxynonenal, pre-eclampsia, HELLP syndrome, IUGR, glutathione, redox signaling, Nrf2, antioxidant enzymes, foetal endothelium, renal diseases, lymphoedema, hyperglycaemia, advanced glycation end-products, cancer*

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Abbreviations: *AD, Alzheimer disease; AGE, advanced-glycation end product; ALT, alanin-aminotransferase; AMA, anti-mitochondrial antibodies; ANA, anti-nuclear antibodies; APP, Amyloid precursor protein; ApoE, apolipoprotein E; AST, aspartate-aminotransferase; BACE1,* β*-site cleaving enzyme; BAP, amyloid beta protein; BMI, body mass index; CTGF, connective tissue growth factor; CML, carboxymethyl-lysine; CNS, central nervous system; CRA, cardio-renal-anaemia; CRP, C-reactive protein; CSF, colonia stimulating factor; CVD, cardiovascular disease; EGFR, EGF receptor; EGR-1, early growth response factor 1; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; ESRD, End-stage renal disease; EPO, erythropoietin; ET-1, endothelin 1; FALDH, fatty aldehyde dehydrogenase i.e. Aldh3a1; FFA, Free fatty acids; GGT, gammaglutamyl-transpeptidase; GLDH, glutamate dehydrogenase; GPx, glutathione peroxidise; GSH, glutathione (reduced form); GST, glutathione S-transferase; HBV, hepatitis B virus; HCC, hepatocellular cancer; HCV, hepatitis C virus; HD, haemodialysis; 4-HHE, 4-hydroxy-2-hexanal; 4-HDDE, 4-hydroxy-2E,6Z-dodecadienal; HDV, hepatitis D virus; HELLP, v haemolysis elevated liver enzyme low platelet syndrome; 4-HNE, 4-hydroxynonenal,12-HpETE:12-hydroperoxyeicosatetraenoic,15-HpETE:15-hydroperoxyeicosatetraenoic acid; 13-HpODE, 13-hydroperoxyoctadecadienoic acid; HSC, hepatic stellate cell; ICAM-1, intercellular adhesion molecule-1; IGF-I, Insulin-like growth factor I; IR, insulin resistance; IRS, Insulin-receptor-substrate; IUGR, intrauterine growth retardation; JNK, Jun N-terminal kinasel; LBD, Lewy body disease; LDH, lactate dehydrogenase; LDL, Low density lipoprotein; 12,15 LO, 12,15 lipoxygenase; LPC, lysophosphatidylcholine; LPO, lipid peroxidation; LVMI, Left ventricular mass index; MCP1, monocyte chemotactic protein-1; MDA, malondialdehyde; MIP-1*β*, macrophage inflammatory protein-1*β*; MMPs, matrix metalloproteinases; MRC, Mitochondrial respiratory chain; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; NFAT, nuclear factor of activated T; NF-*κ*B, Nuclear factor-*κ*B; NOD, non-obese diabetic mice; NOSA, Non-organ specific autoantibodies; NRF-1, nuclear respiratory factor-1; NFT, Neurofibrillary tangles; OH•, hydroxyl radical; OHdG, 8-hydroxy20-deoxyguanosine; OxPLs, oxidized phospholipids; PAF, platelet-activating factor; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PE, pre-eclampsia; PEIPC, 1-palmitoyl-2-(5,6-epoxyisoprostane* E_2*)-sn-glycero-3-phosphatidylcholine; PGC1-expression, PPAR-*γ *co-activator 1-*β *and 1-*α*; PGPC, 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphatidylcholine; PHF, paired helical fragment; PIFKA II, protein induced by vitamin K absence or antagonist II (abnormal plasma prothrombin; PIIINP, N-terminal pro-peptide of collagen type III; PON, paraoxonase; POVC, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphatidylcholine; PPARγ, peroxisome proliferator-activated receptor gamma; PUFAs, polyunsaturated fatty acids; RAGE, receptor for advanced glycation end-products; SMA, smooth muscle antibodies; SOD, superoxide dismutase; SP, senile plaque; SR-B1, scavenger-receptor-B1; T2DM, Type2 diabetes mellitus; TBARS, thiobarbituric acid-reactive substances; THB, tetrahydrobiopterin; TIMP-1, metalloprotease tissue inhibitor-1; TLRs, toll-like receptors; TNF-*α*, tumour necrosis factor-*α*; UPR, unfolded protein response; VCAM-1, vascular cell adhesion molecule-1; VEC, vascular endothelial cell; VEGFR2, VEGF receptor 2.*

Introduction

Accumulating evidence supports the hypothesis that oxidative stress, lipid peroxidation and oxidative damages to tissular and cellular molecules in humans play a major involvement in ageing and most age- and oxidative stress-related diseases. Oxidative stress results from an imbalance between free radicals over-production and a lower degradation rate due to a decreased activity of endogenous defense systems. Reactive oxygen species (ROS) express a variety of molecules and free radicals physiologically generated from the metabolism of molecular oxygen, including superoxide anion, which is the precursor of most ROS and of non-enzymatic reactions with other radicals such as nitric oxide (NO) and a major mediator in oxidative chain reactions. ROS attack/damage all cellular constituents. Lipids (cholesterol, polyunsaturated fatty acids (PUFA) are a main target of oxidative attack and this leads to the formation and the accumulation of lipid oxidation (LPO) products [1,2], in particular oxysterols, hydroperoxides and endoperoxides. The latter undergo fragmentation to produce a broad range of reactive carbonyl intermediates such as α,β-unsaturated aldehydes [4-HNE and acrolein], di-aldehydes [MDA and glyoxal] and keto-aldehydes [4-oxo-trans-2-nonenal (ONE) and isoketals]. These carbonyl compounds, ubiquitously generated in biological systems, have unique properties contrasted with free radicals. Further, the non-charged structure of aldehydes allows them to migrate with relative ease through hydrophobic membranes and hydrophilic cytosolic media, thereby extending the migration distance far from the production site. Based on these features alone, these carbonyl compounds can be more destructive than ROS and may have far-reaching damaging effects on target sites within or outside membranes, as they react with nucleophilic groups in macromolecules like proteins, DNA and aminophospholipids, among others, resulting in their chemical, non-enzymatic and irreversible modification [3–5].

Tissues and organs in which cell turnover is lower or absent (brain, heart, skeletal tissues) tend to accumulate high quantities of LPO products which first behave as physiological mediators. Further, the progressive accumulation of LPO products is a starting point mechanism of tissular and cellular dysfunction involved in ageing and in well-defined diseases of liver, kidney, neurological and cardiovascular systems,

cancers, endocrine and metabolic disorders, diabetes and its complications and other oxidative stress-related pathologies.

The present review is a critical evaluation of the presence and importance of LPO in initiating and/or mediating some aspects of the pathological processes involved in the installment and further complications of these diseases.

Ageing (T Grune)

Ageing and oxidative stress

The 'free radical theory of ageing' was proposed in 1956 by Harman [6]. Since that time this theory was several-fold revised, changed, interpreted and discussed [7–13]. However, the originally created idea, that free radicals or other metabolic side-products are leading to accumulating damages, which in time cannot be repaired and cause physiological pathways dysfunctions and a reduced ability to adapt to stressors and leading finally to cell death, remains the same in quite a few of the revised versions of the free radical theory of ageing. However, Harman et al's [14] theory that production of free radicals is causing cellular senescence and organism ageing seems to be very simplistic. This is especially true on a background of oxidant requirement for adaptational events and today's concept of oxidative-redox status as a balance between pro- and anti-oxidative processes [15]. However, a number of correlations between oxidant production and lifespan seem to exist in mammals and insects [16,17], indicating a causal relationship. Interestingly, in many cases mitochondria seem to play a major role [16–19] in age-related oxidant production, although the accumulating damage itself might cause enhanced oxidant production [20].

Several theories exist, related either to free radical/oxidant production or to damage-accumulation. This includes the 'network theory of ageing' or 'mitochondrial theory of ageing' favouring the accumulation of oxidatively damaged mitochondria and an increasing disturbance of cellular metabolism [21,22]. Nitric oxide (NO) or accumulating lysosomal iron were also proposed as oxidant sources [23,24]. Interestingly, and most importantly, all these theories are proposing disturbances of the cellular homeostasis as the original reason of cellular senescence and the accumulation of damaged material as the consequence and the final reason for cellular death. However, the proposed correlations do not finally prove the causal role of free radicals in the mechanism of the ageing process.

Historical studies on ageing and oxidative stress

The first discovery of an age-related accumulation of damaged, oxidized material was made in 1842 by Hannover [25], describing for the first time age pigments in human neuronal cells. However, years later, Konneff [26] realized that this pigment was connected with the ageing process. Since this time the formation of the age pigment (later called lipofuscin) was described and controversially discussed [27–30]. Besides in humans, the formation of this pigment was also described in model organisms as flies and rodents [31–34]. Interestingly, already in 1976 Harman et al. [14] himself studied the effect of antioxidants on the formation of lipofuscin, implying a connection between age pigment accumulation and oxidative processes. Although lipofuscin has now been known for more than 150 years, the exact formation and role in cellular metabolism remains still largely unknown. It becomes more and more clear that this material is not an inert waste product, but has a multitude of cellular effects, as modulating metabolic pathways [35,36], influencing gene expression [37] or inducing apoptosis [20]. In addition to that, the formation of this material remains obscure. However, lipid peroxidation products might play a key role in the cross-linking event of proteins [38–40], leading to the formation of undegradable, insoluble cross-linked proteins, which is today thought to be the starting point of lipofuscin formation [15]. Therefore, an enhanced lipid peroxidation process during ageing seems important for lipofuscin formation.

Lipid peroxidation in ageing

Early studies on the level of lipid peroxidation during ageing gave contradictory results [41–43]. This seems due to the fact that, although lipid peroxidation has been studied for more than 60 years by biochemists, there is still no easy, accurate and reproducible method for the measurement of lipid peroxidation in serum or tissue. In the literature, a wealth of studies exists investigating the steady state concentration of various lipid peroxidation products in a number of different tissues in humans and model organisms. This includes measurements of MDA by thiobarbituric acid-adduct (TBARS) formation, the measurement of conjugated dienes, of 4-HNE, of protein adducts of lipid peroxidation products and of various other products. Although the results are somehow contradictory—in general—an increase of lipid peroxidation products with age is found. Tissue specificity, cellular involvement or linearity of such an accumulation remains obscure. In addition to that (and resulting from the ageing process itself), cells or organs are changing in cell and extracellular matrix composition, cell size and cell-to-extracellular-matrix-ratio, making the standardization and exact comparisons between young and old tissues difficult. For example, the comparison of an accumulated lipid peroxidation product between young and old liver per gram tissue seems to be odd on the background that old liver is often fibrotic and composed of other cells. Since the measurement of lipid peroxidation products reflects a balance between the actual lipid

peroxidation process and the degradation rate of the measured lipid peroxidation products, it is important to note that the metabolic consumption of lipid peroxidation products is cell type specific. Most intensively this was studied for 4-HNE [44]. Therefore, it is hard to quantify the lipid peroxidation in relation to number of cells, wet weight or protein amount. The clinical use of oxidation parameters is reviewed in Voss and Siems [45].

Nevertheless, numerous studies show an increase in lipid peroxidation during ageing, as a change in the membrane transition temperature in human myelin from white matter of patients was found over the age of 50 [46], an increase in lipid peroxidation in rat brain and liver homogenates [47] and individual regions of the brain [48] were found. Lipid peroxidation was investigated by several authors in various brain regions, e.g. Zhang et al. [49] found an age-dependent increase in hydroperoxide level in the striatum of gerbils and not in the hippocampus and cortex, whereas Krisofikova et al. [50] found decreased levels of MDA in rat hippocampus and Hayakawa et al. [48] found increased 4-HNE immunoreactivity in CA1 regions in mice.

More recently, a number of investigations demonstrated not only an age-association of lipid peroxidation, but also an increase of lipid peroxidation in several age-related diseases, such as Alzheimer's [51,52] or in obesity and metabolic syndrome [53]. Due to the existing number of diseases in all cohorts of elderly human beings, therefore, the measurement of pure age-related lipid peroxidation is even more difficult. However, in plasma an age-related increase of MDA, 4-HNE and lipoperoxides was reported [54–56]. The same was also reported for model organisms [57,58]. However, for unknown reasons other groups were unable to demonstrate that [59,60].

In summary, there is good evidence for increased lipid peroxidation product accumulation during the ageing process in humans and other organisms. The development of new more sophisticated, routine detection methods will undoubtly resolve this issue.

Neurodegenerative diseases (K Zarkovic)

Lipid peroxidation and central nervous system

LPO products accumulate with ageing, in particular in oxidative stress-related neurological disorders such as ischemic, inflammatory, metabolic, developmental and degenerative diseases [61–63]. The central nervous system (CNS) is a very sensitive target for the LPO damage because of a high level of polyunsaturated lipids in neuronal cell membranes, high metabolic rate of transitional metals and poor antioxidative defense [64]. Accordingly, oxidative stress is very pronounced in a number of neurodegenerative disorders by abnormal filament accumulation, deposits of an abnormal form of specific proteins in affected neurons, inflammation and mitochondrial dysfunction. While the effects of permanent oxidative stress on post-mitotic neuronal cells are cumulative, causes of neuronal death in neurodegenerative diseases are multi-factorial. Advances in molecular genetics increased the knowledge of hereditary pathogenesis of neurodegeneration, while evidences for the importance of metabolic imbalance and oxidative stress in sporadic forms of neurodegenerative diseases are constantly rising [65]. In some familiar cases of amyotrophic lateral sclerosis (ALS), mutation in the gene for Cu/Zn superoxide dismutase (SOD1) can be identified. In other neurodegenerative diseases, reactive oxygen species (ROS) and subsequent LPO products have usually uncertain roles in pathogenesis or implications on neuronal death, thus it is often unclear whether oxidative damage is a cause or consequence of neurodegeneration [66]. A role for permanent oxidative stress in neurodegenerative disorders is particularly important because neurons are post-mitotic cells and gradually accumulate oxidative damage over time.

ROS induce peroxidation of the cellular membrane lipids or circulating lipoprotein molecules generating highly reactive aldehydes [67]. Aldehydes are highly reactive with macromolecules and much more stable than ROS so they can spread from site of origin and act at a more distant site. One of the most important products of LPO generated from polyunsaturated fatty acids (PUFAs) is 4-HNE [68]. This highly reactive α,β-aldehyde is cytotoxic and is generated during various physiological and pathophysiological conditions based on the production of ROS [67,69–71].

Lipid peroxidation and Alzheimer's disease

Oxidative damage occurs in all human neurodegenerative diseases and seems especially important in Alzheimer's disease (AD). AD is a progressive neurodegeneration characterized clinically by early memory dysfunction, later progression of disorders with dysphasia and dyspraxia to mute and immobility. AD is the commonest cause of dementia and increases in incidence with age. The prevalence of AD is 10% or more after the age of 80, with higher incidence in women. The majority of cases of AD are sporadic and in some patients a familial variant of AD can be recognized. These patients carry mutant genes for membrane spanning amyloid precursor protein (APP). AD is morphologically present by amyloid-containing neuritic (senile) plaques (SP) and neurofibrilary tangles (NFT), predominantly in the hippocampus and frontal cortex. The core of these plaques is composed of amyloid β-protein (BAP), which is derived by proteolysis (β and γ secretase) from a much larger precursor protein APP. In AD the APP molecule is cut at both ends, releasing an intact BAP, which is toxic and accumulates in neuritic plaques as amyloid fibrils

[72]. The increased activity of β-site cleaving enzyme (BACE1) correlates with oxidative stress in Alzheimer's disease [73]. SP formation in AD may be secondary to another pathologic process. NFT are composed of paired helical filaments (PHF) that consist of an abnormal form of normally occurring microtubule-associated protein (MAP), termed tau. Normal tau proteins are microtubule binding proteins that stabilize the microtubular neuronal cytoskeleton and the phosphate groups are rapidly removed by phosphatase in post-mortal tissue. In contrast, tau protein in PHF is phosphorylated on aberrant site and is relatively resistant to post-mortem dephosphorylation [74]. In AD, tau does not associate with microtubules, but instead aggregates in the form of PHF. The link between BAP generation and the formation of NFT is not known. Of possible relevance is the increased neuronal expression of a receptor for advanced glycation end-products (RAGE) and also a receptor for BAP around deposits of amyloid. Soluble oligomers of BAP aggregated with divalent copper bind to cholesterol, adhere to plasma and intracellular membranes and cause radical-initiated LPO. Binding of soluble BAP oligomers with tau results in tau hyperphosphorylation [75]. Another important observation is the accumulation of abnormal ubiquitin-conjugated proteins in affected neurons, suggesting a dysfunction of the proteasome proteolytic system in these cells [76].

Many studies of AD show increased oxidation of brain lipids, carbohydrates, proteins and DNA in NFT and SP. Oxidative modification of macromolecules decreases or eliminates their function and activates inflammatory processes in the brain of patients with AD. In oxidative pathogenesis of AD, a particular role is played by LPO [65], formed from PUFA which build the brain phospholipids. Phosphorus nuclear magnetic resonance of CNS shows a decrease in phosphatidyl-ethanolamine and stearic, oleic, arachidonic and docosahexenoic fatty acids in the hippocampus and inferior parietal lobule of AD patients compared with age-matched controls [77]. On the other hand, isoprostanes (F type prostane ring–F_2IsoP) are formed non-enzymatically by free radical-induced oxidation of arachidonic acid in these patients [78]. Namely, oxidation of docosahexenoic acid leads to the formation of F_2-IsoP-like compounds, so called F_4-neuroprostanes (F_4-NP). Their concentration in CSF of AD patients is significantly elevated if compared with controls [79].

TBARS are elevated in all AD brain regions compared with controls, while the presence of 4-HNE in AD usually shows specific onset of lipid peroxidation, which might reflect the presence of LPO. There are numerous findings supporting an important role of 4-HNE in AD development. 4-HNE causes impaired glucose transport in cultured rat hippocampal neurons and impaired glutamate transport in rat neocortical synaptosomes [80]. Moreover, after administration to the rat forebrain, 4-HNE damages cholinergic neurons [81]. Exposure of cultured hippocampal neurons to H_2O_2 produces zinc and 4-HNE-mediated lysosomal membrane permeabilization, causing neuronal death [82]. 4-HNE is also capable of inducing apoptosis in PC12 cells and cultured rat hippocampal neurons [83]. A significant increase of free 4-HNE in cerebrospinal fluid [84], amygdala, hippocampus and parahippocampal gyrus was detected in the brain of AD patients when compared with control subjects [85]. Moreover, immunohistochemical studies demonstrated that 4-HNE immunoreactivity is present in NFT in some SP in AD (Figure 1). Oxidatively modified proteins by 4-HNE in AD are an early event in progression of dementia [52,86]. 4-HNE modification of BAP results in an inhibition of degradation of oxidized proteins by 20S proteasome [76], while co-existence of 4-HNE-immunoreactivity in the same cells suggests the essential role of oxidative damage in the altered protein accumulation. 4-HNE is toxic for P19 cells, causing cross-linking of tau into high molecular weight substances that are conjugated with ubiquitin [87]. 4-HNE-immunopositivity seems to be associated with the inheritance of apolipoprotein E 4 (ApoE) alleles. ApoE genotype and advancing ageing are interacting risk factors for late onset and for sporadic AD [88]. 4-HNE is a more potent cross-linker than MDA, for purified ApoE3 and ApoE4 in P19 neuroglial cell culture [89]. A significant decrease of glutathione transferase activity and of other antioxidative enzymes in amygdala, hippocampus and inferior parietal lobule in AD patients [90] lead to more pronounced effects of 4-HNE in these brain regions. However, while 4-HNE seems to be of major importance, this aldehyde cannot be considered as the only causative agent in protein aberrations related to LPO. Namely, acrolein, the most reactive could be rapidly incorporated

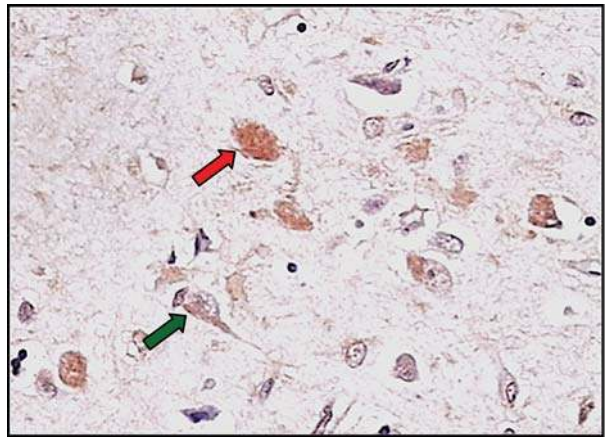

Figure 1. 4-HNE immunopositivity in neurofibrillary tangels. 4-HNE immunopositivity in neurofibrillary tangels within the neuronal cytoplasm (green arrow) and in senile plaques (red arrow) in AD, magnification 400 × Monoclonal antibodies specific for the HNE-histidine epitope (courtesy of Dr Georg Waeg, IMBBM, KF-University, Graz, Austria).

into proteins generating carbonyls or modifying DNA basis [91]. Acrolein preferentially reacts with lysine residues that are prominent components of tau [92] and are present in NFT and dystrophic neuritis surrounding SP in AD [93]. This aldehyde is neurotoxic in a time- and concentration-dependent manner, even more than 4-HNE for hippocampal tissue cultures [90]. Acrolein might be in part responsible for the reactive carbonyl compounds formed during LPO and sugar glycoxidation that increased in the frontal pole, hippocampus and inferior parietal lobule of AD patients when compared with normal aged control subject [94], producing cell dysfunction, inflammatory response and apoptosis [3,95]. Finally, the oxidative nature of protein alterations in AD is supported by immunohistochemical findings of 2,4-dinitrophenyl-hydrazine (DNP) that demonstrated further presence of protein carbonyls in NFT-bearing neurons and glia, when compared with non-NFT-bearing neurons in AD and with normal brains [96].

