Overview of oxidative stress findings in hepatic encephalopathy: From cellular and ammonium-based animal models to human data

D. Simic\textsuperscript{a,b,c}, C. Cudalbu\textsuperscript{a,b}, K. Pierzchala\textsuperscript{a,b,c,*}

\textsuperscript{a} CIBM Center for Biomedical Imaging, Switzerland
\textsuperscript{b} Animal Imaging and Technology, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland
\textsuperscript{c} Laboratory of Functional and Metabolic Imaging, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

**ABSTRACT**

Oxidative stress is a natural phenomenon in the body. Under physiological conditions intracellular reactive oxygen species (ROS) are normal components of signal transduction cascades, and their levels are maintained by a complex antioxidants systems participating in the in-vivo redox homeostasis. Increased oxidative stress is present in several chronic diseases and interferes with phagocytic and nervous cell functions, causing an up-regulation of cytokines and inflammation. Hepatic encephalopathy (HE) occurs in both acute liver failure (ALF) and chronic liver disease. Increased blood and brain ammonium has been considered as an important factor in pathogenesis of HE and has been associated with inflammation, neurotoxicity, and oxidative stress. The relationship between ROS and the pathophysiology of HE is still poorly understood. Therefore, sensing ROS production for a better understanding of the relationship between oxidative stress and functional outcome in HE pathophysiology is critical for determining the disease mechanisms, as well as to improve the management of patients.

This review is emphasizing the important role of oxidative stress in HE development and documents the changes occurring as a consequence of oxidative stress augmentation based on cellular and ammonium-based animal models to human data.

**ARTICLE INFO**

Keywords:
- Hepatic encephalopathy
- Central nervous system
- CNS and systemic oxidative stress
- Antioxidants

1. Introduction

Oxidative stress (OS) is believed to play a role in the pathogenesis of hepatic encephalopathy (HE). Under normal physiological conditions reactive oxygen and nitrogen species (ROS and RNS) (Table 1) are balanced by antioxidants scavenging systems, while a permanent increase in ROS and RNS levels result in OS being harmful on cell- and tissue-homeostasis [1–3]. These antioxidants are low molecular weight reductants such as glutathione (GSH) and ascorbate (Asc) which are accompanied by protein antioxidants such as superoxide dismutase (SOD) that reacts with superoxide anion (O$_{2}^\cdot$), catalase (CAT) and the peroxiredoxins (glutathione peroxidase (GPX)) that catabolize hydrogen peroxide (H$_2$O$_2$) [4–6]. There is growing evidence that the modulation of antioxidant levels in cells is inextricably linked to intracellular OS levels [2,6].

ROS are small messenger molecules that are normal components of signal transduction cascades during physiological processes, but when in excess are also involved in neurotoxicity and neurodegeneration [7–9].

The redox homeostasis is maintained by the balance between ROS generation and elimination by antioxidants (Fig. 1).

Ammonium (NH$_4^+$) is produced by metabolism of amino acid obtained from dietary proteins [10]. Liver plays an important role in ammonium detoxification and in oxidative stress regulation. Therefore, when liver fails, blood ammonium levels increase and its impaired clearance by the diseased liver leads to brain glutamine (Gln) accumulation, causing disturbance of central nervous system (CNS) functions [11–13]. In parallel, a significant decrease in two major liver antioxidant enzymes activity, CAT and SOD, was detected in cirrhotic liver of HE patients [14].

Albumin, which is synthesized by liver hepatocytes and rapidly excreted into the bloodstream, is a very abundant and important circulating antioxidant, accounting for more than 70% of serum’s free radical-trapping activity [15,16]. Albumin has been found to be decreased in cirrhotic patients and has been associated with the development of overt HE as well as risk factors associated with death [17–21]. Epidemiological studies have found that decreased albumin level together with irreversibly oxidized albumin (HNA2) are an independent

\textsuperscript{*} Corresponding author, CIBM Center for Biomedical Imaging, Switzerland.
E-mail address: katarzyna.pierzchala@epfl.ch (K. Pierzchala).

https://doi.org/10.1016/j.ab.2022.114795
Received 11 February 2022; Received in revised form 26 May 2022; Accepted 15 June 2022
Available online 24 June 2022
of the inflammatory process, as well as playing critical functions in the
and neurogenesis, promoting learning, memory, and cognition [27].

ROS facilitate the response to growth factor activation and the formation
to growth factor activation and the formation of the inflammatory process, as well as playing critical functions in the
the immune system and microorganisms eradication [28]. Polymorphonuclear neutrophils (PMN) are the most abundant circulating
insults by releasing a significant amounts of pro-inflammatory

Clinical studies have revealed that systemic inflammation is linked to
clinical studies have demonstrated that long-term human albumin infusion improves the
patients with hypoalbuminemia has been previously shown and was

Ammonium, hyponatremia, and inflammatory cytokines
have been shown to trigger a self-amplifying cycle between astrocyte
endothelial dysfunction and increase the leakiness of the blood
vessels/blood-brain barrier (BBB) [29–31].

Systemic and brain cytokines, in combination with endotoxins (i.e.,
will be activated, contributing to the inflammatory response

ROS and RNS produced during metabolism [5,91].

| Reactive oxygen species-ROS | Reactive nitrogen species-RNS |
|----------------------------|------------------------------|
| **Radicals**               |                              |
| Superoxide O₂              | Nitric oxide NO             |
| Hydroxyl radical NO        | Nitrogen dioxide NO₂        |
| Alkoxyl Radical RO         | Peroxynitrite ONOO⁻         |
| Peroxy Radical ROO         |                               |
| **Non-radicals**           |                              |
| Molecular oxygen O₂        | Peroxynitrite ONOO⁻         |
| Hydrogen peroxide H₂O₂     | Nitrosyl cation NO⁺         |
| Singlet oxygen O₂          | Nitrosyl anion NO⁻          |
| Singlet Ozone O₃           | Dinitrogen trioxide N₂O₃    |
| Organic (lipid) peroxide ROOH | Dinitrogen tetraoxide N₂O₄ |
| Hypochlorous acid HOCl     | Nitrous acid HNO₂           |
| Hypohomocyanate acid HOBr  | Peroxynitrous acid ONOOH    |

The lifetime of certain radicals is affected by the environment, i.e., the lifetime of NO in an air saturated solution may be a few minutes.

ROS, trigger immune mechanisms, which positively regulate neuroplasticity
and neurogenesis, promoting learning, memory, and cognition [27]. ROS facilitate the response to growth factor activation and the formation of the inflammatory process, as well as playing critical functions in the immune system and microorganisms eradication [28]. Polymorphonuclear neutrophils (PMN) are the most abundant circulating immune cells that participate in immune and inflammatory processes (host defense) by releasing a significant amounts of pro-inflammatory cytokines and ROS/oxidative burst. When in excess, these cause endothelial dysfunction and increase the leakiness of the blood vessels/blood-brain barrier (BBB) [29–31].

Clinical studies have revealed that systemic inflammation is linked to neuroinflammation [32] and leads to cognitive deterioration in HE patients [33–35]. TNF-, IL-18, IL-6, endotoxins (i.e., NH₄⁺) levels have been shown to be significantly greater in the plasma of cirrhotic patients with HE [36]. In particular high levels of IL-6, which have been found in brains of BDL rat model of type C HE [2], are associated with ageing [27], the injury response of the CNS [37], impairment of the BBB integrity [38], memory and cognitive functions decline [27,39].

Systemic and brain cytokines, in combination with endotoxins (i.e., NH₄⁺), will set up an inflammatory cascade that exacerbates OS [40–43] and OS related activation of the astrocytes, and microglia [13,44]. Therefore, it is becoming increasingly evident that OS, both systemic and CNS is an important feature in the pathogenesis of HE [42,45]. In addition, impaired brain NH₄⁺ detoxification together with osmotic effect of Gln induces ROS generation [2,7,8,46]. It is worth noting that OS and inflammation are pathophysiological processes that are inextricably linked and interdependent [47]. It is widely accepted that in the presence of OS, inflammatory processes will develop, accelerating the progression of the disease. Similarly, if inflammation is the trigger, an OS response will be activated, contributing to the inflammatory response [48,51]. Ammonium, hyponatremia, and inflammatory cytokines have been shown to trigger a self-amplifying cycle between astrocyte osmotic stress and cerebral oxidative/nitrosative stress [41].

2. Oxidative stress

ROS and RNS are common byproducts of normal aerobic cellular metabolism and serve crucial physiological functions in intracellular cell signaling, homeostasis, cell death, immunological response to infections, and mitogenic response induction [3,54–58]. All metabolic processes involve the oxidant-antioxidant equilibrium required to perform routine molecular and biochemical functions [29,59]. The outcome of oxidative stress depends on the number of affected cells and functioning of the antioxidants systems (enzymatic and nonenzymatic antioxidants), i.e., concentrations of GSH, CAT, SOD, GPX, vit. C and vit. E [Fig. 1] [5,60].

Mitochondria respiratory chain form most of the ROS produced in the body. The superoxide anion (O₂⁻) is one of the most important free radical in biological systems involved in cell signaling. O₂⁻ is generated at the complexes I and III (Fig. 2), acts as a precursor for the synthesis of many other ROS (i.e., H₂O₂ and ONOO⁻), and lipid peroxides [61,62]. Approximately 80% of O₂⁻ is released into the intermembrane space, predictors of mortality [22–26]. The increase of mortality rate of HE patients with hypoalbuminemia has been previously shown and was accounted for 6.7–10.9% for short period of hospitalization and about 42.8–73.9% for the long-term follow up [17–21]. Recent studies demonstrated that long-term human albumin infusion improves the prognosis of cirrhotic patients by lowering the overall mortality and the likelihood of emergent hospitalizations [25,26].

When healthy the environmental/physiological factors, i.e., ROS, trigger immune mechanisms, which positively regulate neuroplasticity and neurogenesis, promoting learning, memory, and cognition [27]. ROS facilitate the response to growth factor activation and the formation of the inflammatory process, as well as playing critical functions in the immune system and microorganisms eradication [28]. Polymorphonuclear neutrophils (PMN) are the most abundant circulating immune cells that participate in immune and inflammatory processes (host defense) by releasing a significant amounts of pro-inflammatory
with most of remainder going to the mitochondrial matrix [63].

In addition, ROS (ie. O$_2^\cdot$) can be generated while endo/exogenous toxin detoxification by microsomal cytochrome-p450 conciliated hydroxylation. When in pathophysiological states the excess production and accumulation of ROS cause signaling pathways deterioration, overwhelm the antioxidant defense mechanisms capacity, cause the redox homeostasis imbalance, form OS state and became toxic [51,64]. Therefore, ROS can cause nonspecific damage, and participate in degeneration of essential cellular components like lipids, proteins, and DNA, potentially leading to cell senescence and death [51,64].

Elevated OS caused by increased levels of O$_2^\cdot$ induce oxidative damage to proteins, lipids, and nucleic acids, compromising cell health, and has been linked to ageing processes and various human pathogeneses such as the development of cancer, neurodegenerative, and cardiovascular diseases [65–67]. ROS are short-lived [68], they react rapidly with first line antioxidants, like GSH, Asc, and SOD [4,5,69,70]. GSH is a major antioxidant in the brain and its decline decrease the ability of CNS cells to counteract the OS and is a common sign in patients with neurodegenerative disorders [71]. Astrocytes synthesize more GSH than neurons and serve as a source of precursors for neuronal GSH synthesis [11,71].

ROS are critical for hippocampal long-term potentiation (LTP), a synaptic plasticity for learning and memory as well as in aging/disease-related impairment. Therefore, when in excess ROS leads to cellular dysfunction, and long-term depression (LTD) [7,8]. In the CNS the oxidative conditions are essential and play a key role in nerve growth factor (NGF) induced cell differentiation. Despite being the longest-living cell type, CNS cells are more vulnerable to OS-mediated injury because of their physiological and biochemical properties, high energy requirements, and unique redox activities:

(i) Neurons generate the highest rate of ROS and utilize ~20% of the oxygen consumed by the body – the majority of which is used for ATP production ($4 \times 10^{12}$ molecules/min) to maintain neuronal intracellular ion homeostasis.

