DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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ASSESSMENT OF NONHAEM FERROUS IRON AND GLUTATHIONE REDOX RATIO AS MARKERS OF PATHOGENETICITY OF OXIDATIVE STRESS IN DIFFERENT CLINICAL GROUPS

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II. Rehema A, Zilmer K, Klaar U, Karro H, Kullisaar T, Zilmer M. Ferrous iron administration during pregnancy and adaptational oxidative stress. Medicina (Kaunas) 2004; 40: 547–552.

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VII. Eisen M, Kaur S, Rehema A, Kullisaar T, Vihalemm T, Zilmer K, Kairane C, Zilmer M. Allergic contact dermatitis is accompanied by severe abnormal changes in antioxidativity of blood. Biomed Pharmacother 2004; 58: 260–263

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ABBREVIATIONS

AD – Alzheimer's disease
CAT – catalase
CD – conjugated dienes
DFO – desferroxamine
DNPH – dinitrophenyl-hydralazine
FDO – 2-furildioxime
Fe – iron
GLN – glutamine
GLU – glutamate
GPx – glutathione peroxidase
GRed – glutathione reductase
GSH – glutathione, reduced
GSSG – glutathione, oxidized
GST – glutathione-S-transferase
H_2O_2 – hydrogen peroxide
HDL – high-density lipoprotein
Hgb – haemoglobin content
HIV – human immunodeficiency virus
HO-1 – haem oxygenase-1
HOxS – high-grade oxidative stress
IRE – iron regulatory element
IRP – iron regulatory protein
LDL – low-density lipoprotein
$LO^{\bullet}$ – lipid alkoxyl radical
$LOO^{\bullet}$ – lipid peroxyl radical
NAC – N-acetylcysteine
NFκB – nuclear factor κB
$NO^{\bullet}$ – nitric oxide radical
$NO_2^{\bullet}$ – nitrogen dioxide radical
NOS – nitric oxide synthase
$O_2^{\bullet-}$ – super oxide radical (anion)
$OH^{\bullet}$ – hydroxyl radical
$ONOO^-$ – peroxynitrite
OxS – oxidative stress
PC – protein carbonyls
PTCA – percutaneous transluminal coronary angioplasty
RBC – red blood cells
RNS – reactive nitrogen species
ROS – reactive oxygen species
$RS^{\bullet}$ – thiyl radical

SOD – super oxide dismutase
TAA – total antioxidative activity
TAS – total antioxidative status
TBARS – thiobarbituric acid reactive substances
TCA – trichloro-acetic acid
TIBC – total iron binding capacity
TNFα – tumour necrosis factor α
UIBC – unsaturated iron binding capacity
UTR – untranslated region

GENERAL INFORMATION

Ferrous (Fe 2+) non-haem iron is a principal pro-oxidant in human organism. By being the most abundant transitional metal in human organism it can initiate and potentate the generation of free radicals through Fenton chemistry and thus propagate free radical reactions. The mechanisms to sequester iron should keep iron bound, but in several pathological conditions the ability to keep iron in harmless state can be compromised locally or even systemically (Crichton et al. 2002, Casanueva and Viteri 2003, Buss et al. 2003).

On the other hand, reduced glutathione is one of the major intracellular antioxidants. Reduced glutathione (GSH) is a tripeptide synthesized from the precursor amino acids cysteine, glutamate, and glycine. It is the most abundant nonprotein intracellular thiol, with multiple roles as an antioxidant agent. Actually, GSH is a major nonenzymatic intracellular antioxidant. One of its main mechanisms of defence is formation of glutathione disulfide (GSSG) by the help of glutathione peroxidase. GSSG must be rapidly converted back to GSH by the combination of glutathione reductase and NADPH. The GSH redox status is important in the regulation of most cellular metabolic processes including transcriptional activation and any depletion of GSH results in high-grade oxidative stress (Moran et al. 2001, Jefferies et al. 2003, Pompella et al. 2003).

These two above mentioned powerful factors (iron and glutathione) reciprocally modulate the critical balance of pro-oxidants and antioxidants – that determines many processes of the cellular function including growth and apoptosis and is crucial in the pathogenesis of numerous diseases like atherosclerosis, cancer, diabetes, neurodegenerative diseases etc. Despite of significance of iron and glutathione (especially its redox status) for human physiology and metabolism their role and indicative power are still among the open problems, and, particularly, simultaneous evaluation of their impact using different clinical groups needs still special investigation.

The current investigation consists of studies that evaluate the level of potentially harmful oxidative stress in several groups of healthy subjects and patients (conditionally healthy adults with alimentary iron overload, pregnant women with borderline anaemia, patients of coronary angioplasty, atopic and contact dermatitis, Alzheimer's disease). Special emphasis is drawn on non-haem iron content and RBC-s glutathione redox ratio as exceptionally indicative and – what is critically important for clinical medicine – modifiable parameters. Nevertheless can either of those or any other parameter in this context be analysed alone, but only in a complex of evaluating oxidative damage on lipids and proteins, and measuring the indices of systemic oxidative defence, so a complex investigation of oxidative damage has been undertaken. The information summarised herein has both fundamental value and practical

outcome for targeted clinical evaluation and modulation of non-haem ferrous iron and glutathione system to predict, avoid and combat high-grade oxidative stress caused pathological events.

Profound (high-grade) oxidative stress

In order to present the material clear and concise it is necessary to define some terms first.

Oxidative stress (OxS) is defined as the shift of balance between antioxidants and pro-oxidants towards pro-oxidants that may lead to potentially harmful processes (Sies 1991, Halliwell and Gutteridge 1999, Zilmer et al. 1999).

"Pro-oxidants" or "oxidative stressors" is a common term for all factors which are able to induce and develop OxS in human organism. Oxidative stressors comprise mainly free radicals and other reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well as ions of transition metals (Zilmer et al. 1999).

Antioxidant is any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell and Gutteridge 1999).

Free radicals are species containing one or more unpaired electrons independent of their route of origin – including ROS and RNS (Halliwell and Gutteridge 1999).

Lipid peroxidation is the main form of oxidative damage to lipids, a process that is determined by the peroxide forming mechanisms and peroxide removing antioxidants.

Under normal physiological conditions, the rate and magnitude of reactive species formation is balanced by the rate and magnitude of their elimination/control. As for the source of pro-oxidants, normal mitochondrial respiration constantly leaks super oxide radicals ($O_2^{\bullet -}$), and monoamine-oxidase activity constantly produces H_2O_2 (Halliwell and Gutteridge 1999). Iron produces free radicals mainly through catalysing the Haber-Weiss reaction generating hydroxyl radicals $OH^{\bullet}$ and NADPH oxidase adds to super oxide generation (Leite et al. 2004).

Human cells generally function in a reduced state but some degree of localised oxidation is needed. Cells have evolved to use reactive oxygen species (ROS), such as super oxide and hydrogen peroxide, as signalling molecules (Yeh et al. 2003, Touyz et al. 2003). Under physiological conditions, ROS are important regulators of cell cycle, protein kinase activity and gene expression. Subtoxic ROS and RNS production can lead to alterations in cellular and extra cellular redox state, and such alterations have been shown to signal changes in cell functions. Numerous cellular processes including gene expression can be

regulated by subtle changes in redox balance. Examples of this include the activation of certain nuclear transcription factors, and the determination of cellular fate by apoptosis or necrosis.

Reactive oxygen species function as important intracellular second messengers to activate many downstream signalling molecules, such as mitogen-activated protein kinase, protein tyrosine phosphatases, protein tyrosine kinases and transcription factors. Activation of these signalling cascades leads e.g. to VSMC growth and migration, modulation of endothelial function, expression of pro-inflammatory mediators and modification of extra cellular matrix. Furthermore, ROS increase intracellular free Ca^{2+} concentration, a major determinant of vascular reactivity (Touyz et al. 2003).

Biological peroxides (i.e. $ONOO^-$ and H_2O_2) have been implicated as regulators of redox sensitive cell signalling pathways. Several studies have shown that H_2O_2 regulates transcriptional and translational events in many cell types, the downstream targets including the mitogen activated protein (MAP) kinases, and nuclear factor kappa B (NF-κB) and hypoxia-inducible factor (HIF). These are important components of numerous redox sensitive signalling pathways that link extra cellular stimuli to gene regulation (Yeh et al. 2003).