Lipid peroxidation and Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder clinically characterized by extrapyramidal movement disturbance: rigidity, bradykinesia, resting tremor and sometimes dysphagia, autonomic dysfunction or dementia. Pathologically, PD is characterized by a loss of dopaminergic neurons from the substantia nigra associated with the presence of intraneuronal inclusions denoted as Lewy bodies. PD typically appears in the sixth to eighth decades of life with no gender differences. Mutation in the gene encoding α-sinuclein leads to an understanding of the mechanisms underlying selective dopaminergic cell death in familiar cases, while such mutation has not been seen in studies of sporadic PD [97]. Thus, aetiology of dopaminergic degeneration remains undefined, especially in the non-familial forms of PD. It has been suggested that aggregates of α-synuclein generate oxidative stress, possibly by interaction with iron [98]. α-synuclein is a pre-synaptic protein that forms the major component of the filament comprising Lewy bodies in PD and in Lewy body disease (LBD). Neurodegenerative disorders characterized by nigral degeneration (such as PD) have high levels of free iron and increase in proteins that store iron in redox-inert forms. H_2O_2 can damage the dopaminergic neurons directly or indirectly through the formation of hydroxyl radicals in the presence of ferrous ions by the Fenton reaction. Possible sources of iron are microglia with ferritin-bound iron and potential increase of iron in astrocytes of substantia nigra [99] combined with an increase in the Fe (III)/Fe (II) ratio and decrease in glutathione (GSH) [31]. Intracellular redox and iron imbalance results in aberrant oxidation of dopamine to 6-hydroxydopamine, which in turn can undergo auto-oxidation to the corresponding dopamine quinone concomitant with generation of superoxide. Reactive quinone can be neurotoxic more than oxygen free radicals for dopamine-induced protein cross-linking in rat brain membrane fraction [100]. The plasma of PD patients, both sporadic and familial form, has elevated levels of LPO and is furthermore susceptible for lipid peroxidation more than normal healthy human plasma [101]. Oxidative stress cascade based on lipid peroxidation can lead to further generation of ROS, which induces oxidative damage of DNA, particularly in substantia nigra, increase in protein carbonyls [102], protein nitration [98] and viciously increased lipid peroxidation. Inhibition of 26S proteasome due to oxidative protein modifications lead to lower degradation of ubiquitinated oxidized or nitrated proteins [103]. The end-products of lipid peroxidation such as 4-HNE acting as second toxic messengers of free radicals give major impact to this. Namely, 4-HNE is present in Lewy bodies and in mitochondria in PD [104] and in diffuse LBD [105]. Therefore, it is not surprising that high levels of glutathione peroxidase (GPx) conjugate with 4-HNE were found present in cerebrospinal fluid of LBD. This indicates that lipid peroxidation may be causatively related to PD not only appearing as an epiphenomenon of the disease [105]. In favour of this possibility are also some experimental findings. Incubation with ranging concentrations of 4-HNE induces a dose-dependent decrease of dopamine uptake and Na/K ATPase activity and a loss of sulphydryl (SH) groups. These data suggest that 4-HNE is an important mediator of oxidative stress that alters dopamine uptake after binding to SH groups of the dopamine transporter and Na/K ATPase. Such toxic effects of 4-HNE contribute to the onset and progression of PD.

Lipid peroxidation and amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by weakness and spasticity, which is pronounced by primary loss of lower and upper motor neurons, secondary neurogenic atrophy of striated muscles and can be associated with variable pathology of non-motor systems. ALS is further classified into common sporadic (sALS) and rare familial (fALS) forms. In sALS a specific loss of the glial glutamate transporter protein EAAT2 is observed in spinal cord [106]. The primary pathogenic processes underlying motor neuron injury in sALS are multifactorial and the precise molecular pathways are unknown. Approximately 10% of all ALS cases are fALS inherited by autosomal dominant or recessive transmission. About one quarter of the fALS cases, which are dominantly inherited, are linked with mutation in the gene encoding SOD1 protein [107]. Most of the mutations in SOD1 protein occur outside the active site and produce only a small decrease in enzyme activity as a result of lower stability and

decreased half-life of mutant protein [108]. SOD1 is a Cu/Zn-binding ubiquitous anti-oxidative enzyme that catalyses the conversion of the toxic superoxide radical to H_2O_2, which in turn is converted to H_2O by the action of glutathione peroxidase or catalase [66]. Similarity between the fALS and sALS suggest that these two sub-types share common pathways of injury by a direct toxic effect of superoxide radicals inadequately scavenged by the mutant enzyme. Weakened affinity for copper in the mutant SOD1 produces competition with other copper and zinc binding proteins, resulting in a diminished amount of active enzyme and insufficient superoxide scavenging [65]. Such changes could allow potential mutant SOD1 to react with various subsidiary activities including peroxidase activity resulting in generation of hydroxyl radicals from H_2O_2 [109] and production of nitrogen ions from tyrosine residues of proteins using peroxynitrite as a substrate [110]. Neurofilament proteins and tyrosine kinase receptors are particularly susceptible to nitrotyrosine damage. Mutant SOD1 A4V protein aggregation and accumulation in the motor neurons of anterior horns in fALS patients was demonstrated in characteristic hyaline inclusions. The same inclusions contain epitopes of ubiquitin and phosphorylated neurofilament protein [111], while a study on autopsy materials indicated that increased level of 4-HNE might be the cause of modification of astrocytic glutamate transporter EAAT2 and impaired glutamate transport in ALS [112]. In patients with sALS, intense immunoreactivity for 4-HNE and 4-hydroxy-2-hexanal (4-HHE) protein adducts, carboxymethyl-lysine (CML), as a lipid peroxidation or protein glycoxidation products, present in the cytoplasm of the degenerated motor neurons, suggest a potential involvement of oxidative stress in the pathomechanisms of this disease. Acrolein protein adducts were not detectable in the spinal cord of sporadic or familial ALS patients [113,114]. Another study demonstrated increased levels of both 8-hydroxy-2-deoxyguanosine (OHdG), as a nucleic acid oxidation product, and protein-bound carbonyl in the motor cortex of sALS cases. Since OHdG immunoreactivity in the cell nuclei was persistent even after RNA digestion, it is believed that the OHdG originated mainly from the nuclear DNA. In fact, protein glycoxidation products imidazolone and pyrraline, formed via reactive intermediates such as 3-deoxyglucosone without oxidation reaction, are present in spinal motor neurons in fALS, imidazolone in cytoplasm and pyrraline in hyaline inclusions [113]. Both oxidative stress with mutant SOD1 and accumulation of specific advanced glycation end products (AGE) occurring in motor neurons needs to be a base for understanding pathomechanisms of ALS.

In conclusion, many experimental and clinical data suggest that oxidative damage could be one important aspect for the onset of neurodegeneration based on persistent oxidative stress associated with lower enzymatic antioxidant defense and increased production of LPO markers in both CNS and the blood of patients with neurodegenerative disease, defining these diseases also as progressive systemic oxidative stress disorders associated with lipid peroxidation.

Atherosclerosis (N Auge, A Negre-Salvayre, G Poli)

Inflammatory reactions in the arterial wall triggered and sustained mainly by lipid oxidation products underlie the initiation and promotion of atherosclerosis and cardiovascular disease (CVD) [115]. Vascular changes in CVD are afterwards favoured by a wide spectrum of events, such as hyperglycaemia, hypercholesterolemia, smoking, all stimuli that are often, if not always, associated with significant changes of redox equilibrium in blood and vascular cells [116]. Various pathophysiological conditions, therapeutic interventions, blood flow modifications and agents from endogenous or exogenous origin contribute to increase the production of ROS in the vascular wall, by modulating the expression and activity of ROS-producing enzymes, including NAD(P)H oxidase, endothelial NO synthase, xanthine oxidase, myeloperoxidase, SODs, catalase and glutathione peroxidase [117,118]. ROS generate vascular dysfunction and remodelling through oxidative damage, which progressively reduce the NO bioavailability, thereby altering endothelium-dependent vasodilatation. ROS and lipid oxidation (LPO) products contribute to alter the balance survival/apoptosis by their wide range of biological properties that lead to inflammatory reactions, endothelial migration and dysfunction, smooth muscle cell vasoconstriction and apoptosis [117,119,120]. Among the most potent LPO products formed during the LDL oxidation process, oxidized phospholipids (OxPLs), 4-HNE and oxidized cholesterol derivatives (oxysterols) were shown to accumulate in atherosclerotic lesions, implicating these lipids as important factors not only in the initiation but also in the promotion of the monocytic inflammation that underlies atherosclerosis [121,122]. At present, however, the signalling and transcriptional mechanisms mediating pro-inflammatory effects of OxPLs, HNE and oxysterols are only partially understood. The same applies to intracellular signalling and gene transcription pathways sustaining cell–cell and cell–matrix interaction within the arterial wall undergoing damage and subsequent remodelling.

Role of oxidized phospholipids in atherosclerosis

The susceptibility of membrane phospholipids to oxidative alterations is related to two inherent traits, the physico-chemical properties of the membrane bilayer

and the chemical reactivity of the fatty acids composing the membrane, which are extremely sensitive to oxidation. Consequently, the high concentration of unsaturated fatty acids in phospholipids not only makes them prime targets for reaction with oxidizing agents but also enables them to participate in long free radical chain reactions. The formation and the properties of oxPLs have been extensively studied [121,123–125]. OxPLs are present and abundant in oxidized LDL, and are thought to play a major role in oxLDL-induced pro-inflammatory properties. The main oxPLs belong to the oxPAPC series, formed upon oxidation of the 1-palmitoyl-2-arachidonoyl-*sn*-3-glycero-phosphorylcholine. They can be separated by chromatography, which allows one to identify 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphatidylcholine (POVPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphatidylcholine (PGPC) and 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphatidylcholine (PEIPC), among the most potent pro-inflammatory mediators present in atherosclerotic lesions. OxPLs also include platelet-activating factor (PAF)-like lipids and lysophosphatidylcholine (LPC), as well as lipids resulting from the arachidonic acid cascade, which implicates phosphatidylcholine hydrolysis by phospholipase A2, arachidonic acid release and eicosanoids synthesis (prostaglandins, prostacyclin and thromboxane), via the activation of cyclooxygenases and peroxidases, and the production of leukotrienes through the activation of 5-lipoxygenase. OxPLs exhibit reactive groups that covalently interact with proteins by forming lipid-protein adducts, such as levuglandins, which are present in atherosclerotic lesions [124]. Such adducts are present in human plasma, on the apoA1 of HDL, and could represent suitable biomarkers for oxidative stress [126].

The HDL-associated enzyme paraoxonase (PON) hydrolyses oxidized fatty acids from phospholipids and thus reduces the accumulation of oxPLs in LDL and their pro-inflammatory response [127]. PAF-acetylhydrolase, another enzyme associated to HDL and involved in PAF hydrolysis, participates in the degradation of oxPLs [128].

Interactions with receptors. Most biological effects of oxPLs in macrophages and vascular cells are due to their interactions with the CD36 receptor. The presence on most oxPLs of a terminal γ-hydroxy (or oxo)-α,β-unsaturated carbonyl, allows their interaction with lysines 164 and 166 of the CD36 receptor binding site [124,129]. The presence of oxPLs in highly Cu-oxidized LDL facilitates their binding to CD36 [130]. Interestingly, plasma circulating oxPLs could bind and activate platelets via CD36, thereby linking hyperlipidemia, oxidative stress and prothrombotic events [131]. The interaction of oxPLs with the VEGF receptor 2 (VEGFR2) stimulates angiogenesis [123], while their binding to the scavenger-receptor-B1 (SR-B1) hinders the binding of HDL and their selective cholesteryl ester uptake from hepatocytes [132]. Berliner and Gharavi [123] reported that the binding of oxPAPC to the prostaglandin receptor EP2 (a G protein-coupled receptor) results in an increased expression of P-selectin in macrophages. Moreover, some oxPLs can bind the PAF receptor, thus mimicking the effects of PAF [133]. Lastly, several reports indicate that some inflammatory properties of oxPLs, such as IL-8 transcription, could result from their interactions with toll-like receptors (TLRs), in particular TLR4 [121,134].

Biological effects in vascular cells. OxPLs are early generated upon oxidation of LDL and plasma membrane phospholipids and accumulate at all stages of atherosclerosis, in fatty streaks and in more advanced lesions. When injected to animal models for atherosclerosis, oxPLs may initiate pro-atherogenic reactions from monocyte recruitment to thrombus formation. OxPAPC, and particularly POVPC and PGPC, are potent activators of endothelial cells, as they trigger an up-regulation of inflammatory cytokines such as IL-6, IL-8 or the chemokine monocyte chemotactic protein-1 (MCP-1). The mechanism evoked by POVPC involves protein kinase A and cAMP [123]. Interestingly, oxPLs trigger an up-regulation of P-selectin, but not of ICAM-1, VCAM-1 and E-selectin [123]. Likewise, oxPLs stimulate the recruitment of monocyte to endothelial cells, but not of neutrophils, thus triggering a selective macrophage accumulation which differs from that exerted by LPS, TNFα or IL-1, which recruit both monocytes and neutrophils [135,136]. OxPLs do not activate the classical pro-inflammatory NF-κB pathway [137]. However, the oxPAPC-mediated inflammatory signalling involves a calcium-dependent activation of the nuclear factor of activated T-cells (NFAT), resulting in over-expression of the prothrombogenic tissular factor, via the phosphorylation of extracellular signal-regulated kinase (ERK), and an induction of growth response factor 1 (EGR-1) [137].

OxPLs trigger the over-expression of IL-8 in endothelial cells, via a src/JAK2/STAT3-mediated pathway [138]. SREBP is involved in IL-8 induction through its binding to a SRE element in the IL-8 promoter [123]. The mechanism of IL-8 induction also implicates the phosphatidylinositol-3-kinase/Akt pathway and e-NOS, as assessed by the inhibitory effect of the e-NOS inhibitor L-NAME [123]. OxPLs are potent agonists for PPARγ and stimulate the formation of lipid vacuoles in macrophages, via a PPARγ-mediated mechanism [139]. OxPAPC promote angiogenesis by interacting with VEGFR2 and inducing the expression of VEGF via a src-mediated pathway [123].

Some oxPLs may bind the PAF receptor and mimic its inflammatory properties, in particular platelet aggregation, monocyte activation, smooth muscle cell migration and proliferation and increased vascular permeability. The PAF-like effects of oxPLs are not regulated, contrary to PAF whose effects are strictly and tightly controlled [125]. In addition, POVPC trigger a de-differentiation of SMC and alter the secretion and composition of the extracellular matrix, thus could contribute to modify the nature of atherosclerotic lesions in the vascular wall [140]. OxPLs stimulate oxidative stress by activating NADPH oxidase [141], but also induce the expression of antioxidant enzymes such as heme oxygenase [142] and of genes and transcription factors associated with the unfolded protein response (UPR) [125,143]. OxPLs activate the intrinsic mitochondrial apoptotic signalling in smooth muscle cells and macrophages [144,145] and contribute to the removal of apoptotic cells by phagocytic macrophages [146].

OxPLs as biomarkers for cardiovascular diseases. OxPLs exhibit a high affinity for lipoprotein Lp(a), which could represent a detoxification mechanism for low Lp(a) concentrations, since Lp(a) is associated with the PAF acetyl hydrolase that degrades OxPLs [147]. However, high levels of oxPLs in Lp(a) are highly pro-atherogenic as they bind the vessel wall, where they exert pro-inflammatory and prothrombotic properties [148]. Such elevated circulating levels of Lp(a), associated with increased oxPLs, are observed in patients affected with cardiovascular diseases [148].

OxPLs plasma levels associated with apoB are elevated in patients with coronary, carotid and femoral artery diseases and could constitute prognostic markers for atherosclerosis and cardiovascular diseases, as well as increased levels in anti-oxPLs antibodies which were reported in patients with hypertension and myocardial infarction [149].

In summary, oxPLs are potent biologically reactive agents, abundantly present in oxidized LDL and in atherosclerotic lesions, where they contribute to modulate inflammation and atherosclerosis development. These agents could also serve as diagnosis markers for the follow-up of coronary diseases and thus represent potential therapeutical targets for cardiovascular diseases.

Role of aldehydes in atherosclerosis

The oxidation of PUFAs generates MDA and highly reactive α,β-unsaturated hydroxyalkenals, such as 4-HNE and 4-HHE [150,151]. Other aldehydes such as α-oxoaldehydes can be generated by lipid oxidation (glyoxal) or through the catabolism of ketone bodies and the fragmentation of triosephosphates (methylglyoxal). The α-oxoaldehyde generation is increased during diabetes and hyperglycaemia and in diabetes-dependent accelerated atherosclerosis [152]. These aldehydes react with amino acids (histidine, cysteine) or lysine residues on proteins to form stable Michael adducts and Schiff-bases, thereby altering progressively the function of circulating, tissular and cellular proteins [4]. Their role in the pathophysiology of cardiovascular diseases has been extensively studied and reviewed [143–147]. Aldehydes are endogenously generated from oxidative attack of PUFA in membranes, lipid-rich tissues and lipoproteins, but high amounts of these compounds are also ingested with fat-rich food [150]. Although many experimental studies in animals point out the atherogenicity and cardiotoxicity of dietary aldehydes present in oxidized fats from deep-fried food, ingested aldehydic LPO are not acutely toxic in humans, contrary to oxysterols [150], this being explained by a low intestinal absorption [153–155]. Moreover, these compounds are rapidly degraded, thus neutralized, by glutathione-dependent enzymes, as reviewed in Esterbauer [150]. It is to note that dietary AGEs may aggravate diabetic complications such as nephropathy and atherosclerosis [156], while fat and carbohydrate-restricted diets ameliorate the antioxidant defenses, decrease age-related cardiovascular complications and increase the lifespan [157].