(ii) Majority of the neuronal cells are nonreplicating therefore more sensitive to OS.

2.1. Approaches to measure oxidative stress

The choice of OS biomarkers and methods to assess the oxidative status in biological samples should be based on the aim of the study and the clinical relevance. This article does not intend to be a survey of detailed methods and assays for OS detection in biological samples, which have been described and reviewed elsewhere [2,50,60–62,65,68,74–91].

Oxidative stress presence may be tested in three ways:

(i) direct detection of ROS (in-vivo, in-vitro and ex-vivo living tissue); Direct detection seem to be the preferred method but depends on local antioxidants concentrations and clearance mechanisms [5,91]. Of note in-vivo steady-state concentrations of ROS range from pico-to nanomolar range [92], with the lifetimes span nanoseconds to seconds (Table 1).

(ii) detection of resulting damage to biomolecules (in-vitro and ex-vivo); Due to some of the challenges encountered by the direct detection some scientists prefer to use techniques based on the detection of final oxidation products and measure the damage on proteins, DNA, RNA, lipids, and other biomolecules.

(iii) detection of antioxidants concentrations, total antioxidants capacity, antioxidants activity (in-vitro and ex-vivo). This approach measures the activity of specific antioxidant enzymes, like CAT,

\[ \text{Neural membranes rich in polyunsaturated fatty acids (PUFA)} \]
\[ \text{are particularly vulnerable to OS, ie. oxidative damage to myelin.} \]

(iv) Modification of ion channels activity, disturbance in Ca$^{2+}$ traffic across neuronal membranes and its intracellular concentration increase often leads to OS.

(v) O$_2^\cdot$ is dismutated to H$_2$O$_2$ by SOD

(vi) H$_2$O$_2$ in the presence of intracellular iron, copper, or manganese ions, which accumulates in brain as a function of age, favors the Haber-Weiss vicious circle/Fenton chemistry, resulting in the generation of OH $^\cdot$, which is among the most active ROS (Fig. 1).

(vii) Antioxidant defense - brain contain low levels of Asc, GSH, CAT, GPX, and vitamin E [29,51,71–73].

---

**Antioxidants**

**Plasma antioxidants:**
- Vit. C, GSH, Uric acid, Bilirubin, Transferrin, Ceruloplasmin, β carotene

**Cell membrane antioxidants:**
- α tocopherol/vitamin E

**Intracellular antioxidants:**
- CuZn-SOD (SOD1), Mn-SOD (SOD2), Vit. C, GSH, CAT, GPX, GR

---

**Fig. 1.** Balance between ROS generation and elimination by antioxidants.
Fig. 2. A) Electron transport chain (ETC) in the mitochondrial intermembrane space (IMS) (adapted from the public domain image, author: LadyofHats). Electrons from NADH and FADH2 pass through the ETC and reduce O2 to form water at complex IV. ROS (O$_2^-$ & H$_2$O$_2$) are produced from the leakage of electrons to form O$_2^-$ at complexes I and III. O$_2^-$ is produced within the matrix at complex I, whereas at complex III it’s released towards both the matrix and IMS. O$_2^-$ is dismutated to H$_2$O$_2$ by superoxide dismutase 1 (SOD1) in the IMS and by SOD2 in the matrix. H$_2$O$_2$ is then fully reduced to water by glutathione peroxidase (GPX). B) Endogenous enzymatic defense systems of all aerobic cells: SOD, GPX, glutathione reductase (GR) and catalase (CAT) - they scavenge directly O$_2^-$ and H$_2$O$_2$ converting them to less reactive species. SOD dismutase O$_2^-$ to H$_2$O$_2$ which is rapidly converted by Fenton reaction into very reactive OH$^-$ radical. GPx neutralizes H$_2$O$_2$ by taking hydrogens from 2GSH molecules resulting in 2H$_2$O and one GSSG. GR then regenerates GSH from GSSG. CAT neutralizes H$_2$O$_2$ into H$_2$O.

Fig. 3. Oxidative stress detection methods: A) EPR in combination with nontoxic cell-permeable CMH spin probe – direct detection of O$_2^-$ in BDL rats [2]. B) Fluorescent microscopy - immunohistochemistry (IHC) of BDL rats brain tissue: Oxo-8-dG – DNA/RNA damage antibody accumulation – sign of elevated HO$^-$ and its interaction with a nucleobase. GPX antibody build-up – sign of an increased production of H$_2$O$_2$ [2]. C) $^1$H-MRS – in-vivo detection of two main antioxidants GSH and Asc – alteration of their concentrations represents an indirect evidence of oxidative stress.
SOD or GPX, and total antioxidant capacity. Furthermore, each individual marker reflects only partially the oxidative status, and therefore an integrative approach is necessary to achieve comprehensive conclusions.

In-vivo and ex-vivo OS detection is a complex task and require probes that are very sensitive, highly selective (i.e., HO⋅, O2−, NO⋅, ONOO−), and fast to react rapidly with ROS/RNS and create a stable secondary radical, which then can be quantified. Most of experimental assays for OS detection provide relative data. The extensively used approaches are electron paramagnetic resonance (EPR) spectroscopy allowing a real time direct detection of ROS, while fluorescence spectroscopy, microscopy, or flow cytometry can detect the final oxidation products or enzymatic activity [50,61,77]. In-vivo antioxidants (GSH and Asc) concentration measurements provide indirect evidence of redox homeostasis imbalance/OS and can be assessed non-invasively using proton magnetic resonance spectroscopy (1H-MRS) [13,93,94].

2.1.1. EPR

EPR is a method for direct detection of unpaired electrons. Free radicals are chemical molecules that have unpaired electron and are primarily formed from molecular oxygen. EPR in combination with nontoxic cell-permeable and resistant to antioxidants spin-traps (covalent bond with the radical by addition reaction) or spin probes (oxidized by ROS without binding) have been recognized as one of the most powerful and exclusive technique that allow direct and reliable detection of the ROS presence in the system under study being less uncertain as compared to other methods (i.e.: immunoassays or UV–Vis spectroscopy) [50,61,75] (Fig. 3A). EPR of sable secondary radicals (paramagnetic/EPR active) formed by adding exogenous spin-traps/probes (diamagnetic/EPR non-active) provides direct information about a variety of biological samples, i.e., living tissue, blood, and other body fluids (in-vitro, ex-vivo in room temperature and frozen) redox state in an accurate, rapid and quantitative manner by the generation of stable nitroxide radicals [50,65,74,95–97].

Individual traps/probes typically exhibit a high selectivity for specific species, allowing for modification of sensitivity to a given radical (i.e., HO⋅, O2−, H2O2, NO⋅, ONOO−) and identification of the species present in the system [79]. EPR studies of the cyclic hydroxylamine spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) revealed that it detects a 10-fold lower O2− radical level than other spin-traps/probes [50,61,79,82,98]. Furthermore, the high cell permeability allows it to detect both extracellular and intracellular ROS production in a reproducible manner, assess antioxidant and drug effects, providing superior specificity and sensitivity for quantification [2,50,61,75,82,98].

2.1.2. Spectrophotometric assays

Involving ultraviolet and colorimetric assays, are based on the interaction of reactive species with redox compounds and change in absorbance [74,99]. Spectrophotometry has found a widespread use in biomedical research, but, like chemiluminescence probes, it does not provide details about ROS/RNS and its localization within the cell (i.e., extracellular or intracellular) unless various probes and/or inhibitors with differing compartmentalization are used [99].

Remarks: Among most extensively applied spectrophotometric assays are: (i) GSH/GSSG as the one of most powerful self-generated antioxidant in the body [100,101] and the (ii) malonaldehyde (MDA) as a marker of lipid peroxidation [102,103].

(i) The major issue with blood ex-vivo GSH and GSSG concentration measurements is the oxidation of GSH after sample collection, and exaggeration of oxidation by increased formation of ROS while the acid deproteinization process. Therefore, leading to an overestimation of GSSG and an underestimating of GSH concentrations and the GSH/GSSG ratio [81]. To address this issue, the N-Ethylmaleimide (NEM) blocking agent, which forms stable, covalent thiether bonds with sulfhydryls, and prevent the formation of disulfide bond, needs to be added immediately to the blood sample after collection [81,100,101].

(ii) MDA is the most commonly used biomarker of OS, which results from lipid peroxidation of polyunsaturated fatty acids [103]. The most often used method for determining MDA in biological fluids is the thiobarbituric acid reactive substances (TBARS) test [68,104]. TBARS test suffer from numbers of limitations. MDA may be produced by other than lipid peroxidation reactions [105] and the false increase could be generated by the heating step of the assay [68]. EDTA treated samples as well as samples stored at −20 °C without addition of antioxidants significantly increase the TBARS levels [102,103,106]. Therefore, the main concerns are: non-specificity of TBA reactivity on MDA (other aldehydes cross-reaction produced from lipid peroxidation), poor reproducibility of analytical results, sample preparation/procedural modifications, storage, and stability of MDA standard solutions [102,103]. Therefore, an expert panel should re-evaluate MDA as an OS biomarker and a validated analytical process should be created [102,103].

2.1.3. The fluorescent redox probes

Are based on sensing mechanisms, and are classified as reaction-based selective probes, and reversible probes which can respond to multiple oxidation-reduction cycles [77]. The advanced fluorescence detection gives the possibility for real-time measurements of large number of samples (plate reader) with high sensitivity, spatial resolution, and specificity [77]. The pattern of oxidation/reduction-induced fluorescence change is crucial in determining the biological significance of a probe. Therefore, it is important to evaluate the probe mechanism: signal enhancement during reduction (nitrooxide-based probes - probing of hypoxia or antioxidants effectiveness) or oxidation (most other classes of probes - ROS/RNS activities) [77]. The fluorescence microscopy enables probe localization (cytology/histology - sub-cellular organelles, proteins, and membrane-components) (Fig. 3B).

However, for measuring in-vitro/ex-vivo living tissue, the intracellular fluorescent probes concentration is critical for correct interpretation of the results. The fluorescent product formation will depend on experimental conditions and probe internalization [107,108], the formation of multiple nonspecific oxidation products, light sensitivity, and probe redox cycling [78]. Therefore, it is critical to recognize these limitations in order to avoid erroneous interpretations.

2.1.4. Proton magnetic resonance spectroscopy (1H-MRS)

Is the only technique able to measure a large number of metabolites simultaneously in-vivo in a non-invasive manner [13,93,94]. Around 19 metabolites can be measured by combining ultra-short echo time localization sequences with an ultra-high magnetic field (≥7T) as also shown in type C HE [13]. As such several processes involved in HE can be studied in-vivo and longitudinally: energy metabolism (lactate (Lac), glucose (Glc), alanine (Ala), phosphocreatine (PCr), Cr), osmoregulation (taurine (Tau), Ins, Cr), myelination/cell proliferation (phosphocholine (PCho), glycerophosphocholine (GPC), phosphoethanolamine (PE), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG)) and neurotransmitters and metabolites involved in neurotransmission (glutamate (Glu), Gln, aspartate (Asp), glycine (Gly), γ-aminobutyrate (GABA)) [13].

As shown in Fig. 3C the two main antioxidants GSH and Asc can also be measured by 1H-MRS in the brain. Of note Asc and GSH are present in low concentration with significant resonance overlap, thus special editing sequences or ultra-high magnetic fields are required [13,70,76,94,109–111]. Changes in these metabolites represent an indirect evidence of OS. Therefore, additional techniques that allows the precise quantification of tissue ROS and identification of different ROS species.
would contribute significantly to the understanding of the pathophysiology of type C HE and potentially offer insight into therapeutic options. As shown above, there are several methods and assays for redox biomarkers detection, making it difficult to select the relevant and most appropriate one. Pros and pitfalls of abovementioned methods are provided in Table 2.