The recognition of ROS/RNS and redox-mediated protein modifications as transducing signals has opened up a new field of cell regulation and provided a novel way of controlling disease processes. One such approach has been proven feasible for gene expression governed by the transcription factor NF-κB. NF-κB is among the most important transcription factors shown to respond directly to oxidative stress (Haddad 2002).

Cellular redox balance is, under normal circumstances, maintained so that overall reducing conditions prevail. Thiols, by virtue of their ability to be reversibly oxidized, are recognised as key components involved in the maintenance of redox balance. Increasing evidence suggests that thiol groups located on various molecules act as redox sensitive switches thereby providing a common trigger for a variety of ROS and RNS mediated signalling events (Moran et al.2001).

Thus, cellular signalling pathways are generally subjected to dual redox regulation in which redox state has opposite effects on upstream signalling systems and downstream transcription factors. Not only are the cellular signalling pathways subjected to redox regulation, but also the signalling systems regulate the cellular redox state. When cells are activated by extra cellular stimuli, the cells produce ROS, which in turn stimulate other cellular signalling pathways, indicating that ROS act as second messengers. It is thus evident that there is cross talk between the cellular signalling system and the cellular redox state (Kamata and Hirata 1999).

Inoue et al. have showed that a cross talk of nitric oxide and oxygen radicals regulates the circulation, energy metabolism, reproduction, and remodelling of cells during embryonic development, and functions as a major defence system against pathogens as well as fates of pathogens and their hosts and that

oxidative stress in and around mitochondria also determines cell death in the development of animals and tissue injury caused by anticancer agents by some carnitine-inhibitable mechanism (Inoue et al. 2003).

It all shows that low-grade OxS has an adaptive and regulative character.

At the same time, any prolonged excess of ROS and RNS or depletion of antioxidants (this situation is called profound or high-grade OxS, HOxS) causes damages of lipids, proteins and DNA.

Apoptosis is accompanied by an intracellular shift towards increased oxidation, but too much oxidation will stop apoptosis by oxidizing and inactivating the caspase enzymes. Transition-metal ions are liberated from metalloproteins as a primary mechanism of injury by oxidative damage (Halliwell 2000).

The oxidation mechanism for disease pathogenesis states that an imbalance in cell redox state alters function of multiple cellular pathways.

Neither $O_2^{\bullet-}$ nor H_2O_2 react with DNA but H_2O_2 crosses the mitochondrial and plasma membranes reaching DNA, where bound transition metals produce in situ $OH^{\bullet}$, thus causing DNA damage. Oxidized bases and single- and double-strand DNA breaks occur and can be detected with biochemical methods (Halliwell and Gutteridge 1999).

Powerful oxidizing agents such as the hydroxyl radical directly modify amino acid side chains of proteins, resulting in a diverse array of altered amino acids. Among these modifications, generation of free carbonyls is considered specific for oxidative damage (Smith et al. 1998). Protein carbonyls result from the interaction of free radicals with amino acid residues oxidating their sulfhydryl groups and hydroxylating tyrosine and phenylalanine (Halliwell and Gutteridge 1999). Under the influence of excess oxidative stress, modifications of proteins and polynucleotides take two forms: (a) adduction reactions by highly reactive intermediate products of lipid peroxidation or glycation, and (b) direct oxidative modification of the macromolecules (Smith et al. 1998).

Fe^{2+} or Cu^{+} can initiate a lipid peroxidation cascade in biological membranes and lipoproteins by the production of $OH^{\bullet}$ (by reacting with unsaturated fatty acids in the presence of O_2). Transition-metal-based radicals may be responsible for initiating the peroxidation of lipids and the formation of conjugated diene double bonds, fluorescent chromo lipids and alkoxyl and peroxyl radicals that are able to propagate lipid peroxidation to final end products such as pentane, ethane, malondialdehyde (reacting with thiobarbituric acid, TBARS), hexanal, isoprostanes etc (Halliwell and Gutteridge 1999).

OxS has been acknowledged to be a pathogenetic and/or etiological factor of numerous pathological conditions. HOxS has a major influence in diseases of aetiology of multifactorial origin. So the role of HOxS has been accepted (and proven) in atherosclerosis, tumours (Jefferies et al. 2003) and neurodegenerative diseases (Perry et al. 2002), but also in diabetes (Haskins et al. 2003) and development of rheumatoid arthritis (Jaswal et al. 2003).

On the other hand it has been observed that in diseases with a definite causative aetiological factor (a specific allergen in allergic contact dermatitis, bacterium in tuberculosis, virus in HIV-infection), the reaction of the human organism, and thus the generation/manifestation of a disease in answer to the causative agent and the following progression of the state, is dependent of the balance between prooxidants and antioxidants.

The oxidative theory of aging is one of many possibilities to explain the process of growing older. It is based on the knowledge that many age-related diseases and diseases that accelerate the aging process are connected to oxidative damage (Polla et al. 2003).

Monitoring OxS can be done indirectly by assaying products of oxidative damages on lipids, proteins and DNA or investigating the potential (total antioxidative potency) of an organism, tissue, cells or body fluid to withstand further oxidation.

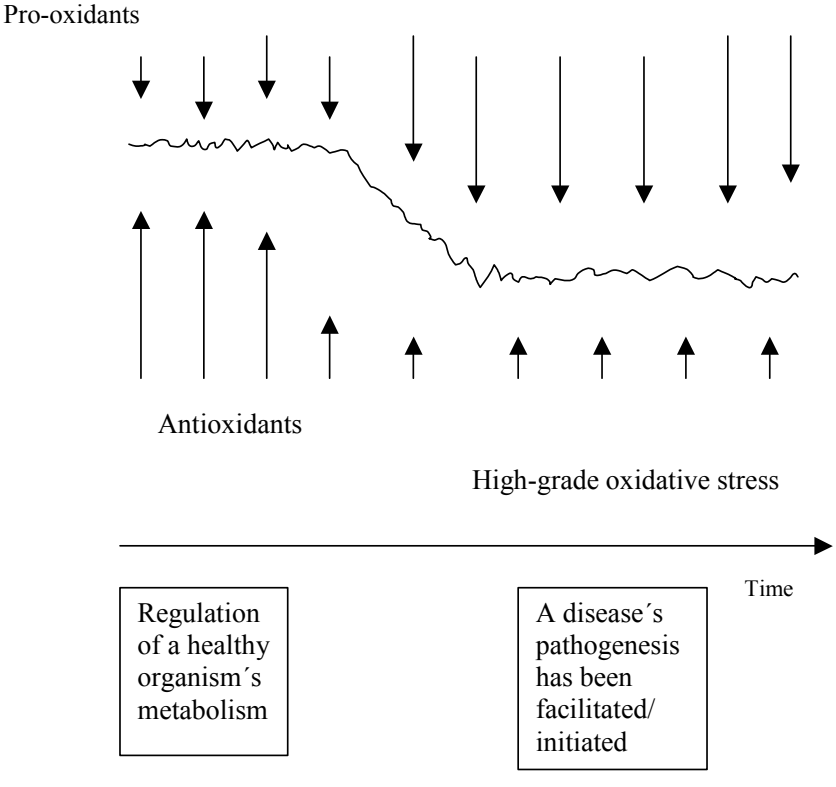

Figure 1: Oxidative stress in the process of disease progression.

More and more scientific investigations are undertaken to define complexes of markers that are cost-effective, easy to use and informative to be used in clinical settings to predict, avoid and combat high-grade oxidative stress caused events. Both the piling evidence reviewed in literature and our sets of experiments indicate that evaluation of ferrous (non-haem) iron status and the integrity of the glutathione system can add valuable information for making clinical decisions.

Oxidative stress and cardiovascular diseases

The free radical theory of aging (free radicals generate molecular damage to lipids, proteins and nucleic acids) (Harman 1956) has been acknowledged to be the first to connect the diseases of the elderly and free radicals.

Animal studies support a fundamental role for ROS in cardiovascular disease (Griendling and FitzGerald 2003). Animal models of atherosclerosis have documented that all the constituents of the plaque produce and use ROS. Atheroma formation is associated with the accumulation of lipid peroxidation (LPO) products and excessive formation of oxidized LDL (OxLDL), induction of inflammatory genes, inactivation of NO resulting in endothelial dysfunction, activation of matrix metalloproteinases (that modulates collagen degradation, which is important in lesion instability) and increased smooth muscle cell growth (Griendling and FitzGerald 2003).