Aldehydes and lipoprotein modifications. LDL oxidation is a main risk factor in atherogenesis according to the oxidative theory for atherosclerosis [158]. LDL oxidation is a slow process occurring in the sub-intimal space and resulting from the oxidative attack of PUFAs by ROS generated by vascular cells [159]. Aldehydes formed during the oxidation process, and particularly MDA, react with the lysine and arginine residues of apoB, which are necessary for LDL recognition by the apoB/E receptor. The modification of apoB by MDA and hydroxyalkenals alters its recognition by the receptor and increases its affinity for the scavenger receptors expressed by macrophages, that are progressively transformed into foam cells [159]. The accumulation of foam cells leads to the formation of fatty streaks which are characteristic of the early atherosclerotic lesions [160]. Like 4-HNE and MDA, methylglyoxal and glyoxal may directly modify LDLs and deviate their metabolism towards macrophages [161].

Cellular effects of aldehydes. The biological effects of aldehydes are modulated by their local concentration and availability which depends on the presence of cellular detoxifying and conjugating systems (such as cellular GSH catalysed by glutathione-*S*-transferase or GST) and the cell ability to degrade modified proteins [162]. The functionality of these systems is

progressively modified by aldehydes, which alters cellular responses [162].

Modification of tyrosine kinase receptors. 4-HNE added to cultured smooth muscle cells are mitogenic at low concentration and induce growth arrest and apoptosis at higher doses [70,163]. The mitogenic signalling of low 4-HNE concentrations (< 1–10 μM) could result in part from a direct modification of tyrosine kinase receptors such as EGF receptor (EGFR) and PDGF receptor (PDGFR) and an activation of their downstream signalling pathway, ERK1/2 phosphorylation and cell cycle progression [164,165]. Higher 4-HNE concentrations (> 10 μM) progressively trigger a desensitization of the receptors to their own ligands and impair cellular responses such as cell migration and proliferation mediated by EGF or PDGF [163,166]. 4-HNE- and acrolein-adducts on PDGFR are observed in atherosclerotic lesions from hypercholesterolemic rabbits, apoE–/– mice and human patients [163], which suggests that aldehydes may potentially disturb PDGFR-mediated responses in the fibrous cap. The same results are observed with α-oxoaldehydes, in particular methylglyoxal which inhibits EGFR and PDGFR, with possible implications in accelerated atherosclerosis in diabetes [167,168].

Inflammation. Aldehydes exert a dual effect on inflammatory signalling. 4-HHE activates the IκB kinase (IKK)/NF-κB inducing kinase (NIK) pathway via p38 MAPK and ERK1/2 kinase, resulting in NF-κB activation [169]. At low concentrations, 4-HNE activates PKCβ-signalling, which results in MCP-1 overexpression and release from macrophages [170]. In contrast, high 4-HNE and acrolein concentrations inhibit the activation of NFκB, either via a direct inhibitory effect on proteasome (which is necessary for the degradation of IκB, the cytosolic inhibitor of NFκB) [171] or the phosphorylation of IκB and its subsequent proteolysis [172] or a modification of IKK β-sub-unit by aldehydes [172].

Redox status, endoplasmic reticulum stress and apoptosis

4-HNE triggers intracellular oxidative stress. At low concentrations, this results in increased expression of heme-oxygenase and thioredoxine-1 [173,174], suggesting that 4-HNE induces adaptive responses to oxidative stress. 4-HNE induces the activation of the endoplasmic resticulum (ER) stress and the unfolded protein (UPR) response in endothelial cells [175]. Moreover, ER stress markers co-localize with 4-HNE protein adducts, in advanced human atherosclerotic lesions [175].

Stress kinase pathways such as Jun N-terminal kinase (JNK) and p38 kinase are activated by 4-HNE and are associated with pro-atherogenic cellular responses including cell proliferation, inflammatory responses and apoptosis [162,176]. Several pathways are involved in cell death triggered by elevated concentrations of 4-HNE or acrolein (> 20 μM), in particular PKCδ and JNK [175]. The phosphorylation of JNK could be mediated by the ER stress sensor pIRE1α, via a prolonged activation of ER stress and UPR, induced by 4-HNE and oxidized LDL [175]. Methylglyoxal and glyoxal are pro-apoptotic via calcium deregulation, GSH depletion, oxidative stress and the activation of stress kinases p38 and JNK [177,178].

4-HNE alters the metabolism of GSH, which induces mitochondrial oxidative stress and calcium-mediated induction of the mitochondrial transition pore [179]. In addition, 4-HHE could directly modify ANT, thereby impairing its function and activity [180].

Aldehydes and cardiovascular diseases. 4-HNE- and MDA-adducts and oxLDLs are detected in atherosclerotic lesions [181,182], while circulating autoantibodies recognizing MDA-modified LDLs can be detected in the plasma (their role remains unknown so far) [183]. These anti 4-HNE or anti MDA-modified LDLs could constitute suitable markers for the follow-up of vascular atherosclerotic process [184]. Circulating modified LDLs are considered as suitable prognostic indicators for acute coronary syndromes, vulnerable plaques, pre-clinical or accelerated atherosclerosis in diabetes [185], while increased levels of MDA and 4-HNE-adducts in dialysed patients constitute an additional risk factor for cardiovascular complications [186]. LDL modified by ALE-pre-cursors are found in the plasma of diabetic patients and play a role in the progression of accelerated atherosclerosis.

Role of oxysterols in atherosclerosis

A very efficient way for cholesterol to promote and sustain the progression of atherosclerosis is to undergo oxidation reactions. Cholesterol oxidation leads to the formation of a number of 27-carbon atoms compounds, termed oxysterols, which may either originate in the blood, cells and tissues, through both enzymatic and non-enzymatic reactions, or derive from the diet. These cholesterol metabolites have been consistently demonstrated to be at least one or two orders of magnitude more reactive than unoxidized cholesterol, showing remarkable pro-inflammatory, pro-apoptotic and pro-fibrogenic effects.

Notable is the ability of oxysterols to cause cell injury in a manner analogous to that of oxLDLs [187]. Indeed, oxysterols are abundant in oxLDLs [188] and are consistently detectable in the core region of an unstable atherosclerotic plaque [189].

Apparently their accumulation is directly proportional to the actual cholesterol content of the plaque [190]. It thus seems highly probable that oxysterols significantly contribute to the complex molecular and cellular events leading to fibrotic plaque formation and overall vascular remodelling that characterize atherosclerosis. In fact, they have been shown to be involved in various key events of this multistep disease process, from endothelial cell dysfunction up to fibrotic degeneration of the arterial wall and vulnerable plaque rupture [190].

Biochemical effects on vascular cells. Many studies point at a role for oxysterols in activating and sustaining monocyte/macrophage-driven inflammation, a process that represents a key mechanism of disease progression in atherosclerosis [191]. In addition, they appear to support excessive fibrogenesis, modulate extracellular matrix content and induce apoptotic death of vascular cells, the latter event becoming crucial in the advanced stages of the atherogenic process.

Inflammation. The potentially strong contribution that pro-inflammatory properties of oxysterols might provide to atherosclerosis progression has attracted over the last years the attention of many investigators. As regards the endothelial dysfunction that initiates the process of plaque formation, 7-ketocholesterol (7-K), 7α-hydroxycholesterol (7α-OH) and 7β-hydroxycholesterol (7β-OH) were shown to over-express vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin in human umbilical vein endothelial cells (HUVECs) [192]. Moreover, incubation with 7-ketocholesterol markedly stimulated expression and synthesis of ICAM-1 and VCAM-1 in human aortic endothelial cells and U937 pro-monocytic cells [193]. Further, oxysterols were consistently proven to up-regulate in human vascular cells the expression of a variety of inflammation-related cytokines and chemokines [190]. In relation to this, multiplex flow cytometry allowed to show 7-K, 7β-OH and 25-OH as potent inducers of IL-8, TNF-α, macrophage inflammatory protein-1β (MIP-1β) and MCP-1 in human monocytic cells [194].

Thus, cholesterol oxidation products should be able to stimulate not only the adhesion of leukocytes to the arterial endothelium but also their transmigration to sub-intimal spaces; in fact, migration, diapedesis and intra-matrix mobility of blood-borne and mesenchymal cells during plaque formation and progression are aspects of a complex process in which at least IL-8 and MCP-1 are considered to play a primary role.

Fibrosis. About a decade ago, a marked up-regulation of both expression and synthesis of the pro-fibrogenic and pro-inflammatory cytokine TGFβ1 was described in human pro-monocytic cells challenged with an oxysterol mixture compatible with that detectable in human hypercholesterolemic plasma [195]. Subsequently, an increased generation of oxysterols was causatively associated to macrophage infiltration and fibrosis of alveolar septa in the lung of rat poisoned with paraquat [196]. Up-regulation of TGFβ1 in macrophages was very recently confirmed to be induced by a biologically relevant mixture of oxysterols [197].

The potential contribution of cholesterol oxidation products to the extensive changes of extracellular matrix occurring in the arterial wall during atherosclerosis was strengthened by the consistent observation of their ability to increase the expression of metalloprotease tissue inhibitor-1 (TIMP-1) both in macrophages [176] and in smooth muscle cells [198].

Apoptosis. Reliable *in vitro* evidence is available that depicts a potential pro-apoptotic effect of the major oxysterols with regard to smooth muscle cells, endothelial cells and monocyte-macrophages [176]. Investigating 7-ketocholesterol-induced apoptosis in cells of the macrophage lineage, Lizard's group [199,200] showed how complicated this process is, as it simultaneously triggered more than one signalling pathway, namely: (1) increase of cytosolic free calcium and activation of calcineurin with eventual dephosphorylation (i.e. activation) of the pro-apoptotic protein Bad; (2) translocation of the pro-apoptotic protein Bim from the microtubule dynein motor complex to mitochondria, interaction and inhibition of the anti-apoptotic element Bcl-2; and (3) involvement of the proline rich tyrosine kinase-2 (PYK2) which transduces survival signals through the activation of MEK/ERK pathway followed by inactivation of Bad through its phosphorylation. Thus, besides the two, often prevailing, pro-apoptotic pathways, the suitable modulation of the MEK/ERK pathway by oxysterols, also demonstrated by other laboratories [176], could be crucial in providing the cells with a possibly valid anti-apoptotic mechanism.

Oxysterol-induced foam cell formation. The hallmark of the atherosclerotic lesion is represented by the uptake of oxidized lipids by activated macrophages and myofibroblasts with the formation of foam cells. Cholesterol oxidation products markedly induce the expression and synthesis of CD36 in cells of the macrophage lineage [201], as well as other LPO products like 4-HNE [202]. The most efficient oxysterols in CD36 over-expression were 7α-OH and cholesterol-α-epoxide, actually two of the most represented cholesterol oxidation products in human atheromas [189]. Further, the oxysterol-dependent up-regulation of this specific scavenger receptor was demonstrated to actually stimulate cellular uptake of Cu-oxidized LDL. Of note, the essential role of CD36

in oxysterol-induced foam cell formation was confirmed by the prevention of the latter process when cells were incubated in the presence of an anti-CD36 specific antibody [201].

Oxysterol-mediated cell signalling The investigation of the molecular signalling behind the aforementioned effects of oxysterols allowed one to demonstrate the involvement of defined components of PKC and MAPK enzymatic families, in particular the MEK/ERK pathway, as well as of redox-sensitive transcription factors like NF-κB and PPARγ. In fact, when U937 human pro-monocytic cells were incubated with a biologically compatible oxysterol mixture, but in the presence of rottlerin, a selective inhibitor of the isoform δ of PKC, CD36 over-expression was fully prevented [201]. The same event was consistently abrogated when U937 cells were treated with PD98059, a selective MEK/ERK inhibitor. As regards oxysterol-signalling downstream of ERK, monocyte treatment with GW9662, a selective PPARγ antagonist markedly prevented the oxysterol-dependent up-regulation of CD36 [201]. Up-regulation of MEK/ERK and NF-kB pathways was shown, underlying the ability of oxysterols to increase expression and synthesis of monocyte chemotactic protein-1 (MCP-1) in macrophagic cells [176].

In conclusion, a very efficient way for LDL lipids to promote and sustain the progression of atherosclerosis is to undergo oxidation reactions. Notably, oxidative stress is a feature that accompanies such a disease process all through its development. Oxidative stress-dependent oxidation of the lipid moiety of LDL generates a large variety of compounds provided with relevant and strong biochemical activities. The main lipid oxidation products that are consistently detectable in atherosclerotic lesions are those stemming from oxidation of LDL phospholipids and cholesterol, but also important appear defined aldehydic end-products of n-6 PUFA peroxidative breakdown, in particular 4-HNE. All these lipid oxidation products exhibit remarkable pro-inflammatory, pro-apoptotic and pro-fibrogenic effects, thus they most likely contribute to the complex molecular and cellular events leading to fibrotic plaque formation and overall vascular changes that characterize atherosclerosis. As regards the way these compounds signal to the nucleus to exert their biochemical effects, they have actually shown a marked ability in modulating the main cell signalling pathways, but with significant specificity of action, among each other. Such a pleiotropic signalling activity of LPO products present in oxLDL and atherosclerotic lesions could represent a real problem for molecular medicine to properly target the mechanisms of progression of atherosclerosis. Monitoring the plasma level of these compounds and/or that of the inflammatory molecules whose over-production they sustain might significantly help the clinical management of the disease.

Foetal vascular dysfunction induced by lipid peroxidation in pre-eclampsia (S Chapple, R CM Siow, GE Mann)

Pre-eclampsia

Pre-eclampsia (PE) is a pregnancy-related disorder associated with systemic oxidative stress in the maternal and foetal vasculature, diagnosed by the onset of hypertension and proteinuria after 20 weeks of gestation in previously normotensive and non-proteinuric pregnant women [203,204]. Worldwide, PE affects between 2–7% of all pregnancies and remains a major contributor towards pregnancy-related morbidity and mortality [203–206]. If left untreated, PE can progress to a convulsive state known as eclampsia and ~ 10% of PE mothers develop secondary haemolysis elevated liver enzyme low platelet (HELLP) syndrome [207]. Foetal health is also compromised with increased incidence of prematurity, intrauterine growth retardation (IUGR) and hypoxemia [208].

The aetiology of PE has been linked to abnormal placental development since the main symptoms of PE are rapidly reversed after parturition. During placentation, insufficient adaptation of the maternal uterine arteries by the invading foetal trophoblasts is thought to result in under-perfusion of the placenta, localized ischemia and the onset of oxidative stress caused by excessive production of ROS [203,204,209]. Accordingly, nitrotyrosine immunoreactivity [210,211] and advanced-glycation end products (AGE) [212] are detected in the placenta in PE, indicative of enhanced ROS generation. ROS in turn induce the formation of lipid peroxidation (LPO) products [213] with both species key mediators of systemic vascular dysfunction and inflammation [201,214,215].

Endothelial dysfunction in the maternal vasculature in pre-eclampsia

Endothelial dysfunction in the maternal circulation is a hallmark of PE [216–219]. An imbalance in anti- and pro-angiogenic factors, such as soluble vascular endothelial growth factor receptor 1 (sFlt-1), soluble endoglin (sEng, CD105 cell surface receptor for TGF-β1 and TGF-β3) [220–223], angiotensin II type-1 receptor auto-antibodies, increased sensitivity to endothelin-1 and thromboxane, reduced synthesis of nitric oxide and prostacyclin and enhanced activation of neutrophils and platelets may also contribute to endothelial dysfunction [224]. The foeto-placental vasculature is often grossly abnormal in PE and chronic exposure to ROS may underlie the increased

resistance of the foeto-placental vasculature [225]. Moreover, umbilical artery blood flow can be severely compromised in PE, as evaluated by Doppler waveform analysis [226].

LPO originating from the placenta extend to the maternal circulation

LPO results from the oxidation of biological membranes by hydroxyl radicals (OH•), which can be generated in the presence of free metal ions, and iron overload has previously been reported in PE pregnancies [227]. Compared to uncomplicated normal deliveries, PE mothers have a reduced iron binding capacity due to increased serum iron content and reduced transferrin levels [227]. In agreement with this finding, as recently reviewed [228], numerous markers of LPO are detected in the placenta and maternal circulation. In the placenta, many studies document increased staining of LPO products including 4-HNE and MDA in syncytiotrophoblasts, endothelial cells and macrophages [212,229–232]. There are numerous reports of decreased activities of antioxidant enzymes such as SOD, catalase and GPx, providing further evidence that PE is associated with systemic oxidative stress [233–235]. Glutathione (GSH) utilizing enzymes such as GPx and the glutathione-*S*-transferases (GST) are critical enzymes for removing lipid hydroperoxides and require selenium for catalytic activity [67,236,237]. Interestingly, selenium deficiency has been reported to increase the risk of developing PE [231,238] and may provide a plausible explanation for increased LPO and reduced GPx activity reported in PE mothers. Propagation of oxidative damage to the maternal and foetal vasculature via lipid peroxides may be exacerbated further by a deficiency in the activity of glucose-6-phosphate dehydrogenase, a key rate-limiting enzyme for NADPH generation required for GSH recycling. While GPx and GST remove LPO end products, the chain-breaking antioxidant α-tocopherol plays a key role in preventing the propagation of LPO within membranes [239,240]. A reduction in α-tocopherol availability or its recycling via ascorbate could therefore potentiate maternal LPO reported in PE pregnancies. Currently, the status of α-tocopherol concentrations (normalized for lipid profile) in the maternal circulation remains somewhat controversial, with the majority of studies reporting decreased α-tocopherol content [241,242] or others negligible changes [243]. It is worth highlighting that other studies appear not to have adjusted α-tocopherol concentrations for differences in lipid profile [232,233,244–247].

Importantly, placental LPO products such as 4-HNE and MDA [67] are capable not only of entering the maternal circulation to induce systemic oxidative stress, but can also cross the maternal–foetal interface and enter the foetal circulation. Although less well characterized, markers of oxidative stress have been detected in the foetal vasculature [248] and an increasing number of studies document that maternal plasma from PE patients induces phenotypic alterations in endothelial cells cultured from the foetal vasculature, e.g. human umbilical vein and artery.

Effects of maternal PE plasma on foetal endothelial cells

As summarized in Table I, a limited number of studies have reported that exposure of healthy foetal endothelial cells (derived from human umbilical vein, HUVEC) to plasma from PE mothers induces the accumulation of intracellular lipids and increases the formation of LPO end products 4-HNE and MDA [249,250]. Interestingly, as may be anticipated from findings in the maternal circulation, increased LPO was not accompanied by a compensatory increase in antioxidant capacity as measured by cellular GSH content [251]. Moreover, maternal serum obtained from normal pregnancies appears to decrease the LPO product content in HUVEC, in line with reports that normal pregnancy is associated with increased cellular antioxidant capacity. Increased LPO product formation will result in dysregulation, perturbation or activation of a number of intracellular signalling pathways involved in modulating inflammation, mitochondrial function, GSH depletion, cell cycle arrest and apoptosis. Although DNA adducts have not been measured directly in foetal endothelium in PE pregnancies, exposure of normal HUVEC to maternal plasma from PE pregnancies increases apoptosis [240], which can be mimicked by treatment of cells with 4-HNE [252]. 4-HNE is known to decrease mitochondrial ATP synthesis and ROS production as a consequence of depolarization of the inner mitochondrial membrane [240]. In addition, 4-HNE has pro-inflammatory actions, and studies by Takacs et al. [250] demonstrate that 4-HNE stimulates intracellular ROS production, induces NF-κB nuclear translocation and transcriptional activation of ICAM-1.

Evidence of LPO in the foetal vasculature of PE pregnancies

Several studies have suggested that treatment of foetal endothelial cells with PE plasma or serum may mimic the effects of LPO products *in utero*, potentially accounting for altered redox regulation in the endothelium of PE offspring. As summarized in Table II, the majority of studies examining LPO products in the foetal circulation report increased plasma concentrations of MDA [231,235,238,247,253]. It should, however, be noted that a limited number of studies, measuring 8-isoprostane concentrations (a more specific marker of LPO), report no change in isoprostane levels [254]. Most plasma/serum samples will have been collected from a relatively small cohort of

Table I. Effects of maternal PE plasma on foetal endothelial LPO and antioxidant status.