3. Oxidative stress in hyperammonemia

*In-vitro* (primary cultures), *ex-vivo* (after intraperitoneal (ip) perfusion) and *in-vivo* (microdialysis) studies showed that high concentrations of ammonium (5–12 mM) are involved in oxidative stress related neurotoxicity (Table 3) [45,112–119]. High concentration of ammonium has been associated with the rise in cellular glutamine synthetase (GS) and synthesis of Gln [45,112].

Administration of high concentrations of ammonium 5–12 mmol/kg (intraperitoneally) or 60 mM (intrastrially) in rats caused acute intoxication and increased generation of free radicals [120,121]. Despite the significant increase in cellular GSH levels, but not mitochondrial, both in cultured astrocytes as well as in microdialyzed cerebral cortex, ROS production was not blocked, suggesting that the increased ROS by ammonium reached levels that were beyond the antioxidant capacity [115,120,122]. Therefore, ROS may induce DNA damage mediated by activation of NMDA receptors [123].

Microglial ROS production increased with ammonium time exposure and in a dose-dependent manner (0.25–5 mM). Treatment with apocynin completely abolished the ammonium induced ROS increase, indicating activation of NADPH-oxidase by ammonium, and therefore suggesting that the OS increase is due to ROS not the reactive nitrogen species (RNS) [112,118].

Cortical mitochondria of hyperammonemic rats exhibited significant O$_2^-$, XO and lipid peroxidation increase accompanied by GPX, SOD2, CAT and GSH depletion, while the plasma CAT increased its activity [121,123–125].

Ammonium-induced RNA oxidation confirmed by Oxo-8-dG accumulation [45,113,114]. Among the oxidized RNA species, mRNA coding for the glutamate/aspartate transporter (GLAST) were identified [113].

4. Oxidative stress in type B and C HE animal models

Over the last several years researchers have demonstrated that oxidative stress is a key factor implicated in the pathogenesis of type B and C HE (Table 4) in animal. Indirect detection of OS using biochemical markers on BDL rats brain homogenates [59,127], as well as direct *in-vivo* brain and *ex-vivo* living brain tissue [2,13] have shown the presence of a strong correlation between chronic liver disease induced HE and OS.

The BDL rat model of type C HE has been shown to support the important role of oxidative stress in pathogenesis of HE by increased CNS OS (O$_2^-$) already at week 2 post-surgery [2], and at week 4 post surgery both, CNS and systemic OS [2,13,127]. GSH and ascorbate (Asc) have been shown to be the first line antioxidants which reacts rapidly with ROS to counteract the deleterious effects of OS [4,5]. GSH and Asc decline in brains of BDL rats correlated with OS increase suggesting an increased ROS scavenging in response to increased production [2,13,127]. Elevated levels of SODs have also been observed in response to increased endogenous and exogenous O$_2^-$ in the same animal model at 4 weeks post-surgery [2]. The nuclear translocation of SOD1 for the glutamate/aspartate transporter (GLAST) were identified [113].

In primary astrocytes, ammonium-induced RNA oxidation has been detected by Oxo-8-dG accumulation [45,84,113,114,128]. Recently a significantly increased formation of Oxo-8-dG, with predominant cytoplasmic localization, was also shown in the hippocampus and cerebellum of BDL rats, indicating interaction of cytosolic and mitochondrial

| Method | Advantages | Disadvantages |
|--------|------------|---------------|
| EPR | - direct detection of free radicals | - ex-vivo – experimental conditions – tissue must be processed fast and in controlled conditions |
|        | - in-vitro and ex-vivo real-time measurements | - sample volume |
|        | - high quality spin-traps/probes that react with short-lived radicals and convert them to long-lived radicals | - blood sample influenced by hemolysis |
|        | - cell permeable and non-permeable spin probes | - if a free radical instantly reacts with a molecule other than the spin-trap/probe agent, no spin adduct is formed |
|        | - direct detection of ROS | - if a spin adduct decomposes, a new spin adduct may be generated |
|        | - single chemical reaction | |
| Spectrophotometry and fluorimetry (colorimetric, fluorimetric, and luminescence-based assays) | - kinetic analyses and real-time measurements of ROS/RNS - reaction products or damage | |
|        | - detection of antioxidants/pro-oxidant property of drugs or food | |
|        | - antioxidant status | |

Table 2

Advantages and drawbacks of methods for OS detection [2,50,60–62,65,68,74–91]. Of note, the concomitant usage of several techniques on the same sample is highly recommended and will lead to complementary information on OS.
nucleic acids rather than nuclear DNA with HO· [2].

It has been shown that several brain regions of BDL rats had impaired enzymes activity of mitochondrial respiratory chain together with increase in mitochondrial reactive ROS generation, and augmentation of lipids and proteins oxidation [127].

However, the PCA model of type B HE has demonstrated conflicting results. At 4 weeks post-surgery no significant differences in plasma ROS, xanthine oxidase (OX), GSH and GSH/GSSG ratio, and CAT levels or in CSF ROS levels have been found between PCA and PCA-sham rats [129,130]. In contrary, in brains of PCA rats at 6 weeks post-surgery ammonium induced an increased brain ROS generation and promoted carbonylation of proteins, together with increased lipid peroxidation [131]. These different results could be related to the use of different animal strains and the length of the experimental design.

In parallel it has been shown that systemic OS correlated significantly with blood NH₃ concentrations [2] in BDL rats. Increase of ROS was represented by increased production of O₂·−, H₂O₂, increased activity of XO and decreased activity of CAT [2,129,130]. Significantly larger amount of O₂·− production in BDL PMN and LYM as compared to the healthy sham controls [2] indicated high resting oxidative burst and hypo-responsivity to the bacterial challenge [2,132].

5. Oxidative stress in human HE and in patients at risk of HE developing

The patients’ diagnoses were determined based on their medical history, physical and clinical examination, laboratory testing (MELD and Child Pugh scores), standardized battery of neuropsychological tests (i.e., the West Haven mental status scale), and histology [112,133-141]. There are few studies on CNS OS in patients with chronic liver disease and chronic liver disease associated HE (Table 5). Evidence of CNS OS presence in HE arose first from the postmortem EM observation of lenticular nuclei [142]. Alzheimer type II astrocytes, the integral neuropathological aspect of HE [143], contain large amounts of lipofuscin pigment aggregates [142] produced by peroxidation of unsaturated fatty acids [144,145]. Further, the IHC of the cerebral cortex of cirrhotic patients with HE supported the presence of oxidative stress in pathogenesis of HE [112]. As such increased expression of neuronal nitric oxide synthase (nNOS) has been accompanied by increased expression of SOD [112], a direct indication of OS presence. The induced RNA oxidation has been detected by increased accumulation of Oxo-8-dG, a direct sign of HO• [84,112,128]. Furthermore, recent studies supported the link between the OS related lipid peroxidation [141,142] and brain atrophy/the grey matter volume (GMV) loss [141].

Liver biopsies of NAFLD patients indicated increased OS accompanied by decreased antioxidant capacity (SOD, GSH and CAT) [14,112].

A significant disorder in redox status was also detected in HE patients’ blood, indicating systemic OS involvement in the disease progression, evidenced by significant enhancement of plasma/serum lipid peroxidation, protein carbonylation together with reduction in antioxidant capacity, i.e., significant decrease of albumin, SOD, CAT and GPX enzymatic activity [14,112,134-137,139,140].

Similarly, to the animal model studies the isolated PMN cells of cirrhotic patients demonstrated significantly higher resting state oxidative burst along with reduced phagocytic capacity [132,136], which was correlated with higher risk of multiple infections and organ failure [136].

6. Oxidative stress consequences

It is now well accepted that OS is implicated in various neurodegenerative disorders, causes neurofilaments (NFL) phosphorylation and leads to proteins aggregates formation [147,148]. To date little is known about OS involvement in neurodegeneration in patients with HE.

Excessive lipofuscin pigment aggregates, a sign of lipids peroxidation, have been detected in the Alzheimer type II astrocytes of the lenticular nuclei of HE patients [142]. OS accelerate the lipofuscin accumulation [144,145,149,150]. Lipofuscin is composed primarily of protein, lipid peroxides, and transition metals [150]. Lipofuscin aggregates at high concentration undergo Fenton reaction, resulting in the generation of OH−, one of the most active ROS [144], which in consequence may generate additional lipid, protein and RNA/DNA oxidation [141,142,151-153] and therefore lead to brain atrophy [141].

Astrocytes protect neurons against the ROS toxicity, by supplying them with the GSH precursors [71,154,155]. Therefore, it has been shown that inhibition of cysteine uptake into astrocytes under chronic ammonium exposure [156] may lead to reduced astrocyte and neuronal GSH levels [14,59,112,127], and place both at risk for oxidative damage.

The loss of glial filaments and dendritic spines of pyramidal neurons, hippocampal and cortical as shown in the BDL rat models [13,96,157], may be related to aforementioned oxidative modification of RNA and may thereby provide another link between OS and ammonium toxicity [2,45,112-114,118,158]. RNA oxidation interferes with translational machinery, gene expression, thereby providing the link between ammonium-induced OS and cognitive decline through impaired protein synthesis [158] and neurotransmission [67].

Filaments loss can be also induced by OS related protein depolymerization [159]. Moreover, ammonium intoxication has been shown to lead to protein nitrotyrosylation and nitric oxide (NO•) overproduction in in-vitro and human brain studies [45,112,114,116,118,123]. NO out-compete with SOD and react with O₂•−, leading to powerful and toxic peroxinitrite (ONOO−) formation, which may lead to DNA damage [123], NFL and actin nitration and disrupt filaments assembly [160]. Similarly, the actin cytoskeleton is a primary target of OS in hepatocytes [161]. Therefore, OS-induced cytoskeleton morphological alterations in hepatocytes will have a negative impact on hepatobiliary functioning.

Postmortem IHC of cortex of NAFLD patients have demonstrated an increase expression of a small heat shock protein-27 (Hsp27) [112], a direct sign of OS presence. During oxidative stress, Hsp27 has an increase expression of a small heat shock protein-27 (Hsp27) [112], a direct sign of OS presence. During oxidative stress, Hsp27 has an increase expression of a small heat shock protein-27 (Hsp27) [112], a direct sign of OS presence. During oxidative stress, Hsp27 has an increase expression of a small heat shock protein-27 (Hsp27) [112], a distress signal to the cells. Oxidative stress, like Hsp27 has an increase expression of a small heat shock protein-27 (Hsp27) [112], a distress signal to the cells. Oxidative stress, like Hsp27 has an increase expression of a small heat shock protein-27 (Hsp27) [112], a distress signal to the cells. Oxidative stress, like
Table 3

Summary of some published work highlighting the presence of oxidative stress in hyperammonemia models.

| Subject | Type of measurements | Method | Findings OS detection | Comments |
|---------|----------------------|--------|-----------------------|----------|
| Rat – hyperammonemia - NH₄CH₃CO₂ (7 mmol/kg)(coma in ~30 min) | Ex-vivo: 15 min after ip perfusion | Mitochondria extraction from forebrain or hemisphere and liver, cytosolic fraction | Blood RBC and plasma | GPX UV-Vis and fluorescence spectroscopy -Enzyme activity |
| Rat (Wistar rats - 200~220 g) – hyperammonemia - NH₄CH₃CO₂ (12 mmol/kg) (coma in ~12 min) | Acute ammonium intoxication | Brain mitochondria (non-specific to region) | Ref |