Atherosclerosis is accompanied by degeneration of vascular endothelial cells and vascular smooth muscle cells due to programmed cell death (Unterluggauer et al. 2003). Oxidized LDL can induce endothelial cell apoptosis in vitro and this effect may be prevented by HDL (Speidel et al. 1990). Unterluggauer et al. have demonstrated on a model system of human endothelial cells that a significant accumulation of ROS is detected in senescent but not young endothelial cells and this accumulation induces growth arrest in the G1 phase of the cell cycle and cell death occurs by apoptosis (Unterluggauer et al. 2003).

In many forms of hypertension, the increased ROS are derived from NAD(P)H oxidases (Landmesser and Harrison 2001, Leite et al. 2004) and it could serve as a triggering mechanism for uncoupling endothelial NOS by oxidants. The mechanism by which oxidative stress is increased by hyperlipidemia could involve the renin-angiotensin system (RAS). In rats made hypertensive by angiotensine II infusion, NAD(P)H oxidase subunit expression and $O_2^{\bullet -}$ production are increased. Both the resulting impaired endothelium-dependent vasodilatation and the hypertension can be corrected by infusion of modified forms of SOD penetrating the vessel wall. Recent data suggest the persistence of low levels of oxidant stress during the vascular repair reaction in neointimal and medial layers (Leite et al. 2004). Another potentially pro-atherogenic effect of angiotensin II, as well as of cytokines produced by inflammatory cells, is up regulation of adhesion molecule expression and expression of receptors for oxidized LDL. Angiotensin II causes also hyper-

trophy of vascular smooth muscle in a ROS-dependent fashion. It has been implied that other mediators can share these properties of angiotensin II (Griendling and FitzGerald 2003). Thus, angiotensin II is a potent stimulator of OxS and RAS blocking agents may reduce the profound OxS in the body.

Another potential effect of elevated ROS in atherosclerosis is a compensatory increase in a mutant form of a functionally active extra cellular SOD (ecSOD) that converts excessive $O_2^{\bullet-}$ into extra cellular H_2O_2. There may still be the possibility that H_2O_2 rather than $O_2^{\bullet-}$ is the main ROS in atherogenesis (Griendling and FitzGerald 2003).

Increased vascular $O_2^{\bullet-}$ production, decreased tissue glutathione, impaired endothelial-dependent relaxation, and increased NAD(P)H oxidase activity leading to uncoupling of eNOS have all been demonstrated in animal models of diabetes as well as in human patients with type II diabetes (Griendling and FitzGerald 2003). All those factors together with disturbances of iron metabolism have an impact on the pathogenesis of CHD.

In balloon-catheter injury models, it has been documented that mechanical injury promotes an initial loss of cellularity, and also that programmed cell death or apoptosis is observed early after balloon distension injury and may contribute to the early medial smooth muscle cell loss. Evidence suggests that members of the mitogen-activated protein kinase family or mediators play an important regulatory role in apoptosis (Pollman et al. 1999). In addition to mediators in the mitogen-activated protein kinase family, ROS may also mediate the cell suicide program as well as cell growth. Pollman et al. documented that balloon injury was associated with a marked reduction in glutathione levels and demonstrated that treatment with antioxidants such as N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) inhibited the acute induction of cell death. They concluded that balloon injury induces acute cell death via a redox-sensitive mechanism (Pollman et al. 1999). The results were consistent with the hypothesis that stress-activated protein kinases activation is part of the redox-sensitive pathway regulating the acute induction of medial smooth muscle cell apoptosis in response to balloon angioplasty injury. After vascular injury, activation of enzymes such as NADPH oxidase leads to a marked increase in super oxide generation, proportional to the degree of injury, which rapidly subsides. Such early super oxide production is significantly greater after stent deployment, as compared to balloon injury (Leite et al. 2004). In addition to animal models, some clinical trials have indicated that antioxidant drugs might have particular efficacy in preventing restenosis in response to balloon injury (Daida et al. 2000).

Oxidative stress and pregnancy

Pregnancy, mostly because of the mitochondria-rich (mitochondrial mass increases with gestational age) placenta, is a condition that favours oxidative stress (Sies 1991, Wang and Walsh 1998, Casanueva and Viteri 2003). Transition metals, especially iron, which is particularly abundant in the placenta, are important in the production of free radicals (Casanueva and Viteri 2003). At the same time placenta is highly vascular and exposed to high maternal oxygen partial pressure (Casanueva and Viteri 2003). In addition NO is also locally produced by the placenta and together with other reactive nitrogen species contributes to potential oxidative stress in the presence of transition metals. The placenta is also rich in macrophages favouring local placental production of free radicals, including reactive chlorine species in which free iron is also implicated (Halliwell and Gutteridge 1999).

Reduced glutathione has been found elevated during pregnancy in erythrocyte lysates (Wisdom et al. 1991), SOD activity in erythrocytes and plasma thiol levels have been found to be lower during pregnancy and coeruloplasmin levels have been found to be higher during pregnancy than in nonpregnant women (Casanueva and Viteri 2003). Serum levels of CD, fluorescent chromo lipids and TBARS increase in normal pregnant women, reaching their maxima in the second trimester and then decline until term. Plasma ascorbic acid concentration varies by intake but generally decreases in normal pregnancy, severely so in preeclampsia (Hubel et al. 1997). Nitriosothiols and other metabolites derived from free radicals are exaggerated in gestational hypertensive diseases and diabetes (Kossenjans et al. 2000).

Defence mechanisms against free radical damage (bilirubin, glutathione, SOD, CAT, GPx, Gred) are also enhanced as pregnancy progresses (Casanueva and Viteri 2003). However, pregnancy is a state where this adaptation and equilibrium between free radicals and protective mechanisms are easily disrupted as evidence by the propensity toward the development of gestational hypertension and insulin resistance that in some cases can lead to gestational diabetes.

Results of Orhan et al. (2003) suggest that oxidative stress and subsequent lipid peroxidation accompany the complications of hypertension, preeclampsia and diabetes mellitus in pregnancy. Hyperglycaemia itself can favour nonenzymatic glycation, which can induce ROS formation in the presence of reactive transitional metals. It also induces reductions in serum transferring and in the activities of CuZnSOD and of coeruloplasmin ferroxidase (Casanueva and Viteri 2003). Evidence of elevated levels of intracellular iron and ROS in insulin resistance (Fernandez-Real et al. 2002) and diabetes and prevention of diabetic foetal anomalies by the reduction of iron and oxidative stress reinforces this concept (Lao et al. 2002).

The supplementation of antioxidants, such as vitamin E and/or C, to prevent eclampsia and diabetes mellitus during pregnancy has been suggested and

several animal models and even clinical trials have been conducted (Chappell et al. 1999).

Both abnormalities in the glutathione system and disturbances in iron metabolism have an impact on non-physiological events during pregnancy. Monitoring both glutathione and iron status in pregnant women is highly important as the primary negative consequences of depletion of GSH or excess of iron have a hidden nature.

Oxidative stress and Alzheimer's disease

Multiple lines of evidence have implicated OxS and free radical damage to the pathogenesis and possible aetiology of Alzheimer's disease (AD). Cellular changes show that oxidative stress is an event that precedes the appearance of the hallmark pathologies of the disease, neurofibrillary tangles and senile plaques (Perry et al. 2002). The damage of oxygen radicals found in AD includes advanced glycation end products, nitration, lipid peroxidation adduction products as well as carbonyl-modified neurofilament protein and free carbonyls (Perry et al. 2002). The OxS-mediated damage (like lipid peroxidation) seems to have regional pattern (Karelson et al. 2001).

The most crucial aspects of the cellular oxidative damage in AD pathogenesis appear to be cytosceletal modifications in neurons susceptible to AD, that cause irreversible cellular dysfunction and ultimately lead to neuronal death (Smith et al. 1995). The oxidative conversion of protein side-chains to reactive carbonyl or acylating moieties, or adduction of bifunctional sugar- or lipid-derived products can result in abnormal protein cross-linking. Covalent cross-linking may in fact represent a neurotoxic event by inhibiting turnover. At the same time, several oxidative modifications may generate chemical species that support further oxidative stress through redox cycling or transition metal sequestration (Perry et al. 2003). Perry has also suggested that as both neurofilament tangles (NFT) and amyloid β (Aβ) show the lowest levels of 8-hydroxyguanosine, a marker of recent ROS attack, but high levels of advanced glycation end products or lipid peroxides, both Aβ and NFT may serve an antioxidant function, and thus be cellular compensations for increased oxidative stress (Perry et al. 2003).