Source	LPO marker	ROS production	Antioxidant status	Other	Reference
Study design: Pooled maternal plasma from 23 normal or 23 PE women matched for GA. HUVEC (Cellworks, P1-10) cultured for 24 h before addition of 2% plasma or fatty acid mixtures (μM) found in normal or PE pregnancies for a further 24 h. Triplicate measurements in a minimum of two passages.					[249]
HUVEC treated with PE vs N plasma or fatty acids	↑ lipid accumulation	↓mitochondrial redox capacity ↓ $\Delta\Psi_m$		↑ apoptosis	
Study design: Maternal serum from 18 non-pregnant, 18 normal pregnant and 18 PE women matched for MA, GA and BMI (pregnant groups). Primary isolated HUVEC (P2) were cultured for 72 h before being exposed to serum for a further 24 h.					[251]
HUVEC treated with N vs NP serum	↓		↔ [GSH]		
HUVEC treated with PE vs NP serum	↔		↔ [GSH]		
HUVEC treated with PE vs N serum	↑		↔ [GSH]		
Study design: Maternal plasma from normal or severe[§] PE women matched for MA and GA. Primary isolated HUVEC (P6-7) were exposed to plasma for 48 h.					[250]
HUVEC treated with PE ↑ vs N plasma		NFκB activation inhibited by α-tocopherol or NAC		↑ NFκB activation ↑ICAM-1 expression	

NP, non-pregnant; N, normal pregnant; PE, pre-eclamptic; HELLP, haemolysis elevated liver enzyme low platelet syndrome; LPO, lipid peroxidation; MDA, malondialdehyde; HNE, 4-hydroxynonenal; ROS, reactive oxygen species; GSH, glutathione; MA, maternal age; GA, gestational age; BMI, body mass index; BP, blood pressure; ICAM-1, intracellular adhesion molecule-1; $\Delta\Psi_m$, mitochondrial membrane potential; NAC, N-actetyl cysteine.
PE defined as BP >140/90 mmHg on two occasions 6 h apart and proteinuria 0.3 g/24 h or +1 protein after 20 weeks gestation. [§]Severe PE defined as BP>60/110 mmHg, proteinuria>5 g/24 h or HELLP.

patients and possibly following different modes of delivery. Thus, there is a need for further studies using larger populations and measuring multiple markers for oxidative stress and LPO in age- and gestation-matched maternal cohorts.

Intracellular ROS production and antioxidant status have been determined as indices of foetal LPO and/or oxidative stress [255,256]. Many studies report that PE is associated with increased cellular ROS production, with uncoupled eNOS and mitochondria the two most likely intracellular sources. In contrast, antioxidant capacity is thought to be reduced in foetal plasma from PE pregnancies [235,247,256]. As in the maternal circulation of PE mothers, the status of ascorbate and α-tocopherol levels in the foetus remains poorly characterized. Decreased plasma ascorbate levels with negligible changes in α-tocopherol (uncorrected for foetal lipid profiles) have been reported in cord blood from PE pregnancies [232,247]. The majority of studies also report reduced selenium content and GPx activity; however, the molecular mechanisms underlying alterations in GPx expression require further characterization [231,235]. Moreover, reduced activity of catalase and reduced, unaltered or increased SOD activity in foetal plasma from PE pregnancies have been reported [234,235,253]. Taken together, these findings suggest that, similar to the maternal circulation, foetal endothelial cells will be subjected to increased ROS production. We have shown that foetal endothelial cells from PE pregnancies are unable to up-regulate antioxidant defences when challenged (data not shown), conferring a pro-oxidant phenotype [257].

PE is also associated with alterations in maternal and foetal fatty acid profiles, which may prime the foetal vasculature for enhanced LPO. While arachidonic acid and linoleic acid fatty acid oxidation result principally in the formation of 4-HNE, the production of MDA can be achieved by the oxidation of arachidonic acid or docosahexaenoic acid [67]. In the maternal circulation, fatty acid profiling suggests that, although arachidonic acid content is unaltered, PE is associated with a marked increase in membrane linoleic acid and docosahexaenoic acid content, known to induce intracellular HNE formation [232,249,252]. In comparison, foetal fatty acid profiling from the umbilical cord reveals no change in arachidonic acid or docosahexaenoic acid content, although γ-linoleic acid concentrations are enhanced, suggesting an increased disposition towards increased endogenous 4-HNE production in the foetal vasculature [232]. Another study, measuring fatty acid composition in umbilical arteries from PE pregnancies, reported a lower long chain polyunsaturated fatty acids (PUFA) content, which the authors suggested could lead to impaired foetal blood flow due to a reduction in prostaglandin synthesis [258].

In studies documenting increased LPO in foetal plasma, the severity of maternal symptoms is positively correlated with foetal MDA levels [235]. These findings imply that the maternal circulation and placenta

Table II. Alterations in fetal LPO and redox status reported in PE

	LPO marker						Antioxidant activity					
Source	MDA	8-IP	PAC	GPx	SOD	Catalase	[GSH]	[α-tocopherol]	[ascorbate]	[Selenium]	Other	Reference
Study design: 30 normal, 19 PE and 25 severe[§] PE women												[235]
PE plasma	↑		↓	↓	↓			↔*	↓			
Severe PE plasma	↑↑			↓↓	↓↓							
Study design: 55 normal, 60 PE women matched for MA and GA												[232]
Plasma	↑							↔*	↓		↑ γ-linoleic acid ↔ Arachidonic acid	
RBCs											↔ Arachidonic acid↑ Omega-6:omega-3 ratio	
Study design: 13 normal and 13 PE women matched for MA and GA												[224]
Plasma	↑			↓	↔	↓						
Study design: 33 normal and 19 PE women delivered by fasting c-section. Matched for MA and BMI (before and during pregnancy)												[254]
Venous plasma		↔	↑					↑*				
Arterial plasma		↔	↑					↔*				
Study design: 9 normal and 18 PE[†] women matched for MA and GA (all pre-term < 36 weeks)												[253]
Plasma	↔			↔	↑		↔					
Study design: 23 normal and 18 PE women matched for MA and GA												[247]
Plasma	↑		↓					↔*	↓			
Study design: 22 non-pregnant, 27 normal pregnant and 25 PE women, matched for MA. BMI significantly ↑ in pregnant vs non-pregnant women at booking. GA significantly ↓ in PE vs normal pregnancies.												[231]
Plasma	↔			↓						↓		
Study design: 33 normal and 29 PE women matched for MA and GA												
Plasma	↑										↑ XO activity ↑ ADA activity	
Study design: 16 normal and 20 PE women												
Plasma	↑										↔ $[PO_2]$mmHg ↑ $[PCO_2]$mmHg ↓ [pH]	

PE, pre-eclampsia; LPO, lipid peroxidation; MDA, malondialdehyde; 8-IP, 8-isoprostane; PAC, plasma antioxidant capacity; GPx, glutathione peroxidase; SOD, superoxide dismutase; GSH, glutathione; RBC, Red blood cell/erythrocyte; MA, maternal age; GA, gestational age; BMI, body mass index; BP, blood pressure; ADA, adenosine deaminase; XO, xanthine oxidase.
PE defined as BP>140/90 mmHg on two occasions 6 h apart and proteinuria 0.3 g/24h or +1 protein after 20 weeks gestation. [†]PE defined as BP>40/90 mmHg on two occasions 6 h apart with proteinuria>0.3 h/24 h OR oedema. [§]Severe PE defined as BP>160/110 mmHg. *[α-tocopherol] not adjusted for lipid content.

may be major contributors to foetal vascular dysfunction and notably foetal plasma LPO levels are higher in the umbilical vein compared to the umbilical artery [254]. Thus, increased ROS generation and impaired antioxidant defences in the foetal vasculature may arise primarily due to increased LPO products crossing the placenta and forming DNA and protein adducts, leading to foetal endothelial dysfunction.

Long-term impact of LPO on PE infants and adolescents

Accumulating evidence suggests that an adverse environment during development *in utero* is associated with an increased susceptibility to cardiovascular disease and diabetes in adulthood [259–262]. In the case of PE, abnormal placentation and placental hypo-perfusion and reperfusion injury may result in long-term 'foetal programming' of impaired vascular function in adulthood [263]. LPO is well known to enhance ROS production, NF-κB activation, protein nitrosylation, apoptosis and to alter the thiol redox status leading to an increased demand on cellular antioxidant systems [67]. In cultured PE HUVEC and umbilical artery smooth muscle cells, which have been removed from the pro-oxidant environment of the placenta and cultured *in vitro* for several passages, we and others have identified alterations in Ca^{2+}-handling, NO production, cGMP formation, ROS production and arachidonic acid metabolism [257,264–266]. As these changes persist in culture, we hypothesize that sustained oxidative stress during

the course of PE leads to *in utero* programming of impaired redox signalling and vascular function in adulthood. Under these circumstances, increased LPO may further exacerbate a weakened redox balance.

Studies monitoring adolescents from PE pregnancies also indicate impaired vascular function. Following exposure to PE *in utero*, children aged 12–17 years old show a consistent elevation in blood pressure compared to their peers. When accounting for body mass index (BMI), which largely appears unaltered, systolic pressure was always found to be significantly elevated [267–271]. Recently a longer-term study of PE offspring concluded that *in utero* exposure to PE increases the risk of developing hypertension and incidence of stroke in later life [272]. While these data suggest that PE is associated with 'programming' of impaired vascular function in adulthood, this may not be the only explanation. As recently reviewed by Ferreira et al. [273] and Oglaend et al. [271], several factors can confound recorded blood pressure measurements in PE adolescents, such as sudden weight gain in underweight PE neonates, socio-economic variables and increased maternal BMI post-delivery. It may in fact be difficult to dissect out potential confounding variables, particularly in relation to maternal parameters which may be altered after the development of PE [274].

In conclusion, although the primary symptoms of PE resolve upon delivery, increased maternal LPO and impaired antioxidant defences persist in the months following parturition [235], enhancing their risk of cardiovascular disease in later life [274]. The foeto-placental vasculature is often abnormal in PE and Doppler waveform analysis reveals that umbilical artery blood flow is severely compromised [226]. There is increasing evidence that PE and other pregnancy-associated diseases cause alterations in foetal endothelial function. Increased LPO products in PE can activate NF-κB and ICAM-1 expression in foetal umbilical vein endothelial cells. As phenotypic changes persist in culture [257,265,275–278], this may have implications for long-term 'programming' of the foetal cardiovascular system [260], as implied by a study in which men whose mothers suffered from PE were found to be at increased risk of developing hypertension in adulthood [279].

Diabetes

Lipid peroxidation in human diabetes: From the beginning to the end (R Pamplona, J Serrano, J Boada, V Ayala, A Negre-Salvayre, R Salvayre, M Portero-Otin)

The prevalence of diabetes mellitus (DM) will reach 300 million in 20 years, in a close relationship with over-weight. This is mainly due to consumption of high-energy diets unbalanced with physical activity. Both chronic hyperglycaemia and hyperglycaemic peaks during post-prandial periods constitute a factor for increased oxidative stress in diabetes. In this short review we will depict the evidences in human studies of the importance of lipid oxidation in the development of diabetes from impaired fasting glucose up to DM chronic complications.

Extracellular evidences of lipid peroxidation in human diabetes. MDA levels are increased in T2DM patients with complications compared with those non-complicated [280], a fact also present in comparison with non-DM individuals [281–284]. Besides, other aldehydes directly related to hyperglycaemia [285] as glyoxal and methylglyoxal, also increase in T1DM patients as a very early event after hyperglycaemia [286,287]. Interaction between hyperglycaemia and lipid peroxidation also includes the fact that MDA increases *in vivo* modification by glycation, as recently shown in chronic renal failure patients [288]. Other lipoxidation markers, as the isoketal F2-isoprostanes [289], oxidized cholesterol [290] and increased conjugated linolenic acid [291] also increase in DM patients. It has also been shown that TBARS increase with the degree of metabolic impairment, from healthy patients to T2DM in serum [292]. However, this later marker has been criticized in the context of human diabetes, due to lack of specificity [287]. Furthermore, hyperglycaemia, before insulinization, leads to increased lipid peroxidation in humans [293–295]. Recent data show that HDL from T2DM patients is modified by lipid oxidation, in a niacine-preventable fashion [296]. Similarly, T1DM patients exhibit elevated concentration of spin-trapped alpha-phenyl-tert-butylnitrone adducts, measured by electron paramagnetic resonance spectroscopic detection, as well as lipid-derived oxygen-centred alkoxyl. This was accompanied by increased levels of lipid hydroperoxides, with diminished levels of lycopene and retinol [297]. These findings are in accordance with those present in T2DM, where the same adducts were increased, in the form of alkoxyl free radicals derived from the peroxidation of lipid membranes [298]. Since direct spectroscopic evidences of lipid peroxidation are correlated with lipid hydroperoxides, it is suggested that metal-induced decomposition of those species are the precursors of alkoxyl radicals that are thermodynamically able to further propagate oxidative damage to proteins.

In plasma, those lipids may arise from phospholipid moieties belonging to either endothelial cells or erythrocytes, even from circulating lipids. The implication of erythrocytes is sustained by increased MDA levels in erythrocyte ghost membranes of T1DM, when compared with euglycemic individuals [283]. In line with this, pioneering work of Jain and colleagues [299,300] led to the identification of lipid peroxidation as an important factor increasing erythrocyte fragility in human diabetes with a good correlation with glycaemic control.

The initiation agent in the absence of mitochondrial activities (i.e. in plasma) should be the superoxide

resulting from glucose and glycation products decomposition [301], in accordance to pioneering work by Hunt et al. [302]. This work evidenced an increase in hydroxyl radicals in the presence of glucose (at concentrations present in human diabetes), in a process termed glucose auto-oxidation. This process, even in non-diabetic states, may contribute to the increased lipoxidative stress evidenced during oral glucose tolerance tests [303]. In accordance with this, T1DM patients show increases in plasma MDA levels after standardized meals [304]. Not only do diabetes patients show increased lipoxidation: it has been demonstrated that MDA and F2-isoprostanes in plasma correlate with glycaemic indexes of meals, suggesting that even in euglycaemia post-pandrial increases in oxidative stress lead to increased lipid peroxidation [298,305].

Besides nutritional supplementation, also correcting in some instances lipid peroxidation, treatment aimed at tackling basic mechanisms of chronic diabetes complications, such as aldose reductase pathway, also show some effect in lipid peroxidation. Thus, the use of an inhibitor of aldose reductase could specifically reduce lipid hydroperoxides in erythrocytes in T2DM patients after 3 months of treatment. This reinforces the concept of an adequate choice for an indicator of lipid peroxidation damage, as other markers, such as plasma TBARS, MDA-modified LDL or even vitamin E were not affected by this treatments [306].

In all these evidences reported for human subjects, it is demonstrated that in a close relationship with glycaemic control, both T1DM and T2DM leads to increased extracellular lipid peroxidation, potentially contributing to their development and complications. Besides obvious solutions, as insulinization, nutritional and advanced pharmacological therapies could also improve lipoxidative status, aimed at preventing long-term complications of DM.

Intracellular evidences of lipid peroxidation in diabetes: The skeletal muscle case and involvement in insulin resistance. Since the key works led by Brownlee and colleagues [307–309] in which oxidative stress was identified as an unifying mechanism behind other recognized pathways between hyperglycaemia and chronic complications in endothelial cells, several other works have extended this concept to other cells and tissues. High glucose levels, in T2DM high fatty acid content in muscle cells, are associated both as a cause and/or a consequence of mitochondrial function [310]. As a cause, in humans, even a short consumption of high-fat diets led to increases in intracellular lipids in skeletal muscles in healthy individuals [311]. Also, in individuals with relatives suffering of T2DM, a small reduction in mitochondrial function is accompanied by an unexpectedly high accumulation in intracellular lipid contents [312,313]. As a consequence, lipids would induce impairment of mitochondrial function. In T2DM patients, plasma FFA levels are significantly and negatively correlated with mitochondrial function, leading to impaired ATP production [314]. Thus, diminished PGC1a levels—a key regulator of energy metabolism—in insulin resistant individuals could be induced by accumulation of intramuscular lipids [315,316].

Mitochondrial ROS (mitROS) can rapidly react with mitochondrial DNA, protein and lipids, thereby leading to oxidative damage. As an example, in skeletal muscle, the fatty acids present in excessive amounts in T2DM patients can be very prone to ROS-induced oxidative damage, resulting in the formation of lipid peroxides. Especially accumulation of fatty acids in the inner mitochondrial membrane of mitochondria, at the site where ROS are formed, would be susceptible to peroxidation, subsequently inducing oxidative damage to the mitochondrial machinery. In this line, it should be remarked that the percentage of total electron flow directed to free radical generation in mitochondria is not constant in different tissues and different conditions inside a given tissue, which suggests that mitROS generation is more than a simple byproduct of mitochondrial respiration, as frequently assumed, and should be better viewed as a homeostatically controlled variable. Oxygen radical generation at the respiratory chain has been classically attributed to complex III semiquinone [317] and complex I flavin mononucleotide [318,319] or complex I FeS clusters near the rotenone-binding site [320]. Besides those structural characteristics, adaptable variables such as entry of substrates could also play a key role in determining mitROS production. Thus, β-oxidation controls entry of fatty acids into mitochondria, although this system does not prevent completely the interaction of fats with mitochondria and hence its contact with mitROS sources. This is due to the fact that physical interaction, via 'flip-flop' mechanism, could allow matrix membrane entry of excess of fatty acids [321]. Since in this location they cannot enter energy production due to the absence of acyl-CoA synthetase, they remain into the inner mitochondrial membrane, exposed to mitROS. Alternatively, and analogously with the mitochondrial changes induced by high glucose concentrations, increased fatty acid efflux to mitochondria could directly increase mitROS production, more glucose being oxidized in the TCA cycle. This situation drives one to push more electron donors (NADH and FADH2) into the electron transport chain, thus leading to an increase in ROS generation [307]. In this situation, there is a higher degree of reduction of complexes I and III, increasing their rate of ROS production. Likewise, in the insulin resistance syndrome, there is an increased free fatty acids flux from adipocytes into arterial endothelial cells that would result in increased fatty acid oxidation by mitochondria [322,323]. Since both β-oxidation of fatty acids and oxidation of fatty

acid-derived acetyl CoA by the TCA cycle generate the same electron donors (NADH and FADH2) generated by glucose oxidation, increased FFA oxidation may cause mitochondrial over-production of ROS by the mechanism above described for hyperglycaemia.

The mechanism notwithstanding, muscle cells from obese pre-diabetic patients show increased intramyocellular lipid peroxidation [324]. As in ageing, where high lipid peroxidation is associated to lower lifespan by increased oxidation-derived damage to proteins and nucleic acids [325], it is suggested that lipid peroxidation in mitochondria from insulin resistant individuals could lead to mitochondrial function impairment [310], thereby generating a vicious circle. In accordance with this, high fat diets induced increased mitROS production [326].

Losing to win: Uncoupling mechanisms for mitROS control in DM. Mitochondrial uncoupling through proton leakage has been proposed as a mechanism to lower mitROS. This is especially interesting as the relationship between proton gradient and mitROS is exponential, so a small loss in ATP production could have a high trade-off value [327]. In human ageing, this mild mitochondrial uncoupling could explain why some muscles show more age-related losses than others [328].