### Table 3 – Continued

- **O₂⁻**¹ 118%, (a.u.): HA 3.64 ± 0.55 vs. control 1.635 ± 0.15, p < 0.01
- **H₂O₂** 31%, (a.u.): HA 0.18 ± 0.06 vs. control 0.26 ± 0.03, p < 0.01
- **GPX** activity 47%, (nmol/min/kg protein): HA 31.5 ± 9.8 vs. control 61.25 ± 7, p < 0.001
- **Gr** activity (nmol/min/mg protein): HA 1.5 ± 0.4 vs. control 1.25 ± 0.4, p < 0.001
- **Ca** activity (a.u.): HA 3.9 ± 0.5 vs. control 2.93 ± 0.24, p < 0.001
- **XDH XO** activity (a.u.): HA 7.5 ± 0.6 vs. control 10.66 ± 0.5, p < 0.001
- **XDH(XO) ratio** indicating conversion of XDH to XO (a.u.): HA 2.1 ± 0.36 vs. control 3.75 ± 0.45, p < 0.001
- **AO** activity (a.u.): HA 7 ± 0.4 vs. control 42.5 ± 5, p < 0.001
- **MAO-A & B** activity (a.u.): HA 1.53 ± 0.07 vs. control .096 ± 0.01, p < 0.001

**Brain cells nuclei**

- **NAD⁺** ↓ 55%, (pmol/mg protein): 13.7 ± 2.9 before and 14.9 ± 1.7, 11.7 ± 2.5 and 6.2 ± 1.5 at 5, 8 and 11 min after ammonium injection
- **O₂⁻**² ↑ (nmol/min/mg protein): HA 0.46 ± 0.08 vs. control 0.27 ± 0.55, p < 0.05

**Brain**

- **GPX₃**, 38% decrease in cytosol and 47% in mitochondria, (nmol/min/mg protein): cytosol - HA 8 ± 9 vs. control 130 ± 30 p < 0.05, mitochondria - HA 16 ± 8 vs. control 30 ± 4 p < 0.05
- **GR** no change, (nmol/min/mg protein): cytosol - HA 41 ± 5 vs. control 45 ± 7, mitochondria - HA 14 ± 2 vs. control 19 ± 1
- **SOD₁** 40% in cytosol and 37% in mitochondria, (U/mg protein): cytosol - HA 6 ± 0.9 vs. control 10 ± 0.9 p < 0.05, mitochondria - HA 2.7 ± 0.1 vs. control 4.3 ± 0.0 ns
- **CAT** 52% in cytosol and 58% in mitochondria, (s⁻¹/mg protein): cytosol - HA 5.5 ± 0.42 vs. control 11 ± 1.8 p < 0.05, mitochondria - HA 1.55 ± 0.15 vs. control 3.7 ± 1.8 p < 0.05

**Liver**

- **GPX₃**, 24% in cytosol and 35% in mitochondria, (nmol/min/mg protein): cytosol - HA 1040 ± 100 vs. control 1370 ± 160 p < 0.05, mitochondria - HA 212 ± 32 vs. control 328 ± 22 p < 0.05
- **GR** no change, (nmol/min/mg protein): cytosol - HA 103 ± 6 vs. control 109 ± 10, mitochondria - HA 51 ± 11 vs. control 51 ± 7
- **SOD₁** 32% in cytosol and 41% in mitochondria, (U/min/mg protein): cytosol - HA 36 ± 3 vs. control 53 ± 46 p < 0.05, mitochondria - HA 4.7 ± 0.8 vs. control 8 ± 0.9 p < 0.05

(continued on next page)
Table 3 (continued)

| Subject | Type of measurements | Method | Findings OS detection | Comments |
|---------|---------------------|--------|-----------------------|----------|
| Rat - hyperammonemina - NH₄Cl (5 mM and 10 mM) | In-vitro: Primary cultures of astrocytes from neonatal cerebral cortex, In-vivo: brain microdialysis - cortex | UV-Vis and fluorescence spectroscopy, Enzyme activity | ROS GSH GSSG SOD | − CAT↓ 42% in cytosol and 27% in mitochondria, (s⁻¹/mg protein): cytosol - HA 0.18 ± 0.016 vs. control 0.31 ± 0.03 p < 0.05, mitochondria - HA 0.8 ± 0.09 vs. control 1.1 ± 0.08 p < 0.05 RBC - GPX ↓ 25%, (μmol/min/ml of cells): HA 94 ± 23 vs. control 125 ± 23 p < 0.05 - GR no change, (μmol/min/ml of cells): HA 66 ± 16 vs. control 67 ± 12 - SOD ↓ 69%, (U/min/ml of cells): HA 286 ± 90 vs. control 932 ± 62 p < 0.05 - CAT↑ 29%, (s⁻¹/ml of cells): HA 1.7 ± 0.1 p < 0.05 Plasma - GPX no change, (nmol/min/ml): HA 5690 ± 600 vs. control 5330 ± 200 - GR no change, (nmol/min/ml): HA 38 ± 4 vs. control 38 ± 5 - SOD no change, (nmol/min/ml): HA 1.6 ± 0.1 vs. control 1.5 ± 0.1 - CAT↓ 88%, (s⁻¹/ml x 10⁵): HA 3.0 ± 0.8 vs. control 1.6 ± 0.1 p < 0.05 Lipid peroxidation - MDA ↑, mediated by activation of NMDA receptors: cytosol - HA 70 ± 4 vs. control 55 ± 2 (nmol/mg protein)p < 0.001, mitochondria - HA 3.8 ± 0.3 vs. control 2.8 ± 0.2 (nmol/mg protein)p < 0.001 - Blocking NMDA receptors with MK-801 prevents ammonium-induced oxidative stress, XDH to XO conversion and MAO-A activation | [115] [117], [122] Acute ammonium intoxication - Ammonium → ROS ↑, dose dependent - Ammonium → GSH ↑: HA – 3 days NH₄Cl 5 mM 21.4 ± 3.8 nmol/mg protein in cells and 24.1 ± 3.6 μmol/mg cell protein in medium vs. control 13.9 ± 2.6 nmol/mg protein in cells and 11.6 ± 2.7 μmol/mg cell protein in medium, the increase is facilitated by activation of the uptake of the GSH precursor cystine - GSH ↑: 10 mM NH₄Cl for 48 h-80% - SOD supplementation → ROS ↓ in astrocytes exposed to NH₄Cl 5 mM - In vivo, intracerebral administration of ammonium via a microdialysis probe → GSH ↑ in the brain extracellular space → dependent on undisturbed GSH synthesis in astrocytes - basal GSH⁺ concentrations in microdialysates from rat’s striatum were about 10-fold higher than from prefrontal cortex: 1.32 ± 0.54 μM vs. 0.097 ± 0.04 μM and 40 min after infusion 3.7 ± 0.53 μM vs. 0.185 ± 0.05 μM - Ammonium promotes GSH synthesis, export from astrocytes, increases its extracellular degradation → supporting GSH synthesis in neurons. - GSH/GSSG ratio ↓ | [120] Acute ammonium intoxication - HO ↑ contents in microdialysates - HA: 2.5-fold ↑ - HO ↑ contents in microdialysates – NMDA 1 mM infusion: 2-fold ↑ - Taurine (85 mM) co-administration (‘ammonium + Tau’) → the NMDA-induced HO ↑ |
| Rat - hyperammonemina - NH₄Cl (60 mM) – microdialysis → extracellular concentration – 5 mM | In-vivo: brain microdialysis - cortex | Liquid chromatography | HO | |

(continued on next page)
| Subject | Type of measurements | Method | Findings | Comments | Ref |
|---------|----------------------|--------|----------|----------|-----|
| Rat/mice – hypoosmolality, hyperammonemia - NH₄Cl (5 mM) | In-vitro: cultured rat astrocytes - from the cortices of cerebral hemispheres of newborn Wistar rats | Immunostaining | ROS | Acute ammonium intoxication | [116] |
|         | Ex-Vivo: brain slices (cortex) from male mice (adult) | Fluorescence microscopy Western Blot Analysis | | ammonia stimulates ROS ↑ | |
|         | | | | ROS ↑ → NH₄Cl 5 mM →-3-fold ↑ | |
|         | | | | ROS ↑ → NH₄Cl 5 mM + apomycin (300 μM) →-1.5-fold ↑ | |
|         | | | | involvement of NADPH oxidase in hypoosmotic ROS production | |
|         | | | | NADPH oxidase inhibitor apocynin almost completely prevents hypoosmotic ROS production | |
|         | | | | ROS ↑ with decreasing osmolarity →-1-fold ↑ → increased cellular O₂, absent in presence of apocynin | |
|         | | | | astroglia ROS may affect the function of neighboring neurons | |
| Rat, mouse – hyperammonemia - NH₄Cl (0.2–5 mM) | In-vitro: cultured rat astrocytes - from the cortices of cerebral hemispheres of newborn rats isolated RNA | RNA oxidation | Acute ammonium intoxication | [113] |
|         | In-vivo/Ex-vivo: rats brain Ex-vivo: mice brain slices 400–500 μm | UV-Vis and fluorescence spectroscopy RNA Oxidation HIC - confocal laser-scanning microscopy North-Western andSlot Blot Analysis - isolated RNA | | RNA oxidation ↑ in primary astrocytes - 6 h NH₄Cl 0.5 mM →-1.5-fold ↑ and 6 h NH₄Cl 1 mM →-2.5-fold ↑, p ≤ 0.05 | |
|         | | | | RNA oxidation ↑ - cortical mouse brain slices - 6 h NH₄Cl 5 mM →-1.6-fold ↑, p ≤ 0.05 | |
|         | | | | Ammonium-induced RNA oxidation is reversible in-vivo | |
|         | | | | Among the oxidized RNA species, 18S-rRNA and the messenger RNA (mRNA) coding for the glutamate/aspartate transporter (GLAST) were identified | |
| Rat – hyperammonemia - NH₄Cl (5 mM) | In-vitro: cultured rat astrocytes - from the cortices of cerebral hemispheres of newborn Wistar rats | UV-Vis and fluorescence spectroscopy Confofal laser-scanning and epifluorescence microscopy | RNA oxidation | Acute ammonium intoxication | [45] |
|         | | | NO and RNOS production/inhibition | | |
| Rat – hyperammonemia - NH₄Cl(O) 4C1O2 (4.5 mmol/kg) (coma in ~15 min) Astrocytes/microglia - NH₄Cl (0.25–5 mM) | In-vitro: cultured rat astrocytes/microglia - from the cortices of cerebral hemispheres of newborn Wistar rats Ex-Vivo: brain slices (cortex) | Fluorescence microscopy Western Blot | ROS production/inhibition iNOS | Acute ammonium intoxication | [118] |
|         | | | | microglia ROS ↑: time/dose-dependent manner | |
|         | | | | basal/control microglia ROS – not reported | |
|         | | | | microglia ROS ↑ NH₄Cl 1 mM vs. 5 mM (a. u.): 3 h-0.99 ± 0.05 vs. 1.12 ± 0.01, 6 h-1.36 ± 0.02 vs. 1.67 ± 0.05, 20 h-1.56 ± 0.15 vs. 2.92 ± 0.05, p ≤ 0.05 | |
|         | | | | ammonium up-regulates ionized Ca²⁺ - binding adaptor protein-1 | |
|         | | | | pretreatment of microglia with apocynin completely abolished the ammonium induced ROS increase → activation of NADPH-oxidase by ammonium | |
|         | | | | oxidative stress ↑ is due to ROS ↑, but not RNS | |
|         | | | | iNOS ↑ in cultured astrocytes | |
|         | | | | iNOS was not affected by ammonium acetate treatment in vivo in the cerebral cortex | |

HA – hyperammonemia.