Preliminary epidemiological and clinical studies suggest that inhibiting oxidative stress and glycation are effective in reducing the clinical manifestation of neurodegenerative diseases (Munch et al. 1998).

Regional differences in protein damage (Hensley et al. 1995) and dinitrophenyl (DNP) adduct formation has been objectivized in the cell bodies and apical dendrites of the pyramidal neurons of the hippocampus in cases of AD, but not in control subjects. Senile plaques (including dystrophic neuritis, β-amyloid deposits, microglia, astrocytes, and adjacent oligodendrocytes) showed no reaction with DNPH in experiment (Smith et al. 1998). The authors

concluded that their results do not support the senile plaque as the primary site of free radical imbalance but rather implicate an ROS source within the cell body (Smith et al. 1998).

Specific biologic mechanisms are thought to interchangeably interact among patients with AD, producing different phenotypic profiles, which partially depend on a genetic basis (Mosconi et al. 2003). Over 100 mutations of the amyloid precursor protein, presenilin 1, and presenilin 2 genes have been described in the rare, early-onset dominant form of familial AD (FAD) (Rosenberg 2000). Alternatively, in the more common sporadic manifestations of AD (SAD), a genetic locus on chromosome 19, coding for the apolipoprotein E (apoE), has been associated with increased susceptibility (Rosenberg 2000). However, more than 70% of the AD cases in the general population are unrelated to any of these four genes, suggesting that genetic predisposition could act in concert with several epigenetic factors to determine AD onset (Rosenberg 2000).

It has been demonstrated that both haem oxygenase-1 (HO-1) protein and its mRNA are increased in brains of AD patients, indicating the up-regulation of antioxidant enzymes (Perry et al. 2002). An altered iron metabolism and release and glutathione depletion have an impact on neurodegeneration including AD (Perry et al. 2003).

Oxidative stress and other potential problems

Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus (I and II). HOxS appears to be the pathogenic factor in underlying diabetic complications. Free radicals are formed disproportionately in diabetes by glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. Consequently, the free radicals thus generated promote the development of complications of diabetes mellitus. 8-hydroxydeoxyguanosine (8-OHdG) has been suggested to serve as a new sensitive biomarker of the in vivo oxidative DNA damage in diabetes (Wu et al. 2004).

Chronic rheumatic diseases are another area that has been linked to HOxS. Several different pathways can lead to increased formation of reactive oxygen species in inflamed joints. This enhanced oxidation plays a significant role in the tissue damage and inflammation perpetuating process in rheumatoid synovium (Ozturk et al. 1999). Although LPO affects many cellular components, the primary site involves membrane-associated PUFA and protein thiols. It is apparent that patients of rheumatoid arthritis (RA) are exposed to OxS and are more prone to LPO. Accordingly, altered concentrations of some antioxidants have also been reported. The concentrations of whole blood glutathione and

total thiols have been found to be significantly lower in patients of RA, as compared to healthy controls (Jaswal et al. 2003).

Patients with chronic renal failure (CRF) are in a state of HOxS compared with healthy controls. The most informative indices to evaluate the degree of OxS in CRF have been reported to be GSSG level, GSSG/GSH status, LDL lag phase and CD (Annuk et al. 2001).

Intracellular reduction-oxidation status is a primary regulator of cellular growth and development. Evidences that support the involvement of free radicals in tumour promotion include the following facts: (i) a number of free radical-generating compounds are found to be tumour promoters in various animal model systems, (ii) ROS generating systems can mimic the biochemical action of tumour promoters, (iii) some tumour promoters stimulate the production of ROS, (iv) tumour promoters modulate the cellular antioxidant defence systems, and (v) free radical scavengers, detoxifiers and antioxidants inhibit the process of tumour promotion. The role of ROS in the progression stage of carcinogenesis is evident from the fact that a number of different free radical generating compounds enhance the malignant conversion of benign papillomas into carcinoma and their effectiveness may be related to the type of radicals produced into the biological system (Athar 2002).

The causal relationship between the markers of OxS and clinical severity of an infection has not yet been established, but Madebo et al. established lower concentrations of antioxidant vitamins C, E and A and also lower thiol concentrations, particularly of the reduced forms, and higher malondialdehyde (MDA) that was associated with clinical severity in tuberculosis patients compared to endemic control group (Madebo et al. 2003). Aukrust et al. (2003) established that highly active antiretroviral therapy was accompanied by both an improvement of glutathione redox-status and an increase in levels of antioxidant vitamins in patients of HIV infection. Apart from that, glutathione supplementation in vitro increased T-cell prolifration in these patients.

So the heterogeneous spectrum of disabilitating diseases like diabetes, RA and malignancies have all been connected to amelioration of the antioxidant defence systems including the glutathione system and to disturbances of iron metabolism leading to an excess of the pro-oxidant (Salonen et al. 1999, Huang 2003, Jefferies et al. 2003, Jaswal et al. 2003).

The pro-oxidants in human body, special emphasis on ferrous iron

The pro-oxidants are reactive oxygen species (ROS) that are generated during oxidative burst in macrophages, in the leak of electrons in cellular breathing etc., and reactive nitrogen species (RNS) as well as transition metal ions that can take part in free radical processes as initiators or activators. The divalent ferrous iron is one of the major pro-oxidants of human organism (Halliwell and Gutteridge 2003, Polla et al. 2003).

(ROS) can be divided to free radicals (FR), which are based on an oxygen atom (super oxide radical, hydroxyl radical, etc) and oxygen-containing oxidizing non-radical compounds (may be converted to FR) like H_2O_2, hypochlorous acid (HOCl), ozone, singlet oxygen ($^1O_2$), 4-hydroxynonenal, etc.

(RNS) can similarly be FR, which are based on a nitrogen atom (nitric oxide radical, etc) and nitrogen-containing non-radical compounds (peroxynitrite etc) (Zilmer et al. 1999).

The endogenic sources for reactive species production in human organism may be as follows (Zilmer et al. 1999, Halliwell and Gutteridge 1993, Leite et al. 2004):

- Mitochondrial respiratory chain ($O_2^{\bullet-}$)
- Inflammation and phagocytosis ($O_2^{\bullet-}$, $OH^{\bullet}$, H_2O_2, HOCl)
- Xanthine oxidase ($O_2^{\bullet-}$)
- Vascular NAD(P)H oxidase ($O_2^{\bullet-}$)
- Cyclooxygenase ($LOO^{\bullet}$)
- Free iron and copper as transition metals ($OH^{\bullet}$)
- Destruction of senescent bio molecules by peroxisomes
- Reaction between $O_2^{\bullet-}$ and $NO^{\bullet}$ (yields in peroxynitrite)
- Reaction between H_2O_2 and peroxynitrite (singlet oxygen)
- Auto-oxidation of catecholamines
- Ischaemia/reperfusion (ROS and RNS)
- Prolonged severe emotional stress (ROS and RNS)
- etc.

It is important to note that iron is potentially the strongest factor considering the production of different free radicals. During normal metabolism ROS ($O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$) are generated from molecular oxygen (O_2). Through Fenton reaction Fe(II) is oxidized to Fe(III) in the presence of H_2O_2 and a highly reactive $^{\bullet}OH$ is produced.