As known since the early 1960s, fatty acids are capable to induce mitochondrial uncoupling [329]. Our previous data show that a homeostatic mechanism depending on lipid peroxidation controls mitROS production through mild uncoupling. Thus, increased mitROS production generates 4-HNE, which, besides a signalling role, shows also both a propagation role and also an uncoupling role, leading to diminished mitROS production [330]. In T2DM, though speculatively, this mechanism would be overwhelmed, as increased lipid peroxidation derived protein damage has been evidenced in samples from animal models of this disease [331]. Furthermore, expression of uncoupling proteins leads to both diminished lipid peroxidation [332] and lipid peroxidation-derived damage: in mitochondria from UCP3-under-expressing models significantly higher levels of lipoxidative damage than wild-type controls were found, suggesting that UCP3 functions *in vivo* as part of the antioxidant defences of the cell [333], a finding also observed by other authors [334]. Noteworthy, T2DM patients and insulin-resistant individuals show a diminished value of UCP3 protein levels in muscle, suggesting that an impairment in the expression of this protein and, possibly, other mitochondrial mechanisms for mitROS normalization could underlie mitochondrial dysfunction in insulin-resistant and diabetic states [316,335].

In conclusion, these data reveal that not only extracellular lipid peroxidation is important for chronic complications, but also intracellular lipid peroxidation could be a key factor in the relationship between unbalanced energy homeostasis and T2 DM rising incidence.

Protective and detrimental effects of 4-hydroxyalkenals in diabetes (Y. Riahi, G Cohen, O Shamni, S Sasson)

The peroxidation of polyunsaturated fatty acids is intensified in diabetes. The peroxidation of n-3 and n-6 polyunsaturated fatty acids and the production of 4-hydroxyalkenals are intensified in diabetes due to the pro-oxidative environment induced by prolonged hyperglycaemia [336]. Of particular interest are the peroxidation products of n-6 poly-unsaturated fatty acids, namely 4-HNE and 4-HDDE [67,70,337]. The former is the peroxidation product of 15-hydroperoxyeicosatetraenoic acid (15-HpETE) and 13-hydroperoxyoctadecadienoic acid (13-HpODE), which are 15-lipoxygenase (15-LO) metabolites of arachidonic- and linoleic acid, respectively. 4-HDDE is the peroxidation product of 12-hydroperoxyeicosatetraenoic (12-HpETE), the 12-LO metabolite of arachidonic acid [337,338].

The physiological, pathophysiological or cytotoxic effects of 4-hydroxyalkenals depend on their absolute concentrations. The best example is 4-HNE: Esterbauer et al. [67] showed that at concentrations higher than 20 μmol/L it exhibited cytotoxic effects that result from its chemical reactivity and the formation of stable adducts with proteins and nucleic acids, which alter their functions [70]. Bacot et al. [337,339] demonstrated that 4-hydroxyalkenal-induced membrane disorders resulted from the formation of adducts with ethanolamine phospholipids. They also reported that the potency of 4-HDDE in forming such ethanolamine phospholipids-adducts was ~ 3-fold higher than that of 4-HNE [337,338]. Likewise, we found that 4-HDDE induced vascular endothelial cell (VEC) death at significantly lower concentrations than 4-HNE (1 vs 25 μmol/L, respectively) [340]. The higher chemical reactivity of 4-HDDE is attributed to the presence of two double bonds and its higher hydrophobicity in comparison with 4-HNE (Log *p*-values 3.48 and 2.45, respectively) [339].

Normally, the cellular levels of 4-hydroxyalkenals are well-controlled because their precursors, 12- and 15-HpETEs, are effectively reduced by GPx to the corresponding hydroxyl-derivatives 12- or 15-hydroxyeicosatetraenoic acid (12-HETE and 15-HETE) [336]. However, free radicals can slow and even block this pathway by inactivating GPx [337]. This leads to the accumulation of HpETEs, which are then subjected to the free radical-initiated peroxidation process that ends in the generation of 4-HDDE and 4-HNE. Similarly, 13-HpODE is peroxidized to generate 4-HNE. SEVERAL alternative mechanisms for the peroxidation process and the generation of these reactive

aldehydes have been proposed [4,341–346]. Common to these models is the primary hydroxyl radical-induced abstraction of hydrogen atom from 12- and 15-HpETE. The resulting radicals interact with free oxygen and further undergo a chain-breaking reaction to generate the corresponding 4-hydroxyalkenals.

Figure 2 depicts the two main routes of arachidonic acid transformation: the common pathway that occurs in metabolically undisturbed cells and the aberrant peroxidation pathway that is induced by an excessive oxidative stress. This model suggests that tissue-specific production of 4-hydroxyalkenals correlates with the specific pattern of expression of lipoxygenases and the biosynthesis of the corresponding HpETEs. This has been demonstrated by Guichardant's group [337,347] in diabetic rat's retina that expresses 15-LO and generate 4-HNE, but not 4-HDDE. Similarly, Coleman et al. [348] have found that 4-HNE, generated from 15-HpETE, is the major peroxidation product in 3T3-L1 pre-adipocytes. We have found that bovine aortic endothelial cells that express 12-LO generate under hyperglycaemic conditions excessive levels of 4-HDDE, but not 4-HNE [340,349,350]. It is reasonable to suggest that human VEC that also predominantly over-express 12-LO under high glucose conditions preferentially generate 4-HDDE under such conditions [351]. A similar relationship between 12-HpETE and 15-HpETE and the generation of the corresponding 4-HDDE and 4-HNE has been reported by Bacot et al. [337]. We speculate that 4-HDDE is the putative lipid peroxidation product that Bleich et al. [352] have implicated in β-cell dysfunction following cytokine-mediated increased expression of 12-LO. This model also shows that oxidative stress induced by hyperglycaemia diverts the flux of HpETEs from the enzymatic pathway towards the peroxidative arm. This role of free radicals in lipid peroxidation has been demonstrated in several systems [347,353–355].

In addition, hyperglycaemia increases the expression and cellular level of 12- and 15-LO in cells and subsequently the availability of HpETEs to peroxidation. Such induction of 12-LO was discovered in islets of Langerhans from Zucker diabetic fatty rats (a rodent model of T2DM) in comparison with the non-diabetic controls [356] and in islets isolated from others types of diabetic rats [357]. We investigated the pattern of expression of 12-LO in primary cultures of aortic endothelial and smooth muscle cells and discovered that high glucose levels increased the cell content of 12-LO (but not 15-LO) [340,349,350]. Similarly, Natarajan et al. [358] reported that elevated glucose levels increased the expression of 12-LO in porcine aortic SMC.

These findings suggest that the impact of hyperglycaemia on lipid peroxidation is synergetic: the expression of lipoxygenases is elevated in cells and the products of these enzymes are channelled to peroxidation and an augmented formation of 4-hydroxyalkenals. This model predicts an increased abundance of 4-hydroxyalkenals in diabetic individuals. Indeed, Toyokuni et al. [359] found a marked increase in the level of 4-HNE-modified albumin in sera of T2DM patients in comparison with normoglycaemic subjects. These elevated levels were attributed to an increased generation of 4-HNE in peripheral tissues. Similar observations were made in Zucker obese rats [360]. Hitherto, no such determinations of 4-HDDE and 4-HDDE-protein adducts in diabetic patients have been reported. Nevertheless, Guichardant et al. [347] measured significant amounts of 4-HDDE metabolites along with 4-HNE and 4-HHE metabolites in urine samples of healthy individuals.

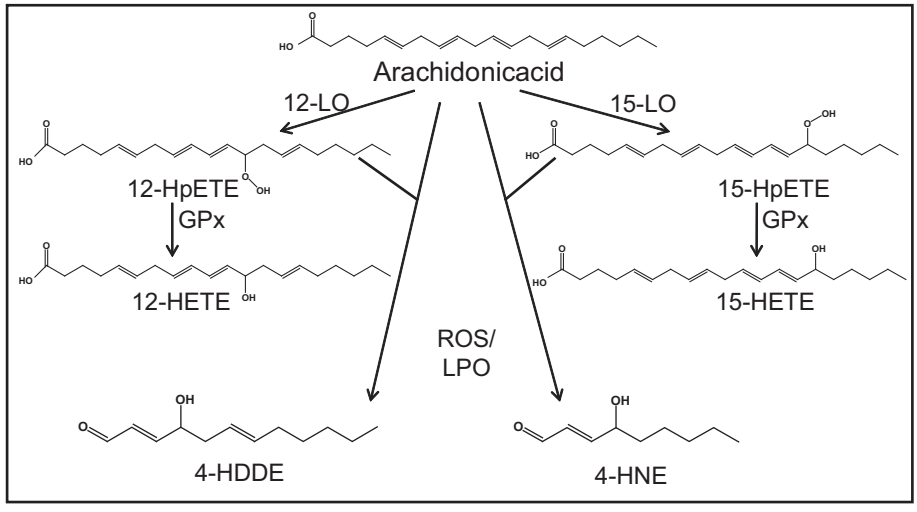

Figure 2. Mechanism of 4-hydroxyalkenal generation. The enzymes 12- and 15-lipoxygenase (12- and 15-LO) metabolize arachidonic acid to the corresponding hydroperoxyeicosatetraenoic acids (12- and 15-HpETE), which are further converted by glutathione peroxidase (GPx) to 12- or 15-hyroxyeicostetraenoic acid (12- and 15-HETE). Radical induced inactivation of GPx diverts 12- and 15-HpETE to the peroxidation pathway to ultimately generate the respective 4-hydoxdodecadienal (4-HDDE) and 4-hdroxynonenal (4-HNE).

Roles of 4-hydroxyalkenals in diabetes. Products of lipid peroxidation can affect cells in diabetic subjects in two major ways, depending on their concentrations in cells and organs. First, high levels of 4-hydroxyalkenals induce detrimental effects and substantial cell damage that lead to severe complications of diabetes. Second, intermediate non-toxic concentrations of these compounds may attenuate cellular signalling and metabolic pathways, which may be either unfavourable or protective.

Role of 4-hydroxyalkenals in β-cell dysfunction. The tendency of 4-hydroxyalkenals to bind covalently to amino acid moieties (i.e. histidine, lysine, cysteine) in proteins, guanine nucleotides in DNA and phospholipids underlies their significant contribution to the development of complications of diabetes. Foremost, the failure of pancreatic β-cells to adequately increase insulin secretion in response to hyperglycaemia is considered, together with peripheral insulin resistance, a critical factor in the development of T2DM. Hyperglycaemia-induced free radical formation is a major contributing factor to the deterioration of β-cell in T2DM. Various studies have alluded to a detrimental role of 4-hydroxyalkenals in this process: Bleich et al. [361] linked the increased expression of 12-LO in islets of diabetic rats to impaired insulin secretory function. Prasad et al. [362] found that over-expression of 12-LO in cultured INS-1E β-cells halved their glucose-induced insulin secretory capacity. Similarly, the incubation of isolated rat islets with a high level of 4-HNE (100 μM) significantly lowered their insulin secretory capacity [363]. Conversely, 12-LO knockout mice did not develop glucose intolerance and β-cell dysfunction when fed a diabetogenic high fat diet [364]. In addition, such 12-LO knockout mice were highly resistant to streptozotocin-induced diabetes and their isolated islets maintained good insulin secretory capacity following treatment with inflammatory cytokines. Collectively, these finding suggest that 12-HpETE, the immediate product of 12-LO, and its peroxidation products adversely affect β-cell function [352]. This idea was confirmed when over-expression of 12-LO in cultured INS-1E β-cells abrogated glucose-induced insulin secretion [362]. Further support comes from the finding that β-cell death in rodent and human islets exposed *in vitro* to inflammatory cytokines (*i*.e. IL-1β, INFγ, TNFα) was prevented in the presence of baicalein, a specific inhibitor of 12-LO, whereas incubation of the cells with 12-LO metabolites reduced basal insulin secretion and increased cell death [365]. It has been suggested that 4-HNE at non-toxic concentrations inhibits cell growth and induces accumulation of cells in the G0/G1 phase of the cell cycle [366]. Plausibly, a similar inhibitory effect of 4-HNE on β-cells replications may reduce their mass in islets, a phenomenon characteristic to diabetic islets.

The autoimmune and cytokine-mediated destruction of β-cells in Type-1 diabetes has also been associated with over-production of reactive aldehydes, such as 4-HNE. The non-obese diabetic (NOD) mice usually develop severe autoimmune insulitis and diabetes. However, the expression of an inactive 12/15-LO in these mice rendered them resistant to the development of diabetes [367]. Similarly, Bleich et al. [352] found that islets isolated from 12-LO knockout mice were resistant to cytokine-induced damage. Suarez-Pinzon et al. [368] also identified a key role for 4-HNE and other cytotoxic aldehydes in the destruction of rat pancreatic islet β-cells by cytokines. Other β-cell lines (RINm5F and HIT-T15) were particularly susceptible to 4-HNE, which caused 75–100% cell death at 50 μM [369]. It was suggested that 4-HNE and other lipid peroxidation products induce apoptosis in RINm5F cells [370]. Noteworthy, not all cells are equally susceptible to 4-hydroxyalkenal-induced damage due to a variable expression of the major detoxifying enzymes glutathione S-transferase (GST, i.e. Gst-μ3 and Gst-ω1) and fatty aldehyde dehydrogenase (FALDH, i.e. Aldh3a1) [348]. GSTs detoxify 4-hydroxyalkenals through conjugative reactions, while FALDH transforms the aldehyde group into a carboxylic moiety to produce 4-hydroxy-2*E*,6Z-dodecadienoic acid (4-HDDA) from 4-HDDE and 4-hydroxy-2*E*-nonenoic acid (4-HNA) from 4-HNE. Both 4-HNA and 4-HDDA were identified in human urine [347] and in various cells and tissues [67,371]. Only few studies addressed the possibility that these metabolites are biologically active. Murphy et al. [372] reported that 4-HNA bound to the γ-butyrate receptor in the CNS, while Echtay et al. [330] suggested that it uncoupled proton conductance in mitochondria. The susceptibility of β-cells to oxidative stress- and ROS-induced damages has been attributed to their inherent poor intrinsic antioxidant defence [373–376]. This, coupled to a progressive 4-hydroxyalkenal-induced inactivation of FALDH and GST, may exaggerate β-cell dysfunction. Of interest is also the report on an impaired hepatic disposal of 4-HNE in diabetic rats [377]. Consequently, the slow clearance may extend the exposure of β-cells to 4-hydroxyalkenals and further intensify β-cell dysfunction.

Role of 4-hydroxyalkenals in macrovascular diseases. Hyperglycaemia is an established major and independent risk factor in the development of cardiovascular disease in diabetes [378–381]. Endothelial cell dysfunction is the earliest event in the development of atherosclerotic lesions in blood vessels. This condition may result from a direct oxidative damage and over-production of 4-hydroxyalkenals that compromise the integrity and functions of the endothelial cell monolayer. For instance, Minekura et al. [382] found that 4-HNE significantly reduced the expression of adhesion molecules

(i.e. ICAM-1, VCAM-1) induced by TNF-α and NF-κB activation in primary cultures of human aortic endothelial cells. Tetrahydrobiopterin (THB), the cofactor of endothelial nitric oxide synthase (eNOS), is particularly susceptible to inactivating interactions with 4-HNE. The inactivation and lack of THB induce uncoupling of the enzymatic reaction of eNOS, which leads to the generation of superoxide radicals that further exacerbate the oxidative damage [383]. Interestingly, 4-HNE has been implicated in macrophage activation and their transformation to foam cells in diabetic blood vessels [5]. This is partly due to the 4-HNE capacity to increase the expression of class A scavenger receptors, which enhances macrophage foam cell formation [384]. Moreover, oxLDL has a considerable capacity to covalently bind 4-HNE [385].

The smooth muscle cell layer in blood vessels is also a target for 4-HNE, which induces a marked mitogenic response, characteristic to the early event in atherosclerosis [386–388]. High levels of 4-HNE were associated with other effects in smooth muscle cells, such as apoptosis [389], autophagy [390], cytotoxicity [391], production of TGF-β [392], impaired PDGF receptor activity [393] and enhanced matrix metalloproteinase-2 production [394,395].

The physiological relevance of these *in vitro* studies has been corroborated by Yamanouchi et al. [396], who studied streptozotocin-diabetic APA Syrian hamsters that developed atheromatous lesions. Immunohistochemical analysis of foam cells in the fatty streaks in blood vessels showed a significant increased abundance of 4-HNE-protein adducts. Similarly, Meng et al. [397] found a high content of 4-HNE-protein adducts in aortae of streptozotocin (STZ)-diabetic rats. Recently, Mattson [53] suggested in a comprehensive review a central role of 4-HNE in obesity, in the metabolic syndrome and in vascular and neurodegenerative disorders. Of particular interest in this review is the suggestion to use 4-HNE as a target to prevent and treat the metabolic syndrome and associated diseases. Two main strategies were put forward: to reduce 4-HNE production by suppressing lipid peroxidation or by its detoxification.

Other complications

The aetiology of other peripheral complications in diabetes has also been linked to aberrant interactions of 4-HNE. For example, Polak and Zagorski [398] found significantly higher levels of 4-HNE and MDA in diabetic patients with retinopathy in comparison with patients that did not develop this complication. The presence of AGEs and 4-HNE-protein adducts was evidenced immunohistochemically in the glomerulus in diabetic nephropathy in humans [399,400]. Others found that 4-HNE mimicked features of diabetic neuropathy by inducing mitochondrial dysfunction and aberrant axonal outgrowth in adult sensory neurons isolated form rats [401,402]. It has been shown that effective quenching of 4-HNE can greatly improve the wound healing process, which is a serious complication in diabetes [403].

Non-toxic interactions of 4-hydroxyalkenals in diabetes. Unfavourable interactions: By their tendency to form covalent bonds with various amino acid moieties, 4-hydroxyalkenals can modify structures of proteins even at non-toxic levels and alter their function. Several reports have addressed such interactions of 4-HNE that affect signalling pathways in cells [404–406]. Some interactions have been associated with cell differentiation, transcription factors regulation, activation of stress kinases, mitogenic response, stimulation of heat shock proteins or modulation of transduction mechanisms. Demozay et al. [407] have demonstrated the role of 4-HNE in attenuating the insulin transduction mechanism and inducing insulin resistance in 3T3-LI adipocytes. Incubation of these cells with non-toxic concentrations of HNE significantly decreased the level of the insulin receptor substrate (IRS)-1/2 proteins. This led to a down-regulated response of downstream targets of the insulin transduction pathway, leading to insulin resistance. The underlying mechanism was the formation of 4-HNE-IRS protein adducts and their rapid degradation. Grimsrud et al. [408] performed a thorough LC-ESI MS/MS analysis of proteins from epididymal adipose tissue of diabetic mice and identified a number of regulatory proteins that bound 4-HNE that were linked to oxidative stress responses and the development of insulin resistance.

Glucose transporters are also targets for 4-HNE. Reagan et al. [409] found an increased level of 4-HNE conjugation with glucose transporter-3 (GLUT-3) in the hippocampus of diabetic rats and linked it to decreased hippocampal neural glucose utilization. It is not clear whether 4-HNE similarly interacts with the ubiquitous GLUT-1, the hepatic and β-cell specific GLUT-2 or the insulin-sensitive GLUT-4.

Protective interactions: We have discovered that VEC exposed to high glucose levels can reduce the rate of glucose uptake and prevent adverse effects associated with an increased influx of glucose. Specifically, these cells reduced the cellular level of their principal glucose transporter GLUT-1 mRNA and protein, as well as its plasma membrane content [349,350,410]. High glucose also increased the expression of 12-LO and the production of 12-HpETE and 12-HETE. Pharmacological inhibition of 12-LO completely blocked these high glucose-induced down-regulatory effects [349–351]. The reduced expression of GLUT-1 in VEC exposed to high glucose resulted from post-transcriptional destabilization and degradation of GLUT-1 mRNA. The protein calreticulin, which was also over-expressed under high glucose conditions, bound to a specific 10-nucleotide sequence in the

3'-UTR of GLUT-1 mRNA, destabilized the molecule and rendered it susceptible to nuclease digestion [411]. We also discovered that the expression of calreticulin was augmented in blood vessels of diabetic animals [411]. Collectively, our studies suggest a functional link between 12-LO and its metabolites to GLUT-1 mRNA destabilization by calreticulin.