* Values approximation – estimation from graphs.
Table 4
Summary of some published work highlighting the presence of oxidative stress in type B and C HE animal models.

| Subject | Type of measurements | Method | Findings OS detection | Comments | Ref |
|---------|----------------------|--------|-----------------------|----------|-----|
| Wistar rats, weighing 250–320 g: BDL – 5- and 10-days post-op | Plasma ammonium: NA | Ex-vivo: cerebral cortex from the frontal lobe, midbrain, and the cerebellum | UV–Vis spectroscopy enzymatic activity | Thiol Redox State: GSH, GSSG Lipid Peroxidation (MDA) | Cortex (μmol TRS component/gram protein): GSH: Sham 7.25 ± 0.35 vs. 5d 5.16 ± 0.28 vs. 10d 2.13 ± 0.45, p < 0.05; GSSG: Sham 0.34 ± 0.02 vs. 5d 0.61 ± 0.09 vs. 10d 0.20 ± 0.02, p < 0.05; and the GSH/GSSG ratio ↓ – 50% | [59] |
| | | | | - lipid peroxidation (μmol MDA/mg protein): Sham 0.130 ± 0.05 vs. 5d 0.134 ± 0.00 vs. 10d 0.246 ± 0.02, p < 0.05 | |
| | | | | Midbrain (μmol TRS component/gram protein): GSH: Sham 3.13 ± 0.13 vs. 5d 2.85 ± 0.29 vs. 10d 1.59 ± 0.28, p < 0.05; GSSG: Sham 0.23 ± 0.01 vs. 5d 0.39 ± 0.06 vs. 10d 0.25 ± 0.15, p < 0.05; GSH/GSSG ratio ↓ – 46% | |
| | | | | - lipid peroxidation (μmol MDA/mg protein): Sham 0.34 ± 0.02 vs. 5d 0.61 ± 0.09 vs. 10d 0.20 ± 0.2, p < 0.05 | |
| | | | | - cerebral cortex | | |
| | | | | - spontaneous oxidative burst ↑ in neutrophils (%) control 8 ± 1.9 vs. ammonium-fed 31 ± 5.3, p < 0.001 | [132] |
| | | | | - brain ROS ↑: 2.5-fold | |
| | | | | - lipid peroxidation ↑ (μmol/mg protein): Sham 0.020 ± 0.010 vs. PCA 0.060 ± 0.020, p < 0.01 | [131] |
| | | | | - protein oxidation ↑ (nmol/mg protein): Sham 2.7 ± 0.4 vs. PCA 3.9 ± 0.4, p < 0.01 | |
| | | | | - BDL Plasma: | [129] |
| | | | | - ROS[H₂O₂|XO] | |
| | | | | - SSAP MAO A + B CAT SOD GR GPX GSH/GSSG Lipid peroxidation (MDA) | |
| | | | | - ROS(μF:): Sham 0.15 ± 0.04 vs. BDL 5.49 ± 1.93, (p < 0.001) | |
| | | | | - H₂O₂: 2.4-fold (μM): Sham 3.35 ± 0.53 vs. BDL 8.02 ± 1.20, (p < 0.001) | |
| | | | | - CAT ↓ 40% → increased activity of XO, (U/ml): Sham 323.4 ± 56.2 vs. BDL 126.5 ± 19.7, (p < 0.01) | |
| | | | | - lipid peroxidation ↑ (μM): Sham 9.87 ± 0.62 vs. BDL 41.91 ± 7.40, (p < 0.001) | |
| | | | | - antioxidant defense ↓ | |
| | | | | - GSH ↓ 44% (mM): Sham 1.37 ± 0.04 vs. BDL 0.77 ± 0.09, (p < 0.001) | |
| | | | | - GSH/GSSG ratio ↓ 2.3-fold (p < 0.05) | |
| | | | | - GR ↑ 4.5-fold, (mU/ml): Sham 9.23 ± 1.02 vs. BDL 42.2 ± 8.94, (p < 0.001) | |
| | | | | - XO ↑ (mU/ml): Sham 6.46 ± 0.75 vs. BDL 30.47 ± 5.04, (p < 0.001) | |
| | | | | Brain: | |
| | | | | - GR ↑ 2-fold (mU/100 μg protein): Sham 0.39 ± 0.03 vs. BDL 0.79 ± 0.03, (p < 0.001) | |
| | | | | - CAT levels was found between PCA and PCA-sham rats or in CSF ROS levels | |
| | | | | - no significant difference in plasma and brain ROS and XO, CAT levels was found between PCA and PCA-sham rats or in CSF ROS levels | |
| | | | | - no significant difference in plasma GSH and GSH/GSSG ratio | |

(continued on next page)
| Subject | Type of measurements | Method | Findings | OS detection | Comments | Ref |
|---------|----------------------|--------|----------|--------------|----------|-----|
| Male Wistar rats weighing between 220 and 250 g – BDL - 4 weeks post-op, brains were removed - cortex, hippocampus, striatum, and cerebellum - homogenized | Ex-vivo: mitochondria – 4w post-op, brains were removed - cortex, hippocampus, striatum, and cerebellum - homogenized | UV–Vis and fluorescence spectroscopy | NADH Succinate Dehydrogenase Cytochrome c Oxidase Lipid peroxidation (MDA) Protein carbonyls ROS GSH/GSSG | - no significant differences in levels of lipid peroxidation Both: | - SOD activity, in both plasma and frontal cortex, showed no significant difference in either of the experimental groups - plasma GR activity ↑ only in of BDL - frontal cortex GR activity ↑ in both BDL and PCA - No changes in GP and MAO A + B activity between the two experimental groups - in both plasma and frontal cortex. Mitochondria Cortex | [127] |
| Male Wistar rats weighing between 220 and 250 g – BDL - 4 weeks post-op, brains were removed - cortex, hippocampus, striatum, and cerebellum - homogenized | Ex-vivo: mitochondria – 4w post-op, brains were removed - cortex, hippocampus, striatum, and cerebellum - homogenized | UV–Vis and fluorescence spectroscopy | NADH Succinate Dehydrogenase Cytochrome c Oxidase Lipid peroxidation (MDA) Protein carbonyls ROS GSH/GSSG | - no significant differences in levels of lipid peroxidation Both: | - SOD activity, in both plasma and frontal cortex, showed no significant difference in either of the experimental groups - plasma GR activity ↑ only in of BDL - frontal cortex GR activity ↑ in both BDL and PCA - No changes in GP and MAO A + B activity between the two experimental groups - in both plasma and frontal cortex. Mitochondria Cortex | [127] |
| Male Wistar rats weighing between 220 and 250 g – BDL - 4 weeks post-op, brains were removed - cortex, hippocampus, striatum, and cerebellum - homogenized | Ex-vivo: mitochondria – 4w post-op, brains were removed - cortex, hippocampus, striatum, and cerebellum - homogenized | UV–Vis and fluorescence spectroscopy | NADH Succinate Dehydrogenase Cytochrome c Oxidase Lipid peroxidation (MDA) Protein carbonyls ROS GSH/GSSG | - no significant differences in levels of lipid peroxidation Both: | - SOD activity, in both plasma and frontal cortex, showed no significant difference in either of the experimental groups - plasma GR activity ↑ only in of BDL - frontal cortex GR activity ↑ in both BDL and PCA - No changes in GP and MAO A + B activity between the two experimental groups - in both plasma and frontal cortex. Mitochondria Cortex | [127] |

(continued on next page)
Table 4 (continued)

| Subject Description                  | Type of measurements | Method                  | Findings OS detection                                                                 | Comments                                                                 | Ref |
|--------------------------------------|----------------------|-------------------------|----------------------------------------------------------------------------------------|--------------------------------------------------------------------------|-----|
| Male Wistar rats weighing between 220 and 250 g − BDL Plasma ammonium: 133 ± 75 μM | In vivo: BDL − 2, 4, 6, 8- and 8-weeks post-op, hippocampus | 1H-MRS, Asc | - GSH ↓ 27%, (mmol/mg protein): Sham 0.26 ± 0.02 vs. BDL 0.19 ± 0.01, p < 0.05
- GSSG ↑ 150%, (mmol/mg protein): Sham 0.02 ± 0.002 vs. BDL 0.05 ± 0.004, p < 0.05
- GSH/GSSG ↓ 70%, Sham 11.86 ± 1.46 vs. BDL 3.59 ± 0.38, p < 0.05
- NADH dehydrogenase ↓ 35%, (nmol NADH oxidized/min/mg protein): Sham 44.95 ± 2.23 vs. BDL 29.21 ± 5.18, p < 0.05
- Succinate dehydrogenase ↓ 53%, (nmol succinate oxidized/min/mg protein): Sham 20.23 ± 2.56 vs. BDL 9.42 ± 1.92, p < 0.05
- Cytochrome c oxidase ↓ 64%, (nmol cytochrome c oxidized/min/mg protein): Sham 3.97 ± 0.54 vs. BDL 1.41 ± 0.51, p < 0.05
- ROS ↑, (pmol DCF/mg protein): Sham 1.56 ± 0.12 vs. BDL 5.19 ± 0.25, p < 0.05
- lipid peroxidation ↑, (nmol of MDA/mg protein): Sham 28.92 ± 0.61 vs. BDL 52.92 ± 3.69, p < 0.05
- protein carbonyls ↓, (nmol/min/mg protein): Sham 118.44 ± 1.87 vs. BDL 183.9 ± 15.58, p < 0.05
- GSH ↓ 38%, (mmol/mg protein): Sham 0.37 ± 0.02 vs. BDL 0.23 ± 0.01p < 0.05
- GSSG ↓ 67%, (mmol/mg protein): Sham 0.03 ± 0.002 vs. BDL 0.05 ± 0.002, p < 0.05
- GSH/GSSG ↓ 63%, Sham 12.62 ± 1.14 vs. BDL 4.60 ± 0.44, p < 0.05
- NADH dehydrogenase ↓ 43%, (nmol NADH oxidized/min/mg protein): Sham 60.07 ± 7.89 vs. BDL 34.35 ± 1.54, p < 0.05
- Succinate dehydrogenase ↓ 51%, (nmol succinate oxidized/min/mg protein): Sham 32.79 ± 4.60 vs. BDL 15.88 ± 1.44, p < 0.05
- Cytochrome c oxidase ↓ 64%, (nmol cytochrome c oxidized/min/mg protein): Sham 6.11 ± 0.83 vs. BDL 2.19 ± 0.61, p < 0.05
- GSH ↓ 27%, (mmol/mg protein): Sham 0.26 ± 0.02 vs. BDL 0.19 ± 0.01, p < 0.05
- GSSG ↑ 150%, (mmol/mg protein): Sham 0.02 ± 0.002 vs. BDL 0.05 ± 0.004, p < 0.05
- GSH/GSSG ↓ 70%, Sham 11.86 ± 1.46 vs. BDL 3.59 ± 0.38, p < 0.05
- NADH dehydrogenase ↓ 35%, (nmol NADH oxidized/min/mg protein): Sham 44.95 ± 2.23 vs. BDL 29.21 ± 5.18, p < 0.05
- Succinate dehydrogenase ↓ 53%, (nmol succinate oxidized/min/mg protein): Sham 20.23 ± 2.56 vs. BDL 9.42 ± 1.92, p < 0.05
- Cytochrome c oxidase ↓ 64%, (nmol cytochrome c oxidized/min/mg protein): Sham 3.97 ± 0.54 vs. BDL 1.41 ± 0.51, p < 0.05 | [13] |

| Male Wistar rats weighing between 220 and 250 g − BDL Plasma ammonium: 88.2 ± 40.8 μM | Ex vivo: brain tissue − cortex, hippocampus, striatum, thalamus, and cerebellum In vitro: whole blood and neutrophils | ESR, UV-Vis and fluorescence, Spectroscopy, Epifluorescence microscopy - IHC, NBT, RNA oxidation | - O2− production ↑, (μmol/g/min): week-2 Sham 39.68 ± 11.31 vs. BDL 64.82 ± 13.34 (+63%, p < 0.01); week-4 Sham 66.84 ± 15.19 vs. 96.05 ± 22.98 BDL (+43%, p < 0.001); week-6 Sham 73.49 ± 16.31 vs. BDL 103.64 ± 19.09 (-41%, p < 0.01); week-8 Sham 81.51 ± 7.29 vs. BDL 111.01 ± 26.07 (+36%, p < 0.05)
- SOD1 ↓, granular layer: week-4 +87.36% (p < 0.001), week-8 +114.41% (p < 0.001) | CNS OS is an early event in type C − 2 weeks post-op | [2] |

CNS OS precede systemic OS
- CNS OS ↓ is due to enhanced formation of intra- and extra-cellular ROS rather than due to reduced antioxidant capacity
- a pattern of intracellular O2− increase in SHAM rats confirms a substantial increase of OS with age