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\bullet}$$

Iron catalyses this reaction

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^\bullet$$

It has been estimated that the rate of oxidation of oxidizable substrate by "$Fe^{2+}+O_2$" could be as much as 10^8 faster than the rate of oxidation by the Fenton reaction (Huang 2003). These results suggest that "$Fe^{2+}+O_2$" chemistry is probably the most important route for free radical biology of iron. In fact, $O_2^{\bullet-}$ and H_2O_2 may be produced directly from dissolved oxygen (O_2) in aqueous media in the Fe^{2+}-mediated autoxidation reactions as follows:

$$Fe^{2+}+O_2 \longrightarrow Fe^{3+}+O_2^{\bullet-} \qquad (1)$$

$$Fe^{2+}+O_2^{\bullet-}+2H^+ \longrightarrow Fe^{3+}+H_2O_2 \qquad (2)$$

$$Fe^{2+}+H_2O_2 \longrightarrow Fe^{3+}+OH^-+OH \qquad (3)$$

In comparison with Fenton/Haber–Weiss reactions where iron is catalytic or redox cycled, iron is consumed in iron autoxidation reactions. For example, oxidants produced by the interaction of Fe^{2+} and O_2 may be quenched by Fe^{2+} itself at the high concentrations as follows:

$$Fe^{2+}+OH^\bullet \longrightarrow Fe^{3+}+OH^- \qquad (4)$$

According to the reactions (1)–(4), a self-quenching reaction can be written as follows:

$$4Fe^{2+}+O_2+2H^+ \longrightarrow 4Fe^{3+}+2OH^-$$

It is known that the activation of oxygen by iron is subject to both kinetic and thermodynamic restraints, and therefore, reactions (1)–(3) as described are oversimplified. For example, it has been suggested that oxidants other than the hydroxyl radical ($OH^\bullet$), such as ferryl or iron oxo, may also be generated (Huang 2003).

Super oxide radical O_2^- is capable of reducing ferritin-bound ferric (Fe^{3+}) iron to the ferrous state (Fe^{2+}), whereupon it is released from ferritin and becomes available to catalyse a self-propagating burst of oxidation (Crichton et al. 2002).

Thus free iron is probably the most important factor in the free radicals of human body via its potent contribution to production of hydroxyl radicals, especially from mild oxidizing agents or a mild reducing agent as H_2O_2. Hydrogen peroxide (H_2O_2) is apparently able to cross cell membranes readily (Halliwell et al. 2000). In chemical terms, H_2O_2 is poorly reactive: it can act as a mild oxidizing or as a mild reducing agent. The danger of H_2O_2 largely comes from its ready conversion to the indiscriminately reactive hydroxyl radical ($OH^\bullet$), either by exposure to ultraviolet light or by interaction with transition metal ions, of which the most important in vivo is iron. H_2O_2 can contribute to

Fenton chemistry not only by being one of the substrates but also by providing the other, e.g. by liberating iron from haem proteins. At sites of inflammation, H_2O_2 generated by activated phagocytes appears to modulate the inflammatory process, e.g. by up-regulating expression of adhesion molecules, controlling cell proliferation or apoptosis and modulating platelet aggregation. It thus seems likely that most or all human cells are exposed to some level of H_2O_2. These data emphasize the importance of metal ion sequestration in preventing the toxicity of H_2O_2 in vivo by decreasing the occurrence of Fenton chemistry, and help explain why a failure of such sequestration can produce devastating tissue damage in almost all organs of the body (Halliwell et al. 2000).

The reactive nitrogen species nitric oxide NO, formed by the nitric oxide synthase, NOS, plays an important role in the cell as an antimicrobial agent. Its reaction with super oxide will form the extremely toxic peroxynitrite species, $ONOO^-$. The increase of cellular iron content may alter the ability of iNOS (that is a haem enzyme) to adequately respond to cellular requirements. INOS expression is also controlled by free iron within the cell (Crichton et al. 2002). The role of the macrophages in liver homeostasis is critical as macrophages are able to phagocytose senescent erythrocytes (predominantly in the spleen). In the macrophage, the free iron, liberated from haem by haem oxygenase, is incorporated into ferritin or released into the circulation to be bound by transferrin. Investigators claim that the presence of excessive amounts of iron in the macrophage may adversely interfere with their role in the activation of NADPH oxidase and the generation of ROS as an inflammatory response (respiratory burst). The production of NO and consecutive $ONOO^{\bullet}$ is one of the major mechanisms in the macrophages defence system. It has been shown that stimulated macrophages from iron-loaded rats had a significantly reduced ability to produce NO (Crichton et al. 2002).

Cells, such as hepatocytes, which have high antioxidant protection are less susceptible to iron, whereas those that have less, like many brain cells, are more sensitive to iron catalysed oxidative stress (Crichton et al. 2002).

LDL cholesterol becomes oxidized when it comes in contact with unstable free radicals, such as the hydroxyl radical (OH^-), singlet oxygen (O_2^-), or hydrogen peroxide (H_2O_2). The intensified generation of ROS is potentially harmful to practically all cellular and extra cellular structures: proteins (both inside and outside the cells), lipids (lipid peroxidation), DNA (cross links and fractioning), mitochondria (decoupling of oxidative phosphorylation) – and all of theses disturbances can be potentated by ferrous iron (Polla et al. 2003).

A substantially elevated production of ROS may be caused by environmental factors (radiation, cigarette smoke) or pathological conditions (ischemia-reperfusion, inflammation and infection) (Polla et al. 2003). In many of these situations the potentiating role of iron has been proven.

Human body iron

The common oxidation states of iron are ferrous (Fe^{2+}) or ferric (Fe^{3+}). At neutral and alkaline pH ranges, the redox potential for iron in aqueous solutions favours the ferric state, at acidic pH values, the equilibrium favours the ferrous state. In organism ferrous iron is mainly the component of functioning iron-protein complexes and ferric iron is mainly present in iron's transportation and storage complexes. All the important steps in iron transportation and storage include reduction and re-oxidation of iron. The reduction of Fe^{3+} to Fe^{2+} is mediated by NADP/NADPH system. Fe^{2+}-s conversion to Fe^{3+} is regulated by ferroxidases, mainly ferroxidase II (Geisser 1998, Crichton et al. 2002).

Iron has an affinity for electronegative atoms such as oxygen, nitrogen and sulphur, which provide the electrons that form the bonds with iron (Geisser 1998, Zilmer et al. 1999). Iron can bind to a variety of macromolecules and catalyse the generation of unstable free radicals. It means that no free iron can be allowed to be present in a living organism. To protect against such reactions, several iron-binding proteins function specifically to store and transport iron. These proteins have both a very high affinity for the metal and incompletely filled iron-binding sites (Geisser 1998, Crichton et al. 2002, Zilmer et al. 1999).

Iron in human organism is almost exclusively a component of proteins: nonhaem proteins (ferritin, transferrin, lactoferrin and redox-enzymes, Fe-S-proteins) and haem proteins (enzymatic like NOS, cytochromes, catalase, peroxidases and nonenzymatic like haemoglobin and myoglobin). Iron is in the functional centre of haemoglobin's haem, enabling the transportation of oxygen from lung capillaries to the tissues and iron is in the functional part of the oxidation chain in the cell as the functional part of cytochromes (Geisser 1998).

The majority of the iron present in an organism is in use as a component of haemoglobin or myoglobin (2–5 g). In normal circumstances 3 to 4 times less is stored in ferritin and only $1/100^{th}$ is – bound to proteins – present in serum (Geisser 1998, Zilmer et al. 1999, Arosio and Levi 2002).

Absorption of iron

The high affinity of iron for both specific and non-specific macromolecules leads to the absence of significant formation of free iron salts, and thus this metal is not lost via usual excretory routes. Rather, excretion of iron occurs only through the sloughing of tissues that are not reutilised (epidermis, hair, gastrointestinal mucosal cells) and physiological/ pathological losses of blood (Geisser 1998, Crichton et al. 2002). In a healthy adult male the loss is about 1 mg/day. Children, premenopausal women and patients with blood loss naturally have increased iron requirements. The lost amount should be substituted.

Cooking of food increases the availability of the metal in the gut. The low pH of stomach contents permits the reduction of ferric ion to the ferrous state (Zilmer et al. 1999).

The major site of absorption of iron is in the small intestine, with the largest amount being absorbed in the duodenum. Humans consume iron in two forms – haem iron (ferrous) is present in fauna i.e. meat, liver, blood etc. Nonhaem iron is mostly present in plants – and the majority of it is ferric. The absorption of haem iron and nonhaem iron from the food is different (Lieu et al. 2001, Huang 2003).

The absorption of haem iron is receptor-mediated (a putative haem receptor on the apical membrane of the enterocyte) and is not dependent on the pH of intestinal lumen or accompanying food constituents. About 20 to 25% of haem iron present in food is absorbed. Only after the absorption into the mucosal cytoplasm the metal is split off from the porphyrin ring with the help of microsomal haem oxygenase in the endoplasmic reticulum.