We have recently found [340] that the augmented expression of 12-LO in VEC under high glucose conditions was accompanied with a marked increase in the generation and secretion of 4-HDDE, but not 4-HNE. Moreover, using pharmacological and molecular approaches we proved that 4-HDDE was an endogenous ligand of the Peroxisome Proliferator-Activated Receptor (PPAR)-δ in these cells. When added to cells at non-toxic concentrations (50 nM) 4-HDDE activated PPARδ, which specifically interacted with a PPAR-response element in the calreticulin gene and augmented its expression. Previous studies have shown that PPARδ preferentially interacts with unsaturated fatty acids and eicosanoids [406,412]. X-ray analyses of crystal structures of the PPARδ ligand binding domain (LBD) identified a network of hydrogen bonds with His^{413}, Tyr^{427}, His^{287} and Thr^{253} involved in the interaction with eicosapentanoic acid [413]. Interestingly, His^{413} has also been implicated in a hydrogen-bonding interaction with the 4-hydroxy group of 4-HNE [348]. The sites involved in 4-HDDE interaction with the LBD of PPARδ have not been identified yet. This natural protective mechanism against the deleterious effects of hyperglycaemia in vascular cells may explain why some diabetic patients never develop cardiovascular complications, as was found in the Adult Treatment Panel III trial [414]. Figure 3 depicts our working hypothesis: patients who moderately increase the generation of 4-HDDE in VEC under hyperglycaemic conditions benefit from its protective interaction with PPARδ and the subsequent reduction in GLUT-1 levels. When 4-HDDE production is not controlled, it adversely interacts with proteins, DNA and phospholipid and causes substantial damage to the cells. Theoretically, low levels of 4-hydroxyalkenals in VEC may result from a moderate increase in 12/15-LO expression and/or an efficient neutralization of 4-hydroxyalkenals.

In summary, lipid peroxidation is a prominent feature of diabetes. Both 4-HNE and 4-HDDE contribute to the development of β-cell dysfunction and the development of peripheral complications of diabetes. Yet, when produced at low and non-toxic levels these molecules may have beneficial roles, such as the protection of VEC against deleterious effects of increased flux of glucose. In addition, less is known and reported on 4-HDDE in comparison with 4-HNE. We strongly suggest considering the former as a potent and equally important product of lipid peroxidation in general, and in diabetes in particular.

Renal diseases (W Siems, I Wiswedel)

It is generally accepted that renal failure is associated with drastic lipid peroxidation [415–417]. Lipid peroxidation contributes to pathogenesis and progression of renal failure. Cardiovascular (CV) injury has been shown to be the most critical factor affecting quality-of-life and mortality in patients suffering from end-stage renal disease (ESRD) undergoing haemodialysis (HD). Lipid peroxidation and oxidative stress

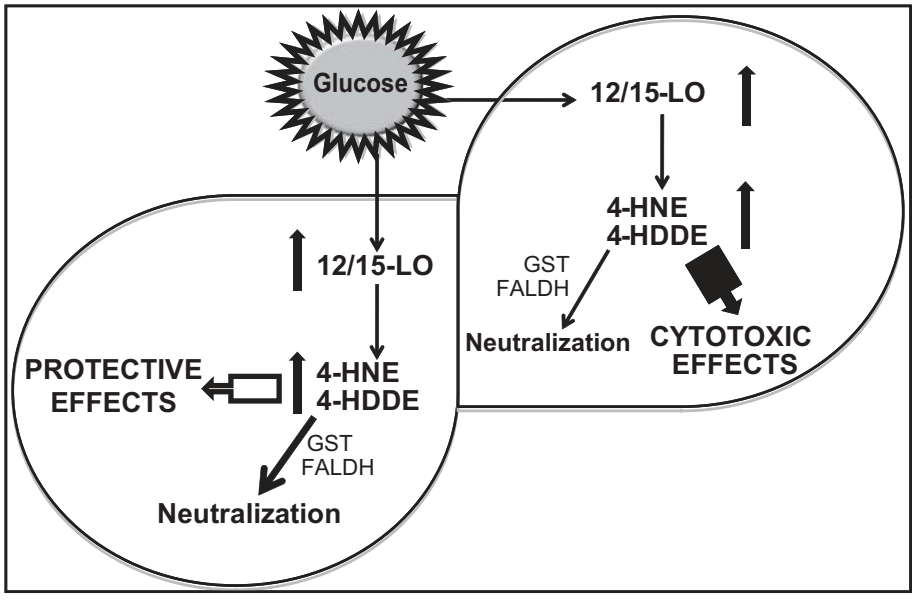

Figure 3. Adverse and beneficial effects of 4-hydroxyalkenals in cells exposed to high glucose levels. The right-hand panel shows a cell that overly increases the expression of lipoxygenases and the downstream generation of 4-hydroxyalkenal, which exerts cytotoxic effects. The left-hand panel depicts a cell that moderately increases lipoxygenase expression and generates non-cytotoxic levels of 4-hydroxyalkenal that may attenuate cell function and even mediate protective interactions.

have been thought to be important risk factors for cardiovascular disorders in renal patients.

Excessive CV morbidity and mortality in kidney insufficiency and uremia can be explained in part by the classical risk factors. However, the pathophysiology of CV lesions is a combination of classical and new emerging risk factors, some of them being strictly related to ESRD [418–421]. Among the new emerging risk factors, anaemia and lipid peroxidation/oxidative stress are two of the most mentioned and studied. These two pathophysiological conditions were already described in the first decline of glomerular filtration rate and several studies have proposed their active role in deterioration of kidney function, especially in diabetic nephropathy [422–429]. We, as others, detected in previous studies dramatic oxidative stress status in ESRD and in patients undergoing haemodialysis [415,427,430–434].

HNE, MDA, protein carbonyls, F2-isoprostanes and cholesterol oxidation products (COP) are increased in plasma of patients with renal failure. The HNE and MDA values were unusually high in the serum of patients. The highest values, e.g. for HNE, in the serum of ESRD patients were around 0.65 μM [430]—comparing with normal physiological values of 0.072 ± 0.020 (40–49 years), 0.083 ± 0.020 (50–59 years), 0.096 ± 0.022 (60–69 years) or 0.107 ± 0.027 μM, respectively (oldest control group with 70–84 years age) [54]. The HNE and MDA values in patients suffering from renal insufficiency were lower after dialysis compared with the values before dialysis. For serum protein carbonyls there was no difference between values before and following dialysis. For HNE, MDA and protein carbonyls there exists a strong direct correlation with the degree of renal anaemia, i.e. the lower the haemoglobin level, the higher HNE, MDA and protein carbonyl levels were [435]. This is shown in Figure 4.

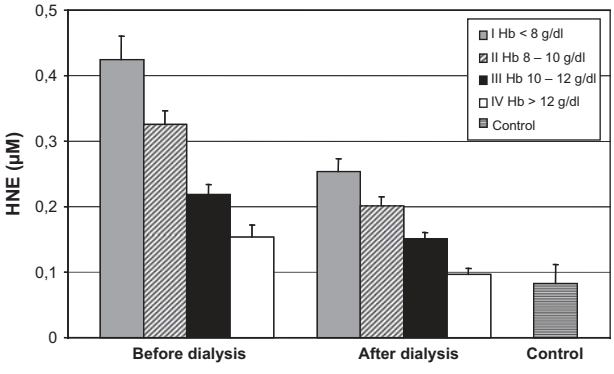

Figure 4. Serum 4-HNE levels in patients with end-stage renal disease. Serum 4-HNE levels in patients with end-stage renal disease before and after dialysis in relation to the degree of renal anaemia (four groups: group I with Hb < 8 g/dl; group II with HB 8–10 g/dl; group III with Hb 10–12 g/dl; group IV Hb>12 g/dl. Total number of HD patients in this investigation was 107, total number of control probands was 80. Data were taken from Siems et al. [435].

F2-isoprostane levels in patients with chronic renal failure related directly to the degree of inflammation, e.g. to C-reactive protein [436] (Figure 5). Besides lipid peroxidation, an increased serum homocysteine level was typical for 80% of patients. Obviously, serum homocysteine levels represent an independent risk factor in ESRD. We found a correlation between serum homocysteine and plasma albumin as a parameter for nutritional status.

Moreover, parameters of lipid peroxidation are a strong indicator for clinical worsening or improvement. There was an almost linear correlation between HNE plasma level and left ventricular mass index (LVMI) of the heart (Figure 6). Both parameters—the CV parameter LVMI and the lipid peroxidation parameter HNE—showed an inverse relationship with haemoglobin concentration. Inter-relationships between renal disease, anaemia and CV injury are called cardio-renal-anaemia (CRA) syndrome [430,437]. Within the CRA syndrome, oxidative stress including lipid peroxidation is a central pathogenetic and pathophysiological event. If lipid peroxidation and oxidative stress exert a central point in the CRA syndrome, the therapeutic strategy has to take into account the minimization of oxidative attack and/or the strengthening of antioxidative defence mechanisms. The treatment of renal anaemia by means of erythropoietin (EPO) in patients suffering from ESRD undergoing HD, therefore, was controlled concerning its effects on the degree of oxidative stress parameters in relation to clinical improvements of the patients.

The correction of renal anaemia by means of EPO led to an efficient strengthening of antioxidative defence. The improvement of antioxidative capacity by means

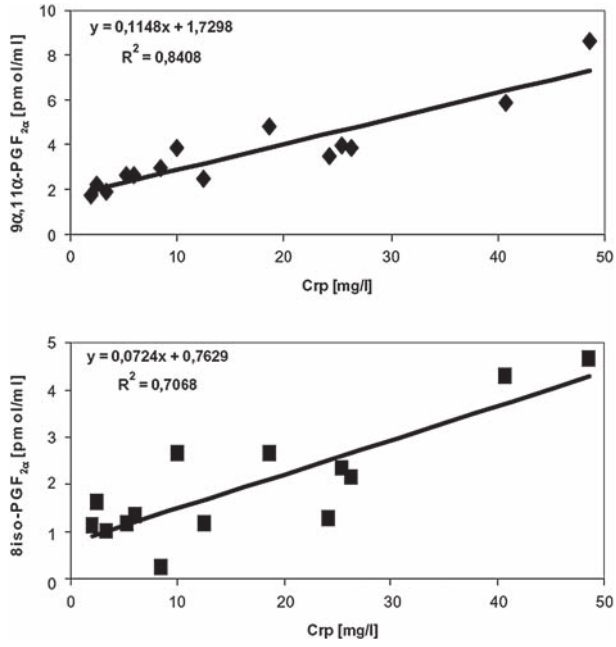

Figure 5. Correlation between serum levels of F2-isoprostanes 8-iso-PGF2α and 9a,11a-PGF2α and C-reactive protein; $n=14$ patients [436].

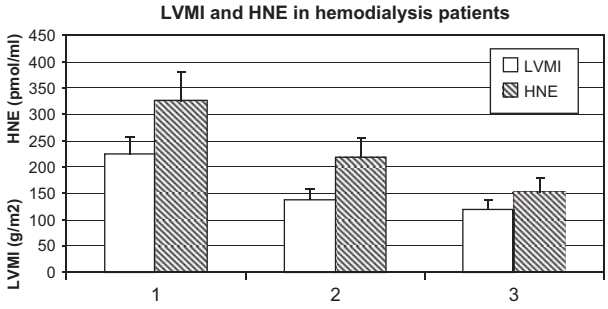

Figure 6. Left ventricular mass index and HNE in relation to the degree of renal anaemia. Left ventricular mass index (LVMI)—as g/m^2—and HNE as pmol/ml serum in relation to the degree of renal anaemia. The values are given for three groups of patients concerning the degree of anaemia: 1 Hb $<$ 10 g/dl; 2 Hb between 10–12 g/dl; 3 Hb $>$ 12 g/dl.

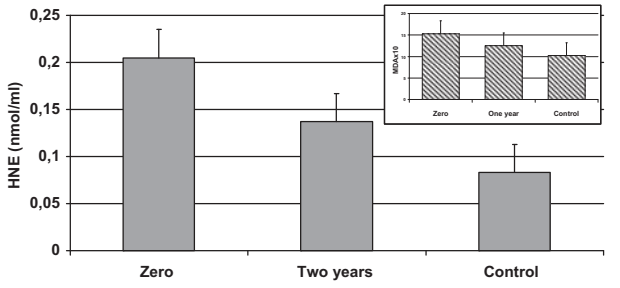

Figure 8. Changes in HNE (main figure) and MDA (inserted figure) serum levels during epoetin treatment of ESRD patients. Average epoetin dose was 60–90 units/kg body weight/week. The data are mean ± SE. Main part of the figure gives the result of Scorrano/Lecce investigation with 56 patients and 80 controls for HNE. Inserted small figure presents the result of Nis study with 20 patients compared to 10 controls, where MDA values were measured (data taken from [434]).

of EPO therapy is of complex nature comprising both enzymatic pathways of erythrocytes as mobile radical scavengers and low molecular antioxidants. The therapy of renal anaemia—even the final target Hb is under discussion—with its reduction both of HNE and other LPO products and of the CV risk is regarded as a complex therapeutical strategy. The correction of renal anaemia as antioxidative therapy leads to significant biochemical and clinical advantages in patients with ESRD [430] (see Figure 7). That the treatment with EPO really leads to improvements in lipid peroxidation was shown in two observational studies [435]: in a 2-year investigation with patients from Scorrano/Lecce (Italy) (56 patients and 80 controls) the decrease of plasma HNE was observed; in a smaller and shorter (1 year) observation with 20 patients vs 10 controls from University Nis (Serbia) the MDA decrease was demonstrated (Figure 8). A further therapeutic application of antioxidative therapy in chronic renal failure was shown by Tepel et al. [438] by using acetylcysteine.

Lymphoedema (W Siems, R Brenke)

Chronic lymphoedema is one of the most frequent and debilitating complications after surgical and radiological tumour treatment. Prevention and therapy of lymphoedema is therefore an important problem of the rehabilitation of those patients. In total—together with the filariasis-induced lymphoedema—there are more than 200 million patients suffering from lymphoedema worldwide. Increased interstitial volume, pressure and oncotic pressure lead to a variety of structural and functional deteriorations both of the lymphatic vessels and the interstitium itself. These include dilatation of lymphatic capillaries, damage of endothelial cells, sclerosis of collector vessels and the reduction of smooth muscle cells. Furthermore, one finds stimulation of fibroblasts, activation of PMNL, an increased and partially abnormal formation of collagen, the sclerosis of connective tissue and secondary inflammatory processes [427,439, 440] (see also Figure 9). First findings on accelerated lipid peroxidation in lymphoedema were from Ohkuma in 1989 and 1993. In the last 15 years, aldehydes, oxysterols and antioxidative compounds were analysed in more detail in this disease.

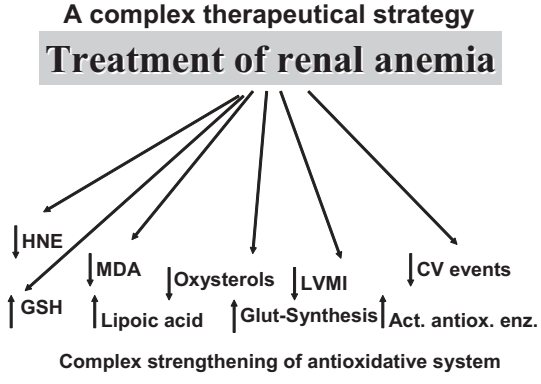

Figure 7. The therapy of renal anaemia is seen as complex therapeutical strategy improving the biochemical and clinical parameters in ESRD.

The blood of patients with chronic lymphoedema contains lower concentrations of GSH and higher levels of disulphides and of MDA and HNE, compared to the control group. MDA was even increased by ~ 3-fold in the serum of the lymphoedema patients [427]. Accelerated free radical formation and lipid peroxidation processes were further demonstrated by the liberation of MDA and HNE into the blood serum after manual lymph drainage [427]. These data demonstrate accelerated lipid peroxidation processes in chronic lymphoedematous tissue. Therefore, it is suggested that the strengthening of antioxidative defence mechanisms could be useful in the adjuvant therapy of chronic lymphoedema [440].

Previously it was found that the concentrations of oxysterols in lymphoedematous tissue and blood of patients suffering from lymphoedema are increased compared to healthy control subjects [441]. Figure 10. A shows differences between 7-keto-cholesterol, α-epoxy-cholesterol and for β-epoxi-cholesterol in

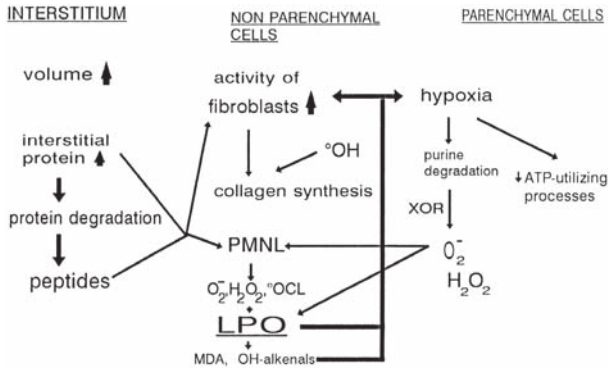

Figure 9. Changes in lymphoedema. Changes in lymphoedema in interstitial matrix and in the cells: activated PMNL and purine degradation as important sources of reactive oxygen species initiating lipid peroxidation in this disease.

patients compared with healthy control subjects. Obviously, increased oxysterol concentration plays a pathogenetic role during tissue remodelling in fibrosis and sclerosis. Those suggestions are in agreement with the role of oxysterols in fibrosis of other organs such as liver [190,442–444]. Additionally, it should be mentioned that the MDA concentration of blood plasma of patients correlates to the volume of the oedema (Figure 10B) and to the reduction of compressibility of lymphoedematous tissue. The higher the MDA, the lower was the reduction of the oedema.

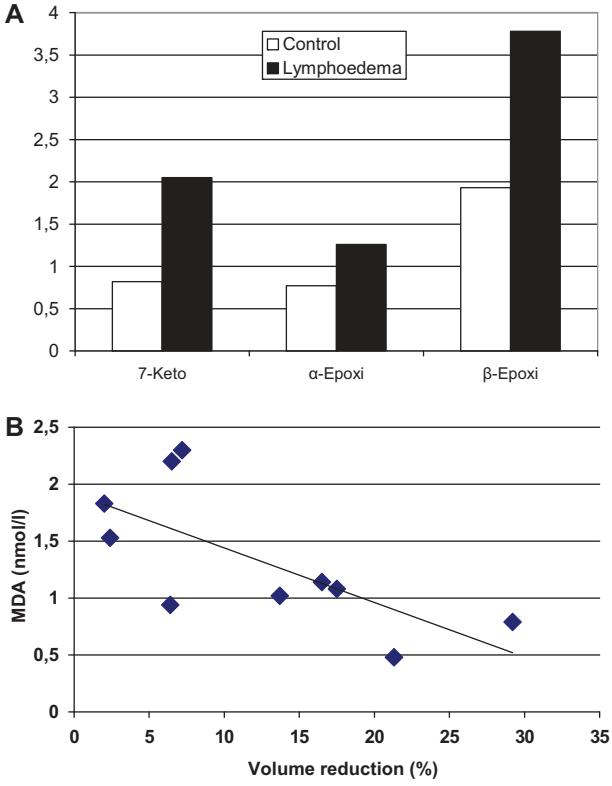

Figure 10. Oxysterols and MDA in patients with lymphoedema. (A) Oxysterols are increased in plasma of patients with lymphoedema compared with controls. Values as mean from 30 patients and 26 controls. (B) As higher MDA as lower was the reduction of the oedema by manual lymph drainage.