Hippocampus
- O2− production ↑, (μmol/g/min): week-2 Sham 39.68 ± 11.31 vs. BDL 64.82 ± 13.34 (+63%, p < 0.01); week-4 Sham 66.84 ± 15.19 vs. 96.05 ± 22.98 BDL (+43%, p < 0.001); week-6 Sham 73.49 ± 16.31 vs. BDL 103.64 ± 19.09 (-41%, p < 0.01); week-8 Sham 81.51 ± 7.29 vs. BDL 111.01 ± 26.07 (+36%, p < 0.05)
- SOD1 ↓, granular layer: week-4 +87.36% (p < 0.001), week-8 +114.41% (p < 0.001) | CNS OS is an early event in type C − 2 weeks post-op | [2] |

(continued on next page)
| Subject | Type of measurements | Method | Findings OS detection | Comments |
|---------|----------------------|--------|-----------------------|----------|
| Cerebellum | | | | |
| - O$_2^-$ production (μmol/g/min): week-6 | | | | |
| Sham 96.2 ± 27.4 vs. BDL 136.8 ± 22.7 ± 19.09 (+42%, p < 0.001) | | | | |
| - SOD1 ↑: granular layer: week-4 + 99.05% (p < 0.001), week-8 + 249.31% (p < 0.001) | | | | |
| - SOD2 ↑: granular layer: week-4 + 22.69% (p < 0.001), week-8 + 31.31% (p < 0.001) | | | | |
| Cortex | | | | |
| - SOD1 ↑: week-4 + 66.29% (p < 0.001), week-8 + 93.55% (p < 0.001) | | | | |
| - SOD2 ↑: week-4 + 44.40% (p < 0.001), week-8 + 96.99% (p < 0.001) | | | | |
| Striatum | | | | |
| - SOD1 ↑: week-4 + 73.71% (p < 0.001), week-8 + 115.62% (p < 0.001) | | | | |
| - SOD2 ↑: week-4 + 4.81% (ns), week-8 + 41.35% (p < 0.001) | | | | |
| Thalamus | | | | |
| - SOD1 ↑: week-4 + 73.71% (p < 0.001), week-8 + 115.62% (p < 0.001) | | | | |
| - SOD2 ↑: week-4 + 4.81% (ns), week-8 + 41.35% (p < 0.001) | | | | |
| - Protective role of SOD1 – translocation into the nucleus | | | | |

**Blood**

- Systemic OS ↑ at week 4 post-BDL
- O$_2^-$ production ↑ + resting oxidative burst ↑
- O$_2^-$ production in blood (μmol/mL/min):
  - Sham 11.07 ± 0.74 vs. week-2 12.85 ± 2.58 (ns) vs. week-4 13.44 ± 2.89 (ns) vs. week-6 16.52 ± 2.89 (p < 0.01) vs. week-8 17.52 ± 3.38 (p < 0.01)
- O$_2^-$ production ↑ at week-8 post-BDL by peripheral PMN – 300%, (p < 0.001), LYM – 112%, (p < 0.006) compromise their functions → continuous neutrophil activation cause hypo-responsivity → phagocytic capacity ↓

**Plasma:**

- GSSG ↓, (μM): BDL 0.76 ± 0.16 vs. Sham 2.42 ± 0.38, p < 0.005
- GSH ↓, (μM): BDL 3.48 ± 0.47 vs. Sham 7.10 ± 1.07, p < 0.005

(continued on next page)
In vitro studies of cultured astrocytes exposed to Gln revealed an increase in OS [165]. Increased ammonium and Gln concentration together with Asc decline correlated significantly with OS increase in BDL rats [2,13]. In addition, the same study demonstrated an elevated expression [2,112] acts as a switch to modulate microglial activation/oxidative burst [34,37,49,53,118,166]. SOD2 overexpression in CNS astrocytes [2] can trigger the proinflammatory cascade as well as increased ROS formation [34,37,49,53,118,166]. SOD2 overexpression [2,112] acts as a switch to modulate microglial activation/inactivation during inflammation [167]. Increase of O$_2^-$ production and overexpression of SOD will lead to an increase of H$_2$O$_2$ concentrations having a negative impact on LTP [7,168,169].

OS and inflammation are strongly linked and interdependent pathophysiological processes [47,170]. In the presence of OS, inflammatory processes will occur, hence assisting in the progression of OS. In parallel, if inflammation is the trigger, an OS response will be generated, contributing to the immune response [48,49]. Previous studies have demonstrated the synergic participation of CNS OS which precedes the systemic OS [2], and inflammation in the progression of HE [2,96,135,138].

Bacterial infection and especially spontaneous bacterial peritonitis is a frequent precipitating factor in HE that contributes to the systemic inflammation [171,172]. Leukocytosis, a hallmark of systemic inflammation and indirect sign of OS, associated with an increased risk of mortality, was identified in both, the BDL rat model [2] and in HE patients [173,174]. Accelerated immune response (increased levels of TNF-$\alpha$, IL-18, IL-6) [24,36] and the oxidative metabolism/oxidative burst of phagocytic cells (neutrophil dysfunction and increased ROS emission) [2,33,129] at the inflamed site will advance tissue injury and immunopathology [175,176]. Furthermore, studies have indicated that high levels of IL-6 have a deleterious impact on the BBB integrity [38]. As a result, high levels of IL-6 in both the peripheral [36] and CNS [2] may increase the BBB permeability, allowing neurotoxic components (ROS/RNS, cytokines, NH$_3^+$, bile acids, bilirubin) to enter the brain and affect neurological functions.

Bile acids have the potential and ability of altering the gut microbiota, resulting in modifications of the total bile acid pool [177,178]. Increased bile acid concentrations have been shown to disrupt tight junctions, permeabilize the BBB via detergent-like cytolytic actions on cell membranes, gain access to the CNS, and contribute to neurological decline [179,180]. Bile acids have been also recognized as a pro-oxidants causing ROS release (interrupt electron transport chain at complex III), which may lead to depletion of antioxidants, thiols groups oxidation and lipid peroxidation [181,182]. Furthermore, bile acids may cause indirectly increase of OS through resident macrophages activation/oxidative burst [183].

The enteric nervous system connects the gut microbiome to the CNS and acts as a key communication route for the gut-liver-brain axis mediated by the activity of the vagal nerves [184]. Therefore, bacterial infection and systemic inflammation may also impact brain function through afferent vagal nerves activation by cytokines/chemokines release at the inflammatory sites [185,186] and impact the cognitive and motor functions [184]. Therefore, OS being related to innate inflammation is a common denominator and therapeutic target for many neurodegenerative disorders associated with cognitive deficits [11,50-53].

Serum albumin accounts for a significant component of total extracellular antioxidant capacity (∼70%). The reduced cysteine residue (Cys34) and a thiol group in serum albumin allow to scavenge the HO and ONOO$^-$ respectively [16,24,171,187-190]. The hypoalbuninemia was shown to increase the mortality risk in HE patients [17-21]. Therefore, a significant decrease of plasma albumin concentrations, an important extracellular antioxidant, and increase in the percentage of oxidized albumin [25,26,140,190] in HE patients will lead to decreased systemic antioxidative capacity and contribute to increase of OS. Furthermore, albumin binds several molecules reversibly, allowing for solubilization and transport (i.e., bilirubin, bile acids, hormones, and endotoxins), controls the immune system, and protects the endothelium [16,24,171,187-190]. In liver cirrhosis bilirubin and bile acids concentrations rise [191,192], while binding capacity of albumin is impaired [140,190,193]. Therefore, decreased albumin synthesis by the diseased liver together with decreased binding capacity will decrease toxin clearance [190], increase of lipid peroxidation [171] and therefore lead to increase of OS, both systemic and CNS, and patient clinical status deterioration.

7. Conclusions

Taken together, the presented findings show that OS is a critical

### Table 4 (continued)

| Subject | Type of measurements | Method | Findings OS detection | Comments |
|---------|---------------------|--------|-----------------------|----------|
| - GSSG/GSH | BDL 0.26 ± 0.04 vs Sham 0.22 ± 0.05, ns |
| Brain: * | CD (nmol/mg protein): BDL 4.74 ± 0.26 vs. Sham 4.49 ± 0.26, ns |
| LP1 (nmol/mg protein): BDL 1.45 ± 0.14 vs. Sham 2.07 ± 0.286, ns |
| MDA(nmol/mg protein): BDL 0.6 ± 0.08 vs. Sham 0.3 ± 0.02, p < 0.001 |
| Heart: * | CD (nmol/mg protein): BDL 0.73 ± 0.15 vs. Sham 0.66 ± 0.14, ns |
| LP1 (nmol/mg protein): BDL 0.32 ± 0.07 vs. Sham 0.21 ± 0.04, p < 0.05 |
| MDA(nmol/mg protein): BDL 0.09 ± 0.005 vs. Sham 0.06 ± 0.003, p < 0.01 |
| Kidney: * | CD1 (nmol/mg protein): BDL 0.68 ± 0.03 vs. Sham 0.38 ± 0.04, p < 0.05 |
| LP1 (nmol/mg protein): BDL 1.43 ± 0.33 vs. Sham 0.63 ± 0.06, p < 0.05 |
| MDA(nmol/mg protein): BDL 0.6 ± 0.06 vs. Sham 0.33 ± 0.03, p < 0.05 |

* Ammonium measured before sacrifice.
* Values approximation – estimation from graphs, DN - dentate nucleus.
Table 5
Summary of some published work highlighting the presence of oxidative stress in humans with chronic liver disease and chronic liver disease associated HE or at risk to develop HE. In these studies, HE has been assessed by standardized battery of neuropsychological tests.