In the absorption of non-haem iron, dietary ferric iron can be reduced to ferrous iron by duodenal ferric reductase, Dcytb prior to its transport into the enterocyte via the divalent cation transporter DMT1 (also known as Nramp2 or DCT1) (Crichton et al. 2002). A ferric reductase is associated with DMT1 and it reduces ferric ions to ferrous ions in the lumen. Thus, ferrous ion uptake prevails but ferric ions can also bind via mucin-solubilized way to a trans-membrane integrin and are carried then to a soluble protein mobilferrin (Zilmer et al. 1999, Aisen et al. 1999, Lieu et al. 2001). From nonhaem iron present in food about 5 % is absorbed.

When iron is absorbed into the mucosal cell, it divides between the ferritin of mucosal cell and the basal membrane of the cell through which (with the help of IREG1, a transmembrane iron transporter protein) iron is bound to transferrin in the capillaries. Hephaestin, a membrane-bound protein, promotes oxidation of ferrous iron to ferric iron, that will allow rapid binding of iron to transferrin and its delivery to cells expressing IRP-regulated transferrin receptors, thus preventing endothelial damage (Crichton et al. 2002).

The major barrier to the absorption of iron is not at the lumenal surface of the duodenal mucosal cell. Whatever the requirements of the host are, in the face of an adequate delivery of iron to the lumen a substantial amount of iron will enter the mucosal cell. Regulation of iron transfer occurs between the mucosal cell and the capillary bed (Zilmer et al. 1999, Crichton et al. 2002, Huang 2003). Hephaestin, Fe-binding chaperones and some other agents help ferroportin1 (an iron export protein FPN1) to obtain iron (both ferrous and ferric), which is probably transported across the basolateral membrane in the ferrous state. Thus, evidently the reductase activity of FPN1 reduces Fe^{3+} to Fe^{2+}. Once the Fe^{2+} reaches the external surface of basolateral membrane it is probably oxidized to Fe^{3+} by the plasma ferroxidase coeruloplasmin and bound to plasma apotransferrin to form an iron transport protein transferrin (Zilmer et al. 1999, Crichton et al. 2002).

Iron transportation, uptake and utilization by mammalian cells

The protein in serum involved in the transport of iron is transferrin, a β_1 glycoprotein synthesized in the liver, consisting of a single polypeptide chain of 78000 Da with two iron binding sites. Transferrin binds ferric, but not ferrous iron. The binding of each ferric ion is absolutely dependent on the coordinate binding of an anion, which in the physiological state is carbonate (Aisen et al. 1999).

In healthy individuals, plasma transferrin saturation is usually 20–35% of maximum meaning that most transferrin molecules have one or two unoccupied iron binding sites and free iron cannot be detected (Crichton et al. 2002). Patients with various malignancies have a diminished capacity to sequester iron and serum free iron can sometimes be detected, especially after chemotherapy and in other conditions with cell death/lysis.

Transferrin binds to specific cell surface receptors (TfRs) that mediate the internalisation of the protein in almost all mammalian cells (Lieu et al. 2001).

The transferrin-transferrin receptor complex is internalised in clathrin-coated vesicles. The vesicles lose their coat, and the resulting smooth vesicles fuse with endosomes. The interior of the endosomal compartment is maintained acidic (approximately pH 5.5). Iron is released from the transferrin-transferrin receptor as Fe^{3+} and transported out of the endosome by the divalent cation carrier DMT1, the transmembrane iron transporter.

Once the iron has been released it is rapidly bound by intracellular protein ligands, notably the iron storage protein ferritin. A major part of intracellular iron utilization is accounted for by the synthesis of iron-containing proteins.

Recent evidence suggests that some types of cells may express a transferrin-independent iron transport system. For example, mice and humans lacking transferrin, while anaemic, show iron overload in parenchymal tissues such as liver and spleen (Huang 2003).

Intracellular iron transport and storage

Organisms require an intracellular storage form of iron that is soluble, non-toxic and bio-available. Ferritin, the principal iron storage protein, is widely distributed in many mammalian cell types. The structural and biochemical properties of ferritins in different organisms – from bacteria to humans – are generally the same, although their roles in metabolism are slightly different (Aisen et al. 1999, Arosio and Levi 2002).

The protein (apoferritin) consists of a roughly spherical hollow shell composed of 24 structurally equivalent subunits. The apoferritin shell encloses an internal cavity of maximum diameter about 80 Å, within which iron is

deposited in small crystalline particles essentially as an inorganic ferric oxyhydroxide polymer with some phosphate (Lieu et al. 2001). This iron core, which can attain a maximum content of 4500 iron atoms within a single molecule, is insoluble at pH 7 in water, but is rendered soluble by the very stable apoferritin protein shell that surrounds it.

In human ferritin two types of subunits are described: H and L. Iron deposition can be divided into stages: (i) oxidation of Fe^{2+} mainly by H-subunits, (ii) migration of Fe^{3+}, (iii) mineralisation (strengthening) of the core, mainly by L-chains.

Human H-ferritin oxidizes iron fast in a reaction also generating hydrogen peroxide (Arosio and Levi 2002). In tissues with the function of iron storage like in the liver and spleen, ferritins contain more H-subunits (Arosio and Levi 2002).

Ferritins are located mainly in the cytosol, thus keeping the stored iron away from the cell nucleus and other organelles (Arosio and Levi 2002). Recently a new, mitochondrial ferritin has been discovered and its described functions resemble considerably H-ferritin (Arosio and Levi 2002). Low amounts of ferritin are also present in serum and secreted liquids. The physiological role of serum ferritin has not been elucidated yet but it has clinical importance as a marker of stored iron (Arosio and Levi 2002, Zilmer et al. 1999). When the amount of iron in the body increases, ferritin synthesis is up regulated on translational level.

Recent investigations have clarified several aspects of the relationship between iron and ferritin – how is the iron core formed and hydrogen peroxide generated, for example. It has been shown that the ferroxidase-catalytical centre is the most important place of control of iron availability – that in turn influences several cellular functions e.g. proliferation and resistance to oxidative damage (Arosio and Levi 2002, Crichton et al. 2002). The answer to the apoptotic stimuli depends on the ferritin expression in a tissue (Arosio and Levi 2002).

The regulation of ferritin expression is mostly post-transcriptional and iron-dependent, based on the interaction of iron regulatory proteins (IRP) with iron responsive elements (IRE) on mRNA. The system is sensitive to both potentially available iron and to the oxidative status of the cell (is activated by $NO^{\bullet}$ and H_2O_2 – Aisen et al. 1999), and it regulates similarly the H- and L-chains. The other, less characterized regulatory system(s) that are not necessarily iron-mediated and are probably transcriptionally regulated, which establish the tissue-specific pattern of H:L ratio (Arosio and Levi 2002).

Ferritin can also be viewed as a member of the group of proteins that respond to stress and inflammation (inflammatory cytokines, particularly tumour necrosis factor α (TNFα) and interleukin-2 (IL-2), up regulate ferritin synthesis in various mammalian cells (Zilmer et al. 1999, Arosio and Levi 2002, Crichton et al. 2002). Most of the stimuli related to inflammation and directed to ferritin synthesis is claimed to up regulate H-ferritin preferentially over the L,

thus determining an increase of the catalytic sites and a reduction of cell iron availability. Ferritin expression is modulated by a variety of conditions associated with oxidative stress that act either directly on gene expression or indirectly via the modification of IRPs activity (Arosio and Levi 2002). Abnormalities in ferritin expression not apparently related to alterations in iron metabolism have been observed in many types of cancer. Evidence has been presented that oncogenes may regulate ferritin expression (Arosio and Levi 2002).

Ferritin capacity to prevent iron pro-oxidant activity by oxidizing and sequestering the metal inside the shell remains the most likely explanation for its anti-oxidant activity (Arosio and Levi 2002). The protein shell of ferritin is notably stable at pH above 5–6. However, some substances like super oxide radical and some types of radiation are capable of releasing iron from ferritin. Physiological reductants such as ascorbate and glutathione do not release iron from ferritin at significant rates. It has been demonstrated that xanthine oxidase is able to mobilize iron from ferritin in the presence and in the absence of oxygen, that iron release is mediated by super oxide (Biemond et al. 1986). Reduced flavins and many redox-cycling xenobiotics have also been shown to release iron from ferritin (e.g. paraquat, adriamycin) (Galey 1997).