Hepatic diseases (J Feher, G Lengyel)

The role of oxidative stress is very important in different liver diseases, mainly in alcoholic and non-alcoholic steatohepatitis and liver cirrhosis, furthermore in chronic viral hepatitis, especially in chronic hepatitis C as well as in primary hepatocellular cancer (HCC). The main processes producing free radicals in mammals are the following: mitochondrial oxidative metabolism providing energy supply, microsomal drug-metabolizing enzyme system, biosynthesis of prostaglandins, constitutive and inducible NO-synthase activity, free radical-producing reactions of phagocytes, monocytes, macrophages and Kupffer's cells and autooxidation of H_2O_2 produced in peroxisomes. Another consequence of free radical activity is lipid peroxidation [445]. In the mechanism of oxidative stress and also in its biological consequences, free radicals can attack small molecules containing thiol and amine groups as well as macromolecules building up the cells. Due to peroxidative damage of the lipids in the cell membrane, permeability changes may occur. Nucleic acid injury can lead to mutations and neoplastic processes of the cells.

The healthy organism is able to prevent the overproduction of free radicals. Low oxygen tension of the tissues is a basic condition. Its value is ~ 26 mmHg or less. The primary line of antioxidants consists of representatives of the enzymatic defence. It is supplemented by antioxidant vitamins with scavenger property (vitamins C, A, E, K), the cofactors, compounds containing thiol, phosphor, amine, polyamine, phenols, quinolines, ubiquinone (coenzyme Q), flavonoids, polyenes, glucose, urate, bilirubin, etc.

Non-alcoholic steatohepatitis and liver cirrhosis

Non-alcoholic fatty liver (NAFLD) is a multi-factorial liver disease. It includes a wide spectrum of liver damage characterized by histological changes of alcoholic origin (ranging from uncomplicated fatty liver to steatohepatitis, fibrosis and cirrhosis) in non-alcoholics (< 20 g/day ethanol consumption). The *non-alcoholic steatohepatitis* (NASH) is part of this disease spectrum, which can progress to *hepatic cirrhosis* and liver failure. Its primary forms have been proposed to be a manifestation or consequence of the metabolic syndrome, closely related to insulin resistance. It can be caused by several other metabolic factors and viral infections, like hepatitis C. The history of non-alcoholic fatty liver disease is not always benign, sometimes it can be accompanied by hepatocellular carcinoma [446].

Researches have identified the factors that can play a causative role: oxidative stress, lipid peroxidation, abnormal cytokine production, fatty acid metabolic disturbance and insulin resistance. The pathophysiology of non-alcoholic fatty liver involves insulin

resistance and production of reactive oxygen species, which stimulate the synthesis of several cytokines (Figure 11) through the up-regulation of their transcription by nuclear factor-κB (NF-κB). The combination of these events causes hepatocyte injury via direct oxidative injury, TNF-α induced apoptosis or inflammation [447].

Role of free fatty acids and insulin resistance. Free fatty acids (FFA) have a decisive role in the development of insulin resistance by being transformed into acyl-CoA intracellularly. In addition to free fatty acids, TNFα and hyperinsulinaemia also play a role in the activation of one of the mechanisms responsible for the negative control of signal transduction of insulin—namely the Ser/Thr-phosphorylation of insulin receptor and insulin-receptor-substrate (IRS) protein—that blocks the signal transduction processes of insulin. In this way muscle cells, hepatocytes and adipocytes all become resistant to insulin. The activity of mitochondrial respiratory chain complexes is reduced in patients with NASH, showing a positive correlation with TNFα levels, insulin resistance (IR) and body mass index (BMI) values.

Inherited defects in the mitochondrial oxidative phosphorylation are supposed to exist in the offspring (diagnosed with IR) of patients with T2DM, leading to disorders in the intramyocellular fatty acid metabolism. The genes dependent on nuclear respiratory factor-1 (NRF-1) that code enzymes, that play crucial roles in the oxidative metabolism and mitochondrial function, are expressed to a lesser extent in diabetes mellitus and IR, possibly due to the reduction of PGC1-expression (PPAR-γ co-activator 1-β and 1-α). In the metabolic syndrome, FFA are released from the abnormal mesenteric tissue to a certain extent depending on the ratio of the β-3- and α-2 receptors in the given fat tissue, as β-3-adrenoreceptors and α-2-receptors are responsible for the induction and the inhibition of the lipolytic process, respectively. Polymorphism of the β-3-receptor has been connected with IR and visceral obesity for a long time, most recently with NAFLD. IR is of decisive importance in how NASH is expressed in a patient, especially in the presence of a reduced mitochondrial oxidation. According to recent research, this involves increased lipolysis in the peripheral tissues, an increase in fatty acid uptake by the hepatocytes, the β-oxidation of the fatty acids taken up by the hepatocytes increases, and the uptake of lipoprotein by the tissues is reduced, due to insufficient lipoprotein lipase. IR is much more severe in patients with NASH than in those diagnosed with 'simple' fatty liver.

Mitochondrial dysfunction in NAFLD. Mitochondrial abnormalities are closely related to the pathogenesis of NAFLD, which raised the possibility that NAFLD is a mitochondrial disease [448]. Many genes encoding mitochondrial proteins in skeletal muscle and fat are negatively correlated with body mass, mtDNA depletion in hepatocytes impairs mitochondrial function and causes hepatic steatosis and other liver injuries. Multiple enzymes are involved in mitochondrial β-oxidation and their deficiency may lead to the development of hepatic steatosis.

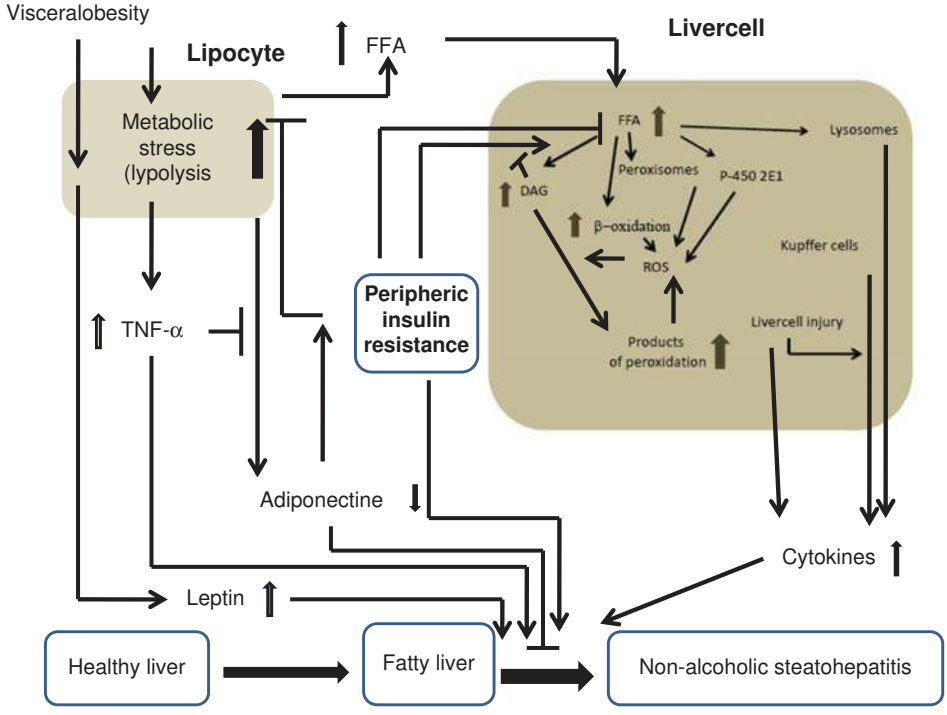

Figure 11. Progression process of non-alcoholic fatty liver. FFA = free fatty acid; DAG = diacylglycerol; ROS = reactive oxygen species (modified from [512]).

A number of mechanisms can be considered to explain the mitochondrial dysfunction found in NAFLD patients and animal models. Possible mechanisms include (a) excessive ROS production, (b) increased TNFα expression and (c) altered PGC-1 expression. MRC dysfunction can directly lead to the production of ROS. If electron flow is interrupted at any point in the respiratory chain, the preceding respiratory intermediates can transfer electrons to molecular oxygen to produce superoxide anion and H_2O_2. MDA and 4-HNE can inhibit mitochondrial cytochrome *c* oxidase by forming adducts with this enzyme. ROS-induced depletion in mtDNA can severely lower mitochondrial number and function, leading to steatosis and liver lesions. Mitochondrial dysfunction may not only cause fat accumulation, but also may generate ROS and cytokines, contributing to the progression of NAFLD by inducing hepatic inflammation and fibrosis [448].

Non-organ-specific autoantibodies (NOSA); positive fatty liver. Non-organ specific autoantibodies (NOSA) include anti-nuclear antibodies (ANA), smooth muscle antibodies (SMA) and anti-mitochondrial antibodies (AMA). They occur in a variety of non-autoimmune chronic liver disease. Their production could be secondary to or triggered by hepatocellular inflammation and necrosis. NOSA positivity in NAFLD has been found to be more prevalent compared to the general population. We have studied the prevalence of non-organ-specific autoantibodies and their correlates with cytokine release and free radical-antioxidant balance in non-alcoholic fatty liver disease [449]. Prevalence of NOSA positivity was found to be 55% in our NAFLD patients. Autoantibody positive patients showed lower antioxidant capacity: lower SH-group concentration, decreased total antioxidant status and elevated chemiluminescence intensity. We suppose that, similar to alcoholic liver disease, the aldehyde products of lipid peroxidation, such as MDA, can react with proteins and form stable protein adducts, which are very immunogenic and capable of inducing immune response, resulting in generation of antibodies. NAFLD with autoantibody positivity may have a worse prognosis because of the impaired antioxidant status. It can be supposed that the appearance of autoantibodies in NAFLD is triggered by free radical reaction, but further investigations are needed to understand the significance, role and the exact mechanisms of NOSA production in NAFLD [449].

Fatty liver in childhood

According to studies, the idiopathic steatohepatitis is characteristic mostly of obese children of peripubertal age. The development of fatty liver can be attributed to several factors. Pathogenetic causes of lipid accumulation in the hepatocytes—likely in adults—may include (1) reduced oxidation and expenditure of fatty acids; (2) increased synthesis of fatty acids; (3) reduced release of triglycerides from the liver; and (4) increased release of fatty acids from the peripheral fat depots [6]. The pathomechanism has a characteristic two-step form: at first fat is accumulated in the liver cells and then this induces a fibrous reaction. Accumulation of ROS has an important role in both steps. Probably the FFA reaching mitochondria cause saturation of mitochondrial beta-oxidation, creating ROS and lipid peroxides, as well as they activate the CYP2E1 isoform of cytochrome P450 enzyme in hepatocytes and Kupffer cells. Children with NAFLD or NASH have an increased disposition to cardiovascular complications, hyperlipidemia and insulin resistance—or, in the case of alcohol consumption, to alcoholic liver disease—in adulthood. The principal treatment in obese children consists of a diet which is low in fat and poor in refined carbohydrates, as well as physical exercise of medium intensity. Blood glucose and lipid levels should be monitored during the treatment [450].

Fatty liver and hepatitis C viral infection

The significance of fatty liver alteration in the hepatitis C virus (HCV) infection [451] is important because of the decreased viral response to the antiviral drug therapy in these cases. Among patients with HCV genotype 1 infection, the grade of steatosis was correlated with host-related factors, mainly with the presence of the metabolic syndrome. In genotype 3, steatosis degree correlates with liver HCV quantification and serum viral load. In genotype 1, steatosis depends on leptin levels and insulin resistance.

Insulin resistance and hyperinsulinaemia have been found in association with liver fibrosis in hepatitis C [451]. The mechanisms through which insulin resistance promotes fibrosis progression include: oxidative stress, lipid peroxidation, fat accumulation in the hepatocytes, hyperleptinaemia, increased TNFα production and impaired expression of PPARγ receptors. Liver cell-related steatosis induces fibrosis progression also through ROS accumulation. Hyperleptinaemia with insulin resistance may play a role in the activation of hepatic stellate cells and fibrosis progression. In insulin resistance the TNFα production is enhanced in patients with hepatitis C and the increased level of TNFα initiates the fibrosis progression, owing to their ability to activate HSC and promote collagen deposits. Moreover, TNFα may inhibit PPARγ activity. An impaired expression of PPARγ receptors was found in patients with hepatitis C. PPARγ agonists inhibit inflammation and fibrosis progression by blocking the activation of the redox-sensitive transcription factor NF-κB and TGFβ1 [452].

The presence of steatosis not only significantly influences the natural history of patients with chronic hepatitis C, but the anti-viral response in these cases is highly decreased. While the sustained virological response is ~ 60% of patients with hepatitis C infection without liver steatosis after 1 year peginterferon-alpha plus ribavirin treatment, this effect is only 30% with liver steatosis in HCV infection with genotype 1. Hepatic steatosis is a high risk factor for reduced response to anti-viral treatment and for evolution towards fibrosis. Obesity and fibrosis represent major therapeutic targets, in association with standard anti-viral regimes, like insulin-sensitizing drugs and free radical scavengers (silymarin, ursodeoxycholic acid, metadoxine); furthermore, the life mode with weight loss can help in therapy efficiency [447,453]. Body-weight reduction and hepatoprotective drugs might have increased the effectivity of combined peginterferon and ribavirin treatment, and thus the sustained virological response.

Alcoholic liver disease

Both acute and chronic alcohol consumption enhances the production of ROS, with the peroxidation of lipids, proteins and DNA. The mechanism by which alcohol causes cell injury is very similar to those observed in non-alcoholic steatohepatitis. Many pathways have been demonstrated in the literature for the alcohol effect, like the oxidative stress, acetaldehyde production, mitochondrial alterations, membrane injury, apoptosis, ethanol-induced hypoxia, effects on the immune system and altered cytokine production, increased endotoxin levels and activation of Kupffer cells, mobilization of iron, changes in the antioxidant defence, particularly mitochondrial glutathione (GSH), one electron oxidation of ethanol to 1-hydroxy-ethyl radical and induction of CYP2E1. These pathways are not exclusive of one another and it is likely that many systems contribute to the ability of ethanol to induce a state of oxidative stress [447,453,454].

Hepatocellular carcinoma

Hepatocellular carcinoma can develop in all kind of chronic liver diseases. The connections of the different factors are shown in Figure 12. In NAFLD the possible follow-up of the pathogenetic trends is: increased fatty acid fluxus, the increased fatty acid content in the liver (VLDL over-production, dyslipidaemia), advanced oxidation and peroxidation in fatty acids, highly increased free radical production (insufficient antioxidant capacity), flow out of inflammatory and immune reactive mediators (the changes in transcription and translation helping the progression of fibrosis) and finally the carcinogenesis.

In conclusion, oxidative stress plays a very important role in most chronic liver diseases, mainly in alcoholic and non-alcoholic steatohepatitis and liver cirrhosis, furthermore in chronic viral hepatitis, especially in chronic hepatitis C as well as in primary

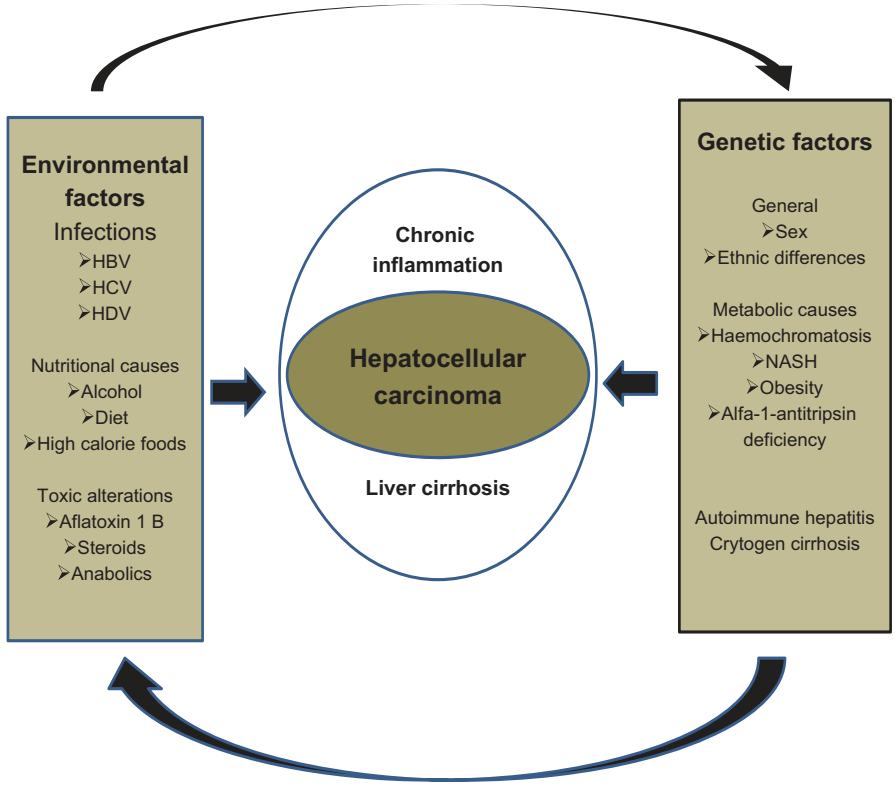

Figure 12. Factors influencing the progression of chronic liver disease to liver cirrhosis and hepatocellular carcinoma.

hepatocellular cancer (HCC). The diagnostic biomarkers to the clinical praxis can be seen in Table III.

Lipid peroxidation and cancer (H Basaga, N Zarkovic)

Cancer is an abnormal mass of tissue the growth of which exceeds, and is uncoordinated with that of the normal tissue, continuing its destructive spread even after the stimuli that initiated it have ceased. Although cancer is not generally considered to be a typical LPO-associated disease, as are neurodegenerative or cardiovascular disorders, not only carcinogenesis but also cancer therapies are strongly influenced by oxidative stress and by LPO. Namely, harmful carcinogenic/mutagenic effects of free radicals play important roles in malignant transformation, while cytotoxicity of oxygen free radicals is important for various anticancer therapies, such as radiotherapy and doxorubicin chemotherapy. On the other hand, intensive lipid metabolism is necessary for dynamic proliferation of cancer cells, while lipid peroxidation products, in particular 4-HNE, are known to act not only as second toxic messengers of free radicals, but also as signalling molecules and growth regulating factors influencing proliferation, differentiation and apoptosis of cancer cells. Therefore, some fundamental aspects of cancer association to LPO will be given in this paper.

Oxidative stress and cancer. Carcinogenesis is thought to occur in stages which could be in general denoted as initiation stage and promotion stage followed usually, but not necessarily, with cancer progression. In the initiation stage, physical, chemical or biologic agents cause an alteration in the molecular structure of the cellular DNA. This molecular injury is followed by a promotion stage, in which the expression of the genes that regulate cell growth (proliferation and differentiation) is altered. While initiating agents usually react directly with the genetic material, promoting agents usually do not act this way, but alter gene expression by indirect mechanisms. Some compounds are 'complete' carcinogens since they possess both initiating and promoting activity, thus by themselves could cause cancer. Among the complete carcinogens are various agents (tumourigenic viruses, carcinogenic chemicals and ionizing radiation) that often cause oxidative stress. ROS play a role mostly in the promotion phase of carcinogenesis [455]. Polycyclic hydrocarbons are complete chemical carcinogens whose harmful activities are correlated with their ability to form free radicals [456]. At least four different radicals might be formed from benzo(a)pyrene (BaP): the benzo(a)pyrene anion radical (BaP^-), benzo(a)pyrene cation radical (BaP^+), radical species formed by heating BaP (related to the radicals formed in cigarette smoke) and oxygenated BaP radicals. The most relevant from the last group are 6-oxyBaP radical and radicals formed from BaP quinones [457]. The production of BaP^+ and oxygenated BaP radicals can be considered as an ubiquitary and frequent process since they can be formed by the activity of the enzyme cytochrome P450 or peroxidase-hydrogen peroxide. Based on the activity of these radicals the binding of BaP to DNA has been implicated as the first step of carcinogenesis caused by BaP. The chromosome breaking (clastogenic) effect of the 'tumour promoter' phorbol myristate acetate (PMA) is mediated by the production of ROS [458]. Additionally, carcinogenic activities of tumour promoters are associated with a decrease of the cellular antioxidant defences, such as the key enzymes defending from the oxidative stress, catalase and superoxide dismutase (SOD).