| Subject | Type of measurements | Method | Findings OS detection | Comments | Ref |
|---------|----------------------|--------|-----------------------|----------|-----|
| NAFLD patients (steatosis (S) and steatohepatitis (SH)) (age 18–55 y) | Liver biopsies 2cm²/liver homogenates and plasma | UV-VIS Spectroscopy | SOD | Liver | [14] |
| Plasma ammonium: NA | | | CAT | | |
| | | | GPX | | |
| | | | GSH | | |
| | | | Protein carbonyls | | |
| | | | | protein carbonyls [(nmol carbonyls/mg protein)]: S 4.88 ± 0.96 (–403%) and SH 1.77 ± 1.12 (188%) vs. healthy 1.04 ± 0.18, p < 0.05 | |
| | | | 1280% increase in the ratio of hepatic protein carbonyl/GSH content (nmol carbonyls/nmol): S 0.48 ± 0.10 vs. control 0.0375 ± 0.007, p < 0.05 | |
| | | | SOD [(U/mg protein)]: S 12.9 ± 1.49 (–48%) and SH 8.68 ± 0.5 (–64%) vs. healthy 24.8 ± 1.49, p < 0.05 | |
| | | | CAT [(U/mg protein)]: S 0.56 ± 0.054 and SH 0.32 ± 0.04 (–48%) vs. healthy 0.62 ± 0.01, p < 0.05 | |
| | | | GSH [(nmol/mg protein)]: S 11.65 ± 2.28 (±57%) and SH 0.32 ± 0.04 (±27%) vs. healthy 19.1 ± 1.49, p < 0.05 | |
| | | | GPX [(nmol/mg protein)]: S 0.084 ± 0.011 vs. SH 0.067 ± 0.06 vs. healthy 0.08 ± 0.03, ns | |
| Postmortem Brain Tissue - liver cirrhosis and HE | Ex-vivo: Brain - intersection parietal to occipital cortex (17 ±8 h (5–35 h)) | IHC - confocal laser-scan microscopy | RNA oxidation | | [112] |
| Plasma ammonium: NA | | | NOS | | |
| | | | SOD | | |
| | | | | HE but not cirrhosis triggers oxidative stress in the cerebral cortex | |
| | | | | INOS mRNA levels in the cerebral cortex = not significantly different between and patients with cirrhosis | |
| | | | | Oxidized RNA ↑ 2-fold increase vs. control and a small heat shock protein-27 (Hsp27) ↑ 4-fold increase vs. control → marker for oxidative stress | |
| | | | | neuronal nNOS ↑ and SOD ↑ in the cerebral cortex - non significantly | |
| | | | | GS activity ↓, but not the GS protein level | |
| Neutrophils from patients blood (age 55.3 ± 7.5y) | In-vitro: neutrophils | Flow cytometry | Oxidative burst | | [132] |
| Plasma ammonium: 74 ± 6 μM | Whole blood incubated with 75 μM NH₄Cl for 90 min and then neutrophils were isolated | | | | |
| Patients-hepatogenic coma (male, age 67 - biliary obstruction; male, age 42 - alcoholic cirrhosis) | Ex-vivo: Brain tissue postmortem - lenticular nuclei | Electron microscopy | Lipid peroxidation | | [142] |
| Plasma ammonium: NA | | | | Lipid peroxidation ↑ - excess of lipofuscin aggregates accumulated in astrocytes | |
| Patients - liver cirrhosis (alcoholic) (age 50y (range 25–60)) | In-vitro: blood samples – serum | CAT | | | [134] |
| Plasma ammonium: NA | | | GPX | | |
| | | | Lipid peroxidation | CAT [(pmol/mg protein): CLD 1.5 ± 0.2 vs. healthy 2.2 ± 0.2, p < 0.032 | |
| | | | | GPX [(nmol/mg protein): CLD 0.011 ± 0.002 vs. healthy 0.020 ± 0.003, p < 0.04 | |
| | | | | Lipid peroxidation [(nmol/mg protein): CLD 0.074 ± 0.007 vs. healthy 0.046 ± 0.005, p < 0.035 | |
| | | | | proteins content (Apo A1 and Apo B100) (mg/ mL): CLD 200 ± 88 vs. healthy 415 ± 64, p < 0.0024 | |
| Patients - liver cirrhosis and HE with high and normal systemic ammonemia (age 56.0 ± 10.4 y) | In-vitro: blood samples – serum | UV-VIS Spectroscopy | SOD | | [139] |
| Plasma ammonium: 35 ± 4 μM | | | GPX | | |
| | | | Lipid peroxidation (MDA) | Oxidative stress ↑ in HE → significant decrease of serum antioxidant enzymatic activity | |
| | | | | SOD ↓: healthy 1.35 ± 0.08 vs. CLD 0.90 ± 0.08 U/mL | |
| | | | | GPX ↓: healthy 0.093 ± 0.06 vs. CLD 0.006 ± 0.008 U/mL | |
| | | | | Lipid peroxidation ↓: healthy 35.94 ± 1.37 vs. CLD 68.90 ± 5.68 nmol/mL | |
| | | | | Systemic ammonemia ↑ in HE associated with the present antioxidant enzymes activity | |
| | | | | Compensatory increase in the activity of SOD and GPX in high ammonemia, as compared with HE patients bearing normal ammonemia | |

(continued on next page)
Table 5 (continued)

| Subject | Type of measurements | Method | Findings or OS detection | Comments | Ref |
|---------|----------------------|--------|--------------------------|----------|-----|
| Patients - Liver cirrhosis (alcoholic (A) and viral (V)) (Age 38-75 y) Plasma ammonium: NA | In-vitro: blood – plasma/serum and blood cell fractions were separated | UV-VIS Spectroscopy | Lipid peroxidation (MDA): CLD 23.7 ± 1.2 and CLD + AH 47.58 ± 2.75 (p < 0.001 vs. healthy and CLD) vs. healthy 10.6 ± 1.52 | - Protein carbonylation (%mol/mg protein): CLD 393 ± 133 vs. healthy 126 ± 47 | [137] |
| Patients - Liver cirrhosis and HE (non-alcoholic and alcoholic) (Age 59 ± 6 y) Plasma ammonium: 95-345 μM | In-vitro: blood – plasma | UV-VIS Spectroscopy | Fluorescence spectroscopy | Lipid peroxidation (MDA): CLD 23.7 ± 1.2 and CLD + AH 47.58 ± 2.75 (p < 0.001 vs. healthy and CLD) vs. healthy 10.6 ± 1.52 | - Protein carbonylation (%mol/mg protein): CLD 393 ± 133 vs. healthy 126 ± 47 | [137] |
| Patients with cirrhosis and alcoholic hepatitis (AH) (Age 50.3 ± 1.3 y) Plasma ammonium: NA | In-vitro: blood, neutrophils in a whole blood or after isolation | UV-VIS spectroscopy | Fluorescence-activated cell sorting | Lipid peroxidation (MDA): CLD 23.7 ± 1.2 and CLD + AH 47.58 ± 2.75 (p < 0.001 vs. healthy and CLD) vs. healthy 10.6 ± 1.52 | - Protein carbonylation (%mol/mg protein): CLD 393 ± 133 vs. healthy 126 ± 47 | [137] |
| Patients – chronic NALD (age 37.61 ± 2 y) Plasma ammonium: NA | Blood - RBC | UV-VIS Spectroscopy | SOD | Lipid peroxidation (MDA): CLD 23.7 ± 1.2 and CLD + AH 47.58 ± 2.75 (p < 0.001 vs. healthy and CLD) vs. healthy 10.6 ± 1.52 | - No significant differences were noted | [146] |
| Patients with MHE (Age 63 ± 10 y) Plasma ammonium: 117 μM | In-vitro: blood samples – serum | UV-VIS Spectroscopy | GSH/GSSG/GPX | Oxidative stress † | - Protein carbonylation (%mol/mg protein): MHE 0.186 ± 0.013 vs. healthy 0.150 ± 0.008 | [136] |
| Patients with cirrhosis (Age 54 ± 12 y) Plasma ammonium: NA | In-vitro: blood samples – plasma | Chromatography | Albumin: HMA, HNA1, HNA2 | Oxidative stress † | - Oxidative stress † | [136] |
| Patients with cirrhosis (Age 49.29 ± 9.48 y) Plasma ammonium: 35.53 ± 10.09 μM | In-vitro: MRI | Grey matter volume (GMV) Lipid peroxidation - serum MDA Albumin | MRI - voxel-based morphometry (VBM) | Lipid peroxidation (MDA): CLD 23.7 ± 1.2 and CLD + AH 47.58 ± 2.75 (p < 0.001 vs. healthy and CLD) vs. healthy 10.6 ± 1.52 | - increased serum MDA levels were associated with GMV loss in patients with CLD → negative correlation r = –0.378, p = 0.036 | [141] |

(continued on next page)
component of HE pathogenesis even at an early stage and that a lack of defense exacerbates CNS status. According to recent studies, CNS OS occurs before systemic OS, suggesting that increased BBB permeability in the latter stage of disease progression may play a substantial role and contribute significantly to the increase of ROS/RNS in the brain. Therefore, further work on cell culture, animal models and postmortem brain tissue of patients with type C HE is required to elucidate the relationship/synergy between OS, ammonium, Gln, inflammation and the pathogenesis of HE. Moreover, additional longitudinal, multi-parametric, and multimodal studies combining direct/indirect in vivo/ex-vivo/in-vitro techniques to study OS, brain regional differences in parallel with the relationship between OS, brain metabolic functional alterations, cellular changes, and neurological manifestations in HE are needed. Because of the complexity of oxidative damage within the CNS, identifying OS biomarkers in clinical samples of HE patients is critical for a better understanding of OS-induced processes (molecular mechanisms) and to develop an appropriate diagnostic strategies.

Despite significant research on oxidative stress related damage to the cells biomolecules (RNA/DNA, lipids, and proteins) and antioxidant status in biological samples, the literature on direct measurement of OS in clinical samples is limited, and screening and surveillance for OS biomarkers are not yet routine procedures in the healthcare sector. Furthermore, sample preparation should be done with caution to ensure sample stability and to reduce the possibility of oxidative damage to tissue/cells/biomolecules during collection. Because there is no gold standard for defining redox status, complementary techniques must be used when screening for OS biomarkers to eliminate methodological biases and to obtain clinically comprehensive (diagnostic and prognostic) information with high sensitivity and specificity to pathological alterations. It is critical to conduct a comprehensive panel analysis of both pro- and antioxidants, as well as inflammation biomarkers, which should be defined by the study’s goal and provide an overall redox state in specific conditions.

Authors’ contributions

KP, CC and DS have participated in conceptualization and manuscript preparation and have read and approved the final manuscript.

Acknowledgements

Graphical abstract: Created with BioRender.com.

Supported by the SNSF projects no 310030_173222 and 310030,201218. We acknowledge access to the facilities and expertise of the CIBM Center for Biomedical Imaging founded and supported by Lausanne University Hospital (CHUV), University of Lausanne (UNIL), École Polytechnique Fédérale de Lausanne (EPFL), University of Geneva (UNIGE) and Geneva University Hospitals (HUG).

References

[1] S.K. Bardaweel, et al., Reactive oxygen species: the dual role in physiological and pathological conditions of the human body, Eurainn J. Med. 50 (3) (2018) 193–201.

[2] K. Pierzchala, et al., Central nervous system and systemic oxidative stress interplay with inflammation in a bile duct ligation rat model of type C hepatic encephalopathy, Free Radic. Biol. Med. 178 (2022) 295–307.

[3] J. Roy, et al., Physiological role of reactive oxygen species as promoters of natural defenses, Faseb J. 31 (9) (2017) 3729–3745.

[4] J.A. C.A. P.W. A, D.T. E, Glutamine and ascorbate during ischemia and postischemic reperfusion in rat brain, J. Neurosci. 35 (5) (1980) 4.

[5] A. Phanindra, D.B. Jestadi, L. Periyasamy, Free radicals: properties, sources, targets, and their implication in various diseases, Indian J. Clin. Biochem. 30 (1) (2015) 11–26.

[6] T. Finkel, Signal transduction by reactive oxygen species, J. Cell Biol. 194 (1) (2011) 7–15.

[7] L.T. Knapp, K. Klann, Role of reactive oxygen species in hippocampal long-term potentiation: contributory or inhibitory? J. Neurosci. Res. 70 (1) (2002) 1–7.

[8] L.T.K.E. Knapp, Potentiation of hippocampal synaptic transmission by superoxide requires the oxidative activation of protein kinase C, J. Neurosci. 22 (3) (2002) 657–662.

[9] M. Schiefer, N.S. Chandel, ROS function in redox signaling and oxidative stress, Curr. Biol. 24 (10) (2014) R453–R462.

[10] D.S. D, Ammonia metabolism and the urea cycle function and clinical, J. Vet. Intern. Med. 8 (2) (1994) 73–79, Mar-Apr.

[11] M. Skowrotka, J. Albrecht, Oxidative and nitrosative stress in ammonia neurotoxicity, Neurochem. Int. 62 (5) (2013) 731–737.

[12] V. Lachmann, et al., Precipitants of hepatic encephalopathy induce rapid astrocyte swelling in an oxidative stress dependent manner, Arch. Biochem. Biophys. 536 (2) (2013) 143–151.

[13] O. Braissant, et al., Longitudinal neurometabolic changes in the hippocampus of a rat model of chronic hepatic encephalopathy, J. Hepatol. 71 (3) (2019) 505–515.

[14] L.A. Videlis, et al., Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients, Clin. Sci. (Lond.) 106 (3) (2004) 261–268.

[15] M.A. Rothschild, M. Oratz, S.S. Schreiber, Albumin synthesis, Int. Rev. Physiol. 21 (1980) 249–274.

[16] M. Roche, et al., The antioxidant properties of serum albumin, FEBS Lett. 582 (13) (2008) 1783–1787.

[17] Z. Bai, et al., Association of serum albumin level with incidence and mortality of overt hepatic encephalopathy in cirrhosis during hospitalization, Therap Adv Gastroenterol 12 (2019), 175628481981302.

[18] J. Mestamante, et al., Prognostic significance of hepatic encephalopathy in patients with cirrhosis, J. Hepatol. 30 (5) (1999) 890–895.