Mitochondrial ferritin has the potential of being an important regulator of local iron trafficking and defence against the possible interaction between free iron and ROS, both present in the organelle (Arosio and Levi 2002).

Usually degradation of ferritin takes place in the "secondary lysosomes", encapsulated in the membrane, and that is supposed to be the source of haemosiderin – the storage form of iron, from which iron can not be taken up any more.

Intracellular iron metabolism

Once iron has been assimilated by a mammalian cell, it can undergo a series of intracellular transfers. In most mammalian cells, the bulk of the intracellular non-haem iron is found in ferritin and its lysosomal degradation product, haemosiderin, or in a number of non-haem iron proteins (oxygenases, ribonucleotide reductase, electron transport iron-sulphur proteins) (Arosio and Levi 2002).

There remains however an intermediate pool of chelatable low-molecular-weight or "transit" iron, which is speculated to be "in transit" between the extracellular (transport) and intracellular (storage) forms of the metal. The effects attributed to the transit iron pool are numerous. Such low molecular weight iron can act as a catalyst in the Fenton reaction to potentate oxygen toxicity by the generation of a wide range of free radical species, including hydroxyl radicals (Crichton et al. 2002). Using the metal sensitive fluorescent probe calcein, the estimated concentrations of low-molecular-weight iron are in

the range of 0.2–1.5 μM for resting erythroid and myeloid cells and about 10 μM in rat hepatocytes. Yet, levels of low-molecular-weight iron in healthy human's serum have not been reported or estimated (Huang 2003).

An increase in intracellular iron stimulates apoferritin synthesis and, in hepatocytes, diminishes transferrin synthesis. It may be the source of iron for the terminal step in haem biosynthesis, source of iron for incorporation into ferritin etc. (Crichton et al. 2002). It has been stated that Fe-S clusters are synthesized only inside the mitochondria and are locally used by mitochondrial enzymes or exported for insertion in cytosolic and nuclear enzymes (Lill and Kispal 2000).

In normal human subjects some 40 mg of tissue iron is mobilized per day. About 80% of this represents the recycling of iron (derived from the catabolism of RBCs). Most of the remaining 7–8 mg of tissue iron mobilized daily in normal subjects is derived from hepatocytes. In clinical situations characterized by parenchymal iron overload the mobilization of hepatocyte iron is of paramount importance (Aisen et al. 1999).

In mammals the balance between iron uptake and intracellular storage (and utilisation) is achieved predominantly at the level of protein synthesis (translation of mRNA into protein) rather than at the level of transcription (mRNA synthesis). Regulatory sequences (iron regulatory elements – IREs) are located in the non-coding or un-translated regions (UTRs) of the mRNA, at either the 5'– and 3'-extremities of the coding part of the mRNA sequence: IREs in the former are usually associated with initiation of translation, in other words ribosome binding, whereas those at the 3'-UTR are associated with mRNA stability and degradation (Crichton et al. 2002). Two cytosolic IRE-binding proteins (known as iron regulatory proteins – IRPs), found in many cell types, act as iron sensors, essentially exist in two conformations (Crichton et al. 2002).

Haem proteins are conjugated proteins that contain haem. They are divided: (i) nonenzymatic (haemoglobin, myoglobin) and (ii) enzymatic (cytochromes, catalases, peroxydases). In non-enzymatic haem proteins the function of haem is to bind oxygen. In enzymatic haemoproteins the role depends upon the apoprotein – in cytochromes haem is active in electron transport, in catalase haem is a component of active centre (Zilmer et al. 1999, Crichton et al. 2002).

Human organism gets haem from food, but cannot use directly the haem absorbed. There is no transport of haem between organs, so every tissue has to synthesize its own haem for its needs. The only condition needed is the presence of mitochondria in the cells. In all tissues the rate-limiting factor is ALA synthase (aminolevulinic acid synthase). The coenzyme of this enzyme is pyridoxal-phosphate (that derives from B6). Myoglobin reminds 1 Hgb subunit as it has one protein and one haem particle. Some pharmaceuticals and ROS can oxidize haem iron to Fe 3+ and convert myoglobin to metmyoglobin.

A class of ion channel, the large-conductance calcium-dependent Slo1 BK channels, possesses a conserved haem-binding sequence motif. It has been

shown that haem binds to Slo1 BK channels with a high affinity, suggesting that haem may function as an acute cell-signalling molecule producing fast-acting non-genomic modulation of protein function. After cellular injury, hypoxia and/or stress, haem is liberated from haemproteins, leading to an increase in the free haem concentration. The extracellular haem released during haemolysis and trauma can be transported across the plasma membrane. In addition, cells may contain other endogenous haem-like molecules that mimic haem action. Inhibition of plasma-membrane Slo1 BK channels is generally expected to exert an excitatory influence on cellular excitability. The existence of Slo1 BK channels in mitochondria, where haem synthesis takes place, has been documented as well (Tang et al. 2003).

The effects of reactions between Hgb and biologically relevant peroxides in the context of cell signalling have not been explored. The concentrations of cell-free Hgb can potentially compete with endogenous reactions that consume the peroxides mentioned. Therefore, the effects of Hgb on cell function may be subtler than oxidative damage mediated by Hgb and involve perturbation of redox sensitive signalling pathways (Yeh and Alayash 2003).

The catabolism of haem-containing proteins presents two requirements to the mammalian host: (i) the development of means of processing the hydrophobic products of porphyrin ring cleavage and (ii) the retention and mobilization of the contained iron so that it may be reutilised. Senescent RBCs are recognized by their membrane changes and removed and haem is degraded primarily by a microsomal enzyme system in reticuloendothelial cells (Crichton et al. 2002).

In certain diseases destruction of RBCs occurs in the intravascular compartment rather than in the extravascular reticuloendothelial cells. In these circumstances transferrin binds free iron and thus permits the reutilization of the metal. Free haemoglobin in the plasma is bound to haptoglobins and delivered to the reticuloendothelial cells (Zilmer et al. 1999, Crichton et al. 2002).

Haem oxygenase 1 (HO-1) is an inducible enzyme that catalyses the rate limiting step in the degradation of heam to biliverdin, carbon monoxide and iron. Activation of HO-1 is an ubiquitous cellular response to oxidative stress (Crichton et al. 2002). HO-1 is considered to be protective against oxidative stress and it inhibits iNOS in activated macrophages by decreasing haem availability for NOS synthesis (Crichton et al. 2002).

Iron deficiency and overload

Iron deficiency is the most widespread mechanism of anaemia and has been acknowledged as a problem centuries ago. It affects about 5% of the population in America and Europe and even more in the third world. Arbitrarily it can be divided in stages: (1) depletion of stores, (2) anaemia and (3) impairment of enzyme function (Crichton et al. 2002). The clinical consequences derive from

the diminished capacity of RBCs to transport oxygen (i.e. anaemia) and, in more advanced stages, from impairment of the function of other haem proteins and proteins with Fe-S clusters.

The causes of iron deficiency may be:

- increased losses of blood
- increased needs (pregnancy)
- strict diets and eating predominantly foodstuffs containing low amounts of iron
- limitations in absorption (chronic gastritis, ulcer patients on medication, gastrectomy).

The defined risk groups for developing iron deficiency anaemia are infants (especially if keen on drinking lots of milk), pregnant women and patients with chronic losses of blood.

Iron overload is a newly recognized problem and could therefore be underestimated as well as overemphasized. It affects about 1% of the population in America and Europe (Crichton 2002). Iron overload is mistakenly believed to be rare. Iron overload is an increase in total body iron generally exceeding 5 g (Huang 2003).

Generally iron overload may be caused by

- increased haemolysis/ transfusions
- increased alimentary intake, especially when combined to vitamin C and alcohol
- genetic haemochromatosis (heterozygote prevalence 3–5%, homozygous state 0.25% of the population, but penetrance not conclusively defined, Beutler et al. 2003).

Two point mutations have been found within the HFE protein in haemochromatosis patients. HFE, originally called HLA-H, is a protein that resembles atypical HLA class I molecules, consistent with the localization of the gene near the HLA cluster. One of the two point mutations, at position 282 of the gene, changing the invariant cysteine to tyrosine (indicated as Cys282Tyr or C282Y), has been found to be mutated in 90% of the American patients studied. The second point mutation, His63Asp, has been found in more diverse ethnic backgrounds and may represent an older mutation. The association between HFE and β_2-microglobulin seems necessary for the interaction of HFE with transferrin receptor and subsequent cellular transferrin iron uptake (Huang 2003).

Any iron overload increases the possibility of free-iron-derived highly damaging events. The effects of iron excess can be local or generalized. Generalized iron excess is observed in chronic primary and transfusion iron overload, the cause may be also chronic intake of supplements containing high amounts of iron (Green et al. 1989). Local iron excess and iron-mediated

oxidative stress have been demonstrated in the intestinal mucosa, liver, spleen, bone marrow and placenta (Casanueva and Viteri 2003). When exposed to excess intake of iron, the intestinal mucosa stores most of it as ferritin, but it is vulnerable to oxidative damage secondary to the continuous presence of a relatively small excess of iron intake (Geisser 1998, Lund et al. 2001). Intestinal mucosal iron accumulation leading to gastrointestinal erosions has been documented in patients at therapeutic doses (Abraham et al. 1999).

In an animal model the production of free radicals in intestinal mucosa in response to therapeutic iron was more prominent in previously iron-deficient subjects (Srigiridhar and Nair 1998). Excessive iron load in the liver, pancreas, skin, and connective tissue has been linked to an increased risk for carcinomas and sarcomas, probably because of increased oxidative stress. Iron has also been reported to be a risk factor for colon cancer. Individuals with serum ferritin levels higher than 70 μg/l have an increased risk of recurrence of colon adenoma as compared to those with lower values (Lieu et al. 2001). A haemochromatosis gene mutation Cys282Tyr and an increased risk of cardiovascular diseases have been connected (Roest et al. 1999, Tuomainen et al. 1999, Garry 2001).

The information that can be gathered by measuring parameters of iron metabolism

Hgb is measured by different methods, but it always expresses the amount of haemoglobin (in erythrocytes as well as in serum – the latter not contributing significantly in normal conditions) in one litre of blood.

RBC= erythrocyte count can be measured under a microscope but nowadays it is mostly counted mechanically

MCV= mean cellular volume describes the mean volume of erythrocytes. In iron depletion MCV is less than normal, as a rule.

MCH= mean cellular/corpuscular haemoglobin, that describes the mean amount of haemoglobin in one erythrocyte. In iron depletion MCH is usually less than normal.

MCHC= mean cellular haemoglobin concentration, a calculative figure from MCV and MCH In iron depletion MCHC can be below normal.

Ht=haematocrit, the percentage of cellular matter in whole blood, mainly measured by centrifuging cells to the lower part of a capillary tube and measuring their volume afterwards

Serum iron (Fe-ser) describes the concentration of iron (bound to transferrin) in serum Serum iron samples must be taken in the morning after an overnight fast as marked daily variation occurs (up to 30%)

TIBC= total iron binding capacity, describes mainly the amount of iron, that can be safely bound to transferrin (**UIBC**=unsaturated iron binding capacity)

+ the amount of iron that is already bound to transferrin (Fe-ser). **% sat**, saturation % of iron-binding proteins, calculated as serum iron/TIBCx100%, corresponds in serum to transferrin saturation (see below).

Transferrin, the transport protein, is measured by the methods suitable for measuring all specific proteins, but mainly by immunoassays.

Transferrin saturation is calculated from the figures of transferrin content and iron content in an amount of serum.

Serum ferritin is believed to be the best figure for evaluating body iron stores, 1ng of serum ferritin corresponding to 8 mg of stored iron – when inflammation, acute MI or other critical event is not present.

RBC ferritin responds to acute changes more slowly and can be therefore used in acute clinical situations, but the correlation between RBC-ferritin and body iron stores is not believed to be so precise.

The soluble fraction of **transferrin receptor** can be measured also. In many situations it is considered to be the best parameter describing iron stores.

A pathological form of iron – so-called **chelatable iron** (desferal-chelatable, bleomycin-chelatable, etc. by detection method) s. nontransferrin-bound iron should not be present in serum, but becomes detectable in critical pathological situations (preterm infants with haemolysis, cancer patients receiving chemotherapy, chronic inflammatory conditions,) and soon after ingestion of an iron bolus (Casanueva 2003).

Iron and atherogenesis

For over 20 years now it has been speculated that the "diseases of civilization" as atherosclerosis, coronary heart disease (CHD), Alzheimer's disease etc. are strongly related to oxidative stress, mediated by free iron (Lieu et al. 2001).

As concerning CHD, in 1981 Sullivan first proposed the so-called iron hypothesis, suggesting that regular menstrual loss, rather than other effects of estrogen, protects women against CHD (Sullivan 1981). He was assisted with the results of Berge et al. more than 10 years later. The parallel rise in serum ferritin, total cholesterol, and LDL cholesterol might contribute to the increased risk of coronary heart disease among postmenopausal women (Berge et al. 1994).

More to that, in has been documented, that estrogens directly alter the iron redox chemistry, this fact probably being involved in the antioxidant effects of these molecules (Ruis-Larrea 1995). These two facts: smaller iron stores and altered redox potential of iron – could really be responsible for the well-known fact that premenopausal women experience only 30–50% of the CHD incidence and mortality of age-matched men. Recent studies have suggested that iron depletion protects endothelial function, an effect that may contribute to the lower disease rates of menstruating women (Sullivan 2003).

The iron hypothesis is strongly supported by the work of J.T. Salonen et al. (starting with Salonen et al. 1992). It is widely believed that oxidation of LDL cholesterol is a pivotal step in atherogenesis. Native LDLcholesterol, whose serum levels correlate well with CHD risk, is only weakly atherogenic. Oxidized LDLcholesterol, on the other hand, is strongly atherogenic, being a chemo-attractant for macrophages, which in turn internalise the lipid components via the unregulated scavenger receptor. Eventually, this unlimited lipid uptake produces immobilized, lipid-laden foam cells which accumulate to sub endothelial raised lesions, the probable precursors of atherosclerotic plaques (Zilmer et al. 1999).

Serum ferritin concentration has been shown to have statistically significant direct correlations with most of the measured oxysterols. Different oxysterols derive from different pools of cholesterol, and are produced both by cholesterol autoxidation and by specific cholesterol oxidizing enzymes. Oxysterols may have a double role in atherosclerosis: they have been shown in vitro to promote cellular damage, possess cytotoxic and apoptotic properties, and in that way progress atherosclerosis, but on the other hand, oxysterols have multiple cholesterol homeostasis regulating functions (Tuomainen et al. 2003).

The role of iron is proposed to be crucial in undermining successful ageing. Increases in ROS may result from environmental exposures, including tobacco smoke, non-ionizing or ionizing radiation, or from pathological conditions such as ischaemia-reperfusion, infection or inflammation. These conditions are linked to a variety of diseases, whether genetically determined or age-related. Iron has been shown to contribute to oxidative stress and the pathological process of many of these diseases.

Exposure to oxidizing stress, endogenous or exogenous, stimulates the activity of endogenous anti-oxidants. During ageing, these endogenous anti-oxidant stores will be depleted, suggesting that exogenous anti-oxidants become more critical in older individuals. Key among exogenous anti-oxidants are those derived from the ingestion of nutrient-rich foods. Iron plays a negative role in this equilibrium and overall tends to accelerate the onset and development of various age-related diseases. The involvement of iron in ageing has been established in many studies using iron chelation or iron deprivation as the experimental approach (Polla et al. 2003).

Iron and pregnancy

Iron-deficiency anaemia is considered the most widespread pregnancy-associated pathological condition. Severe anaemia (Hgb less than 80 g/l) in the first half of pregnancy is proved to be associated with preterm delivery and small-for-gestational-age foetus (Scanlon et al. 2000, Rasmussen 2001, Schümann 2001). In contrast, the values of borderline anaemia (96–105 g/l of Hgb) appear to be related to the minimum incidence level of preterm delivery

(Steer 2000, Malhotra et al. 2002). This is attributed to the fact that high (over 120 g/l) values of haemoglobin might indicate the inadequacy of adaptational plasma volume expansion in the third trimester rather than an iron-replete state of the woman (Scholl and Reilly 2000). Perinatal maternal and foetal complications have been found to increase exponentially once Hgb values decrease further below 90 g/l. On the other hand – the incidence of gestational