Antioxidants are often acting as anti-promoters and anti-carcinogens [459], in agreement with their fundamental role of biological response modifiers of oxidative homeostasis [460]. Thus, suppression of the cancer development by antioxidants on one side and, on the other, carcinogenesis based on oxidative stress should not only cause structural changes in the genome DNA, but also functional changes, i.e. changes of the gene expression. That should be particularly relevant if genes encoding for the enzymes

Table III. Biomarkers of liver cell injury, inflammation, fibrogenesis, fibrosis and hepatocellular carcinoma.

Liver cell injury	Inflammation	Fibrogenesis	Fibrosis and ECM turnover	Hepatocellular carcinoma
ALT	CRP	fibrogenic cytokines	hyaluronan	alfa-fetoprotein
AST	alfa-2-macroglobulin	CTGF	PIII NP	PIVKA II
GGT	haptoglobin	circulating fibrocytes	MMPs	
GLDH	chemokines	CSF	TIMPs	
LDH5		chemokines	laminin	
Liver cell injury caused by: alcohol; drugs, toxin; chemicals; NAFLD; metabolic diseases; HBV; HCV; HDV; autoimmune reaction; cryptogenic; venous obstruction; cholestasis	inflammatory cells	activation of hepatic stellate cells expansion of myofibroblast pool mediators: IGF-I; PDGF; TGF-beta; ET-1; ROS	characterizing in the tissue: collagen; elastin; glycoproteins; proteoglycans; hyaluronan	producted by the liver: tumour cells

involved in detoxification of ROS, DNA repair and in cell growth regulation (proto-oncogenes) would be affected. Hence, the same or similar mechanisms of oxidative stress could lead on one side to depressed expression of the genes involved in defence against ROS and on the other to enhanced expression of the genes stimulating cell growth. This should, although pathologically altered, be relatively balanced to allow not only survival of the cancer cells but also its further growth and progression of cancer. Unfortunately, the mechanisms that guide the carcinogenesis in such a 'controlled' way are not understood [461].

However, fortunately, some recent discoveries on Ral-binding protein 1 (RALBP1), a stress-responsive and stress-protective multi-specific transporter of glutathione conjugates (which is often over-expressed in malignant cells and plays a prominent anti-apoptotic role selectively in cancer cells through its ability to control cellular concentration of pro-apoptotic LPO products such as HNE), not only increase our understanding on how tumour cells escape for growth regulating and anti-cancer effects of LPO but also open novel approaches to cancer therapies. Namely, even in the absence of chemotherapy, inhibition of RALBP1 by specific antibodies causes regression of murine melanoma B16 [462], while inhibition of RALBP1 combined with appropriate chemotherapies could cause regression of human solid tumours that over-express RALBP1 such as non-small-cell lung cancer (NSCLC H358 and H520) and colon SW480 cell lines [463].

Lowered activities of primary antioxidant enzymes, such as the cytoplasmic CuZnSOD (SOD1), are often, but not always, seen in tumours, suggesting that decreased antioxidant protection accompanied by increased ROS production might explain not only essential steps in carcinogenesis, but, more generally, many cancers cell properties. Among the hereditary factors of cancer development there are examples of genetic alterations related to the lack of defence against oxidative stress and harmful effects of ROS. An example are patients with triple chromosome 21 (trisomy 21 or Down's syndrome) in which the tissue content of cytoplasmatic SOD1 is increased for ~ 50%, since the gene encoding for the enzyme is located on chromosome 21. Erythrocyte GSH-peroxidase activity is also increased in patients with Down's syndrome, probably due to the higher H_2O_2 production resulting from the increased SOD activity. However, in patients with trisomy 21, only cytoplasmatic SOD1 is increased while mitochondrial MnSOD (the gene for the mitochondrial SOD2 is located on chromosome 6) is reduced by one third [464]. This suggests the possibility of regulation of mitochondrial SOD2 activity by cytosolic feedback mechanisms of SOD1 and/or superoxide ions. Thus, altered SOD activity pattern may be significant in carcinogenesis because trisomy 21 patients have a 10–30% increased risk of developing acute leukaemia. Hence, a decrease of SOD2 activity may be associated with the acquisition of the cancer. In agreement with this are findings of higher incidence of cancer in patients with Dubin-Johnsons-Sprinz syndrome, an inherited disease which shows deficiency of SOD2 [465].

The relevance of persistent oxidative stress in cancer could be in continuous activation of transcription factors leading to intensive proliferation of the cells through altered expression of c-fos, c-jun and c-myc genes [466]. Additionally, ROS can specifically damage proteinase inhibitors (alpha-1-proteinase inhibitors, alpha-1-macroglobulin, alpha-1-plasmin, etc.) by oxidizing methionine residues of the active sites, which would consequently allow further progression of cancer [467]. Thus, it is obvious that ROS play a relevant role not only in cancer induction and promotion but also in its progression; hence, cancer itself represents a condition of oxidative stress, which is furthermore often associated with another oxidative stress and LPO associated disorder—inflammation.

Chronic inflammation, oxidative stress and lipid peroxidation in the genesis and perpetuation of cancer

A number of chronic inflammatory conditions induced by biological, chemical and physical factors pre-dispose susceptible cells to malignant transformation and cancer progression. Chronic inflammatory processes induce oxidative/nitrosative stress and LPO, thereby generating excessive ROS, reactive nitrogen species (RNS) and DNA-reactive aldehydes. In general, the longer the inflammation persists, the higher the risk of cancer. It has been estimated that chronic infection and associated inflammation contribute to about one in four of all cancer cases worldwide [468]. For example, ulcerative colitis has long been linked with high incidence of colorectal cancer; and chronic gastritis, such as from infection with H. pylori, has been associated with a high incidence of gastric cancer [469,470].

Targets of free radicals in inflammation include DNA, RNA proteins and lipids. Among these, oxidatively modified DNA is a major source of the mutation in living organisms and more than 100 oxidative DNA adducts with purine, pyrimidine and the deoxyribose backbone have been identified [391,471–473]. ROS induced DNA damage can result in single- or double-strand breakage, base modifications, deoxyribose modification and DNA cross-linking. Moreover, cell death, replication errors and genomic instability can occur if the oxidative DNA damage is not repaired prior to DNA replication [474–477]. The most extensively studied and most abundant oxidative DNA lesion produced is 8-hydroxydeoxy guanosine (OHdG) and many studies have demonstrated that OHdG levels are elevated in various human cancers [478–481]. In addition, RNS are produced during the chronic inflammation process, which causes nitrative DNA damage to

form 8-nitroguanine. The formation of 8-nitroguanine has been observed in human samples and experimental evidence has suggested that 8-nitroguanine is also mutagenic to form DNA lesions [482].

It has been shown that mutations in cancer-related genes or post-translational modification of proteins by nitration, nitrosation, phosphorylation, acetylation or poly ADP-ribosylation by free radicals or lipid peroxidation derived reactive aldehyde species such as 4-HNE are some of the events that increase the risk of cancer. For example, oxidative damage to DNA has been linked to aflatoxin B-induced *p53* and *ras* gene mutations in hepatocarcinogenesis [483] and in UV-induced mouse and human skin cancers [484,485]. Most notable among pre-cancerous mutations are those that result in modulated signal transduction pathways, thereby increased expression of oncogenes (e.g. *myc*, *ras*, *abl*, *bcl-2*) or decreased activity of tumour-suppressor genes (e.g. *p53*, *Rb*), conferring a selective growth or survival advantage to the cell. Under circumstances of prolonged stress, such as chronic inflammation, cells lose the ability to turn on and off transiently the genes, e.g. cytP450 enzymes that help to eliminate the toxic chemicals—and a mutation may lock in the growth-advantaged phenotype. Hence, prolonged exposure to stress results in a selection of pre-cancerous cells. Phenotypic changes representative of pre-neoplastic mutations include a decreased need for metabolites and growth factors, abnormal signal transduction, inappropriate expression of receptors for growth factors, dysregulation of cell-cycle checkpoints and resistance to apoptosis. Thus, any agent causing increased cell proliferation increases the risk of neoplastic transformation. Therefore, cancer proneness is a consequence of extensive and sustained free radical stress-related damage in diseases (Table IV).

ROS, RNS and LPO products can also modulate signalling molecules [486] and alter functions of enzymes and proteins involved in inflammation and carcinogenesis [487] such as the nuclear transcription factor NF-κB, iNOS and cyclooxygenase-2 (COX-2). Thus, chronic inflammatory processes facilitate, through deregulation of cellular homeostasis, the initiation of normal cells and their growth and progression to malignancy, through production of various types of DNA damage, impairment of DNA repair pathways, overproduction of pro-inflammatory cytokines and reduced apoptotic rates of damaged cells [488,489].

Table IV. Examples of high cancer risk ROS overload diseases modified from [488].

Haemochromatosis	Hepatitis B	Pancreatitis
Chron's disease	Hepatitis C	Schistosoma japonicum
Ulcerative colitis	Human papilloma virus	Schistosoma hematobium
Prostatitis	Helicobacter infection	

Nutritional aspects

Different lifestyle- and environmental-related factors such as smoking, diet, alcohol, ionizing radiations, biocides, pesticides, viral infections and other health-related factors (e.g. obesity or the ageing process) may be pro-carcinogenic. In all these cases oxidative stress acts as a critical pathophysiological mechanism. The excessive consumption of calories derived from animal protein and fat combined with a poor diet in fruit, vegetables high in carotenoids and green leaf vegetables fosters the occurrence of some cancers, including, e.g. non-Hodgkin lymphoma, colon, prostate, endometrium and breast cancers [490,491]. Fruits and vegetables are rich in antioxidants such as vitamin C, vitamin E, carotenoids, natural flavonoids and other compounds able to remove oxidant species, thus providing a diet-dependent protection system for our organism [492,493]. The World Health Organization attributed insufficient consumption of fruits and vegetables to 19% of the stomach, 20% of the oesophagus, 12% of the lung and 2% of colorectal cancer cases worldwide [494].

New genomic technologies have made possible the investigation of nutritional modulation of the carcinogenesis pathway with nutrients, micronutrients and phytochemicals. Modern studies of nutrient-modulated carcinogenesis involve exploring the effect of nutrients on DNA damage and repair mechanisms. DNA methylation and other epigenetic changes, which influences gene expression and cellular phenotypes; antioxidant redox state and oxidative stress; target receptors and signal transduction pathways; cell cycle controls and check points; cell death mechanisms; and anti-angiogenic processes.

Developments in nutritional genomics, proteomics and metabolomics will facilitate to simultaneously elucidate the biological effects of dietary constituents on cell function and global gene expression. This generation of new knowledge on nutrient–gene interactions provides background for a research framework for diet and cancer prevention focused on identifying and developing new biomarkers for dietary intervention.

LPO products and cancer

While oxidative stress and LPO may play important roles in carcinogenesis, cancer cells are sensitive to the LPO products which are also mediators of oxidative stress. Among them of particular importance is 4-hydroxynonenal (HNE), which has toxic effects for the cancer cells, causing both necrosis and apoptosis, and is therefore considered as a second messenger of free radicals. On mouse colon epithelial cells, HNE has been shown to affect more Apc mutated cells, compared to normal cells, inducing therefore relatively selective apoptosis of these cells [495]. The highly reactive aldehyde 4-HNE is one of the major cytotoxic products of lipid peroxidation, but it could be found also in various tissues even under normal,

physiological conditions influencing proliferation and differentiation of the cells [70]. While physiological roles of 4-HNE have not been entirely clarified yet, this particular aldehyde seems to be an important cell growth regulating factor acting as a signalling molecule interacting with the growth regulating effects of various cytokines [71,496,497]. Unlike free radicals and other ROS, 4-HNE is able to remain stable, not metabolized, after binding to macromolecules, especially proteins. 4-HNE macromolecular conjugates might even be required for the biological activities of 4-HNE and could be detected by the use of monoclonal antibodies (Figure 13). However, one of the essential characteristics of the oxidative metabolism of tumour cells is their resistance to inducers of ROS, resulting in a relative oxidative homeostasis ('steady-state') of endogenous lipid-peroxidation products mostly below the normal values [498]. That is also why some tumour cells could be entirely '4-HNE-free', while other tumours develop in spite of abundant 4-HNE, as can be seen in Figure 12. It is interesting that dividing liver cells in partially-hepatectomized rats accumulate alpha-tocopherol during DNA replication, suggesting that a decrease of susceptibility to lipid peroxidation may be a feature of normal cell division rather than a specific feature of cancer cells [499]. In agreement with that are findings of the increased production of GSH, another key anti-oxidative defence system, in the liver of partially hepatectomized animals [500] and the association of 4-HNE with hepatitis and liver carcinogenesis [501]. Interestingly, the appearance of 4-HNE in the usual form of protein adducts that are membrane-associated in normal cells, even in case of inflammation [502], is transformed into the nuclear membrane appearance in the case of liver pathologies [501]. Similar nuclear association of HNE-protein adducts was recently observed also in ulcerogenic changes in human gastric mucosa [503,504], also indicating persistent oxidative stress and lipid peroxidation accruing in chronic inflammation as in cancer. In this respect, the pro-inflammatory cytokines granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor α (TNFα) and interleukin-1 (IL-1) are of particular interest. Namely, the gene promoter region encoding for these cytokines contain a binding site for the nuclear factor kappa B (NF-κB), a cytosolic 'gene enhancer' protein which is activated by ROS [466], while 4-HNE was denoted to be one of the key factors regulating activity of cytokines involved in various inflammatory and fibrogenic processes [505]. Similarly, oxidative stress activates also potent gene activator protein 1 (AP-1). AP-1 activates the genes encoding for the fibrogenic growth factors (cytokines TGFβ and PDGF, which are activated through AP-1) that promote mesenchymal growth leading to the formation of blood vessels and connective tissue necessary for inflammation and for cancer development. 4-HNE, as a second messenger of ROS (free radicals), activates AP-1, followed by further TGFβ synthesis and fibrogenesis [506]. Hence, inflammation and cancer could be considered as interfering processes which share two common pathophysiological mechanisms: the cytokine network and oxidative

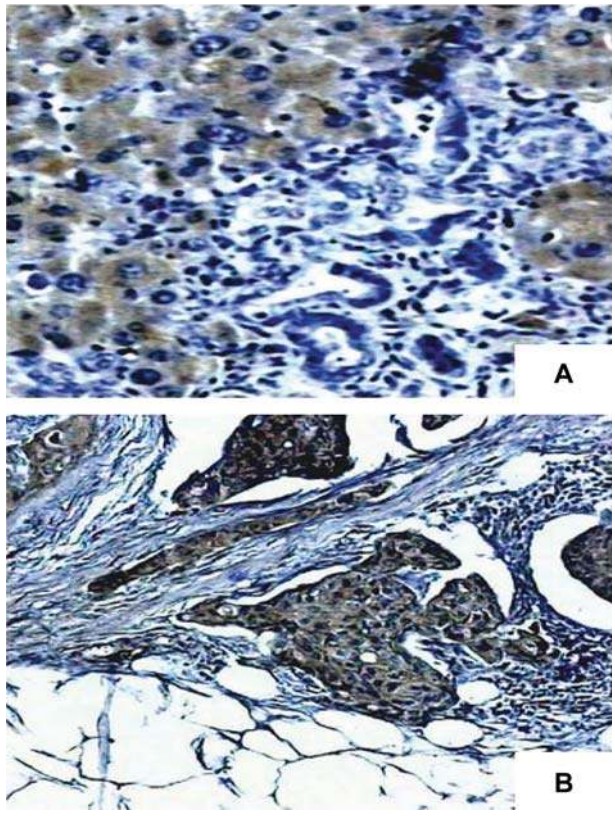

Figure 13. Immunochemical detection of the HNE-histidine adducts. Immunochemical detection of the HNE-histidine adducts in different carcinoma tissues done by specific monoclonal antibodies. The monoclonal antibodies were developed by G. Waeg's group. from culture medium of the clone 'HNE 1g4', produced by a fusion of Sp2-Ag8 myeloma cells with B-cells of a BALB-c mouse immunized with HNE-modified keyhole limpet haemocyanine (501). The antibody is specific for the HNE-histidine epitope in HNE-protein conjugates. HNE lysine and HNE cysteine give 5% and 4% cross-reactivity with HNE Ig4. Visualization of the immunochemical reaction was done by peroxidase labelled secondary antibody (rabbit-anti mouse) with DAB (3, 3-diaminobenzidine tetrahydrochloride) staining giving brown immunopositive reaction, with haematoxylin blue contrast staining. The slides were prepared at Rudjer Boskovic Institute (Zagreb, Croatia) and analysed by Professor K. Zarkovic at Clinical Hospital Centre of the Medical Faculty (Zagreb). (A) Immunohistochemical analysis of LEC rat liver carcinoma. The unique model of LEC rats that accumulate copper (done by Dr F. Gueraud et al., INRA, Toulouse, France) allows monitoring the experimental liver carcinogenesis associated with LPO. The liver carcinoma tissue is entirely negative for HNE (blue), while surrounding liver tissue is strongly HNE-immunopositive (brown) (magnification 400×). (B) Immunohistochemical analysis of human mammary carcinoma. This particular carcinoma has very pronounced stromal component, i.e. connective tissue, within which malignant cells are spread. As can be seen carcinoma cells are strongly positive for HNE (brown), while connective tissue and fat tissue of the mammary gland (lower part of the photo) are entirely negative for HNE (magnification 150×).

stress, while 4-HNE could at the same time promote fibrogenesis and inhibit the cancer growth.

The molecular mechanisms of the growth modifying effects of 4-HNE include the c-fos gene [507]. 4-HNE may induce an increased binding activity of the AP-1 transcription factor [506]. The c-Fos protein (product of the c-fos gene) is a mammalian transcription factor which acts by forming the heterodimer AP-1 with the transcription factor c-Jun. It is generally assumed that the existence of heterodimeric factors is important for fine tuning of transcriptional control. The corresponding genes, c-fos and c-jun, belong to the early response genes, whose induction can occur with no intervening protein synthesis, but requires only the modification of pre-existing transcription modulators. Among the early response genes c-fos is the best characterized gene. Agents that are able to induce this gene include the epidermal growth factor (EGF) and the platelet-derived growth factor (PDGF) or plain serum. The c-fos gene is necessary for cell proliferation, because c-Fos can up-regulate the cell cycle by inducing cyclin D1 which is part of the 'master switch' of the cell cycle and was found to be crucial for the regulating roles of 4-HNE and TGFβ in colon carcinogenesis based on JNK up-regulation [508]. Since 4-HNE acts as bifunctional (stimulating as well as inhibiting) regulator of the c-fos expression that also modifies the effects of humoral factors which promote c-fos transcription resulting in an inhibition of cancer growth, it is likely that this aldehyde could have an essential role in 'turning inflammation against cancer'. In agreement with this are findings of association of 4-HNE and TGFβ deregulation (in particular affecting the R1 receptor of TGFbeta) [509].

However, 4-HNE is probably not the only LPO-generated reactive aldehyde involved in colon carcinogenesis, because acrolein seems to be involved in development of benign colon tumours and their transition to carcinoma and the spread of LPO from malignant into the surrounding non-malignant tissue in relation to the cancer progression [510]. The most recent findings suggest that acrolein might be in a similar way also associated with development of prostate carcinoma, yet, in this particular case, it should be noted that acrolein might also come from spermine/spermidine oxidation, not only as a product of LPO [511].

Further studies on association of LPO and cancer development might therefore lead to novel approaches in cancer therapy based on the biomodulation of cancer and the organism's response to it (tumour–host relationship). This approach will surely involve inflammation as a key pathway of the immune response to disease, antioxidants acting as biological response modifiers of oxidative homeostasis and finally bio-regulating, not only cytotoxic, LPO products and mediators of oxidative stress, such as 4-HNE.

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