[19] J. Ficter, et al., Prognosis and 1-year mortality of intensive care unit patients with severe hepatic encephalopathy, J. Crit. Care 24 (3) (2009) 364–370.

[20] Y. Cui, et al., Establishment and evaluation of a model for predicting 3-month mortality in Chinese patients with hepatic encephalopathy, Metab. Brain Dis. 34 (1) (2019) 213–221.

[21] J. Cordoba, et al., Characteristics, risk factors, and mortality of cirrhotic patients hospitalized for hepatic encephalopathy with and without acute-on-chronic liver failure (ACLF), J. Hepatol. 60 (2) (2014) 275–281.

[22] P. Goldwasser, J. Feldman, Association of serum albumin and mortality risk, J. Clin. Epidemiol. 50 (6) (1997) 693–703.

[23] F.H. Yap, et al., Association of serum albumin concentration and mortality risk in critically ill patients, Anaesth. Intensive Care 30 (2) (2002) 202–207.

[24] M. Bernardi, et al., Albumin in decompensated cirrhosis: new concepts and perspectives, Gut 69 (6) (2020) 1127–1138.

[25] M. Di Pascali, et al., Long-term administration of human albumin improves survival in patients with cirrhosis and refractory ascites, Liver Int. 39 (3) (2019) 98–105.

[26] P. Caraceni, et al., Long-term albumin administration in decompensated cirrhosis (ANSWER): an open-label randomised trial, Lancet 391 (10138) (2018) 2417–2429.

[27] B. Yang, et al., Immune modulation of learning, memory, neural plasticity and neurogenesis, Brain Behav. Immun. 25 (2) (2011) 181–213.

[28] K.H. Krause, K. Bedard, NOX enzymes in immuno-inflammatory pathologies, Semin. Immunopathol. 30 (3) (2008) 193–194.

[29] M. Mittal, et al., Reactive oxygen species in inflammation and tissue injury, Antioxidants Redox Signal. 20 (7) (2014) 1126–1158.

[30] R. Pahwa, et al., Chronic inflammation, in: StatPearls Publishing. 2021 StatPearls Publishing Copyright © StatPearls Publishing LLC, Treasure Island (FL, 2021).

[31] J.M. Robinson, Reactive oxygen species in phagocytes leucocytes, Histochem. Cell Biol. 130 (2) (2008) 281–297.

[32] J. Wang, et al., Connection between systemic inflammation and neuroinflammation underlies neuroprotective mechanism of several phytochemicals in neurodegenerative diseases, Oxid. Med. Cell. Longev. 2018 (2018), 1992714.

[33] T.H. Tranah, et al., Systemic inflammation and ammonia in hepatic encephalopathy, Metab. Brain Dis. 28 (1) (2013) 1–5.

[34] J. Goltart, T.H. Tranah, B.L. Shawcross, Inflammation and hepatic encephalopathy, Arch. Biochem. Biophys. 536 (2) (2013) 189–196.

Table 5 (continued)

| Subject | Type of measurements | Method | Findings OS detection | Comments | Ref |
|---------|---------------------|--------|-----------------------|----------|-----|
| - oxidative damage may be involved in GMV loss | OS adversely affects GMV | - albumin (g/L): CLD 34.85 ± 7.08 vs. healthy 46.50 ± 8.35 | | | |

Values approximation – estimation from graphs.
D. Simic et al.

Analytical Biochemistry 654 (2022) 114795

E. Chroni, et al., Brain oxidative stress induced by obstructive jaundice in rats, C.P. Rubio, J.J. Ceron, Spectrophotometric assays for evaluation of Reactive Oxygen Species (ROS) in serum: general concepts and applications in dogs and humans, M. Erta, A. Quintana, J. Hidalgo, Interleukin-6, a major cytokine in the central nervous system, W. Droge, Free radicals in the physiological control of cell function, Biochim. Biophys. Acta 1822 (6) (2012) 831–842, A. Kumar, B. Yegla, T.C. Foster, Redox signaling in neurotransmission and human disease, Int. J. Biochem. Cell Biol. 39 (1) (2007) 44–59.

S.K. Biswas, Does the interdependence between oxidative stress and inflammation exist in the skin? Br. J. Dermatol. 164 (4) (2011) 749–759.

B. Halliwell, M. Whitman, Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br. J. Pharmacol. 142 (2) (2004) 231–255.

C.L. Hawkins, M.J. Davies, Detection, identification, and quantification of oxidative protein modifications, J. Biol. Chem. 294 (51) (2019) 19683–19708.

B. Veskoukis, et al., Spectrophotometric assays for measuring redox biomarkers in blood, Biomarkers 21 (3) (2016) 208–217.

S. Suzen, H. Gurer-Orhan, L. Sano, Detection of reactive oxygen and nitrogen species by electron paramagnetic resonance (EPR) technique, Molecules 22 (1) (2017).

K.S.B. Korkmaz, D. Gorenben, Detection of 8-OhdG as a diagnostic biomarker, J. Lab. Precis. Med. 3 (2018) 95. B.

A.K. Desgupta, K. Klein, Methods for measuring oxidative stress in the laboratory, in: Antioxidants in Food, Vitamins and Supplements, 2014, pp. 19–40.

S. H, Methods for measurements of increased release of free oxygen radicals, Forch Komplement. 2 (5) (1995) 254–258.

B. Palmieri, V. Bledowski, Oxidative stress tests: overview on reliability and use - Part I, Eur. Rev. Med. Pharmacol. Sci. 11 (5) (2007) 309–342.

E. Cadenas, K.J. Davies, Mitochondrial free radical generation, oxidative stress, aging, Free Radic. Biol. Med. 29 (3–4) (2000) 217–230.

D.H.I. Matsuwa, Magnetic resonance spectroscopy study of the antioxidant defense system in schizophrenia, Antioxidants Redox Signal. 15 (7) (2011) 2057–2065.

F. Bottino, et al., In Vivo brain GSH: MRS methods and clinical applications, Antioxidants 10 (9) (2021).

G. Cheng, et al., Detection of mitochondria-generated reactive oxygen species in cells using multiple probes and methods: potentials, pitfalls, and the future, J. Biol. Chem. 293 (26) (2018) 10363–10380.

K.S. Pierschalla, A. Sienkiewicz, S. Sessa, O. Braissant, V. McIn, R. Gruetter, C. Cudalbu, Hippocampal and Systemic Oxidative Stress in a Rat Model of Chronic Hepatic Encephalopathy, a Multifocal Approach, ISMRM, 2020, p. 2040. D.

V.Y.U. Rani, Free Radicals in Human Health and Disease, Springer, New Delhi, 2015.

S.I. Dikalov, et al., Mitochondrial reactive oxygen species and calcium uptake regulate activation of phagocytic NADPH oxidase, Am. J. Physiol. Regul. Integr. Comp. Physiol. 302 (10) (2012) R1334–R1412.

Chapter 6 - UV-vis absorption and chemiluminescence techniques, in: Frederick A. Villanella (Ed.), Reactive Species Detection in Biology, Elsevier, 2017, pp. 203–231.

P. Di Simplicio, et al., Blood glutathione disulfide: in vivo factor or in vitro artifact? Clin. Chem. 48 (5) (2002) 742–753.

D. Giustini, et al., Pitfalls in the analysis of the physiological antioxidant glutathione (GSH) and glutathione redox cycle (GSSG) in biological samples: an elephant in the room, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 1019 (2016) 21–28.

Khoubsaasab Jafari M, Ansarin K, and J.A. Commentary on “Use of Malondialdehyde and Nitrotyrosine as Biomarker for Assessing Oxidative and Nitrosative Damage in Different Disease Pathologies: a review”. Iran. J. Public Health. 2017; 46(4): 714-715.

M. Khoubsaasabjafari, K. Ansarin, A. Jouyban, Reliability of malondialdehyde as a biomarker of oxidative stress in psychological disorders, Biopsix 5 (3) (2015) 123–127.
[171] V. Arroyo, R. Garcia-Martinez, X. Salvatella, Human serum albumin, systemic inflammation, and cirrhosis, J. Hepatol. 61 (2) (2014) 396–407.
[172] N.S. Poudyal, et al., Precipitating factors and treatment outcomes of hepatic encephalopathy in liver cirrhosis, Cureus 11 (4) (2019) e4363.
[173] J. Vaquero, et al., Infection and the progression of hepatic encephalopathy in acute liver failure, Gastroenterology 125 (3) (2003) 755–764.
[174] N. Weiss, et al., Cerebrospinal fluid metabolomics highlights dysregulation of energy metabolism in overt hepatic encephalopathy, J. Hepatol. 65 (6) (2016) 1120–1130.
[175] P. Kruger, et al., Neutrophils: between host defence, immune modulation, and tissue injury, PLoS Pathog. 11 (3) (2015), e1004651.
[176] T. Narasaraju, et al., Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis, Am. J. Pathol. 179 (1) (2011) 199–210.
[177] E. Williams, C. Chu, S. DeMorrow, A critical review of bile acids and their receptors in hepatic encephalopathy, Anal. Biochem. 643 (2022), 114436.
[178] K.B.M.S. Islam, et al., Bile acid is a host factor that regulates the composition of the caecal microbiota in rats, Gastroenterology 141 (5) (2011) 1773–1781.
[179] M. McMillin, et al., Bile acid signaling is involved in the neurological decline in a murine model of acute liver failure, Am. J. Pathol. 186 (2) (2016) 312–323.
[180] M. Quinn, et al., Bile acids permeabilize the blood brain barrier after bile duct ligation in rats via Rac1-dependent mechanisms, Dig. Liver Dis. 46 (6) (2014) 527–534.
[181] R.J. Sokol, et al., Evidence for involvement of oxygen-free radicals in bile-acid toxicity to isolated rat hepatocytes, Hepatology 17 (5) (1993) 869–881.
[182] R.J. Sokol, et al., Role of oxidant stress in the permeability transition induced in rat hepatic mitochondria by hydrophobic bile acids, Pediatr. Res. 49 (4) (2001) 519–531.
[183] P. Ljubuncic, et al., Effect of deoxycholic acid and ursodeoxycholic acid on lipid peroxidation in cultured macrophages, Gut 39 (3) (1996) 475–478.
[184] Z. Chen, et al., The role of intestinal bacteria and gut-brain Axis in hepatic encephalopathy, Front. Cell. Infect. Microbiol. 10 (2020), 595759.
[185] J. Licinio, M.L. Wong, Pathways and mechanisms for cytokine signaling of the central nervous system, J. Clin. Invest. 100 (12) (1997) 2941–2947.
[186] A.S. Seyan, R.D. Hughes, D.L. Shaverzos, Changing face of hepatic encephalopathy: role of inflammation and oxidative stress, World J. Gastroenterol. 16 (27) (2010) 3347–3357.
[187] E. Bourdon, D. Blache, The importance of proteins in defense against oxidation, Antioxidants Redox Signal. 3 (2) (2001) 293–311.
[188] S. Prakash, Role of human serum albumin and oxidative stress in diabetes, J. Appl. Biotechnol. Bioeng. 3 (1) (2017).
[189] M. Sitar, S. Aydin, U. çAkatay, Human Serum Albumin and its Relation with Oxidative Stress, vol. 59, Clinical Laboratory, 2013, 09-10/2013.
[190] J.R. Carvalho, M. Verdelho Machado, New insights about albumin and liver disease, Ann. Hepatol. 17 (4) (2018) 547–560.
[191] S. DeMorrow, Bile acids in hepatic encephalopathy, J. Clin. Exp. Hepatol. 9 (1) (2019) 117–124.
[192] H. Ohkubo, et al., Role of portal and splenic vein shunts and impaired hepatic extraction in the elevated serum bile-acids in liver-cirrhosis, Gastroenterology 86 (3) (1984) 514–520.
[193] R. Jalan, et al., Alterations in the functional capacity of albumin in patients with decompensated cirrhosis is associated with increased mortality, Hepatology 50 (2) (2009) 555–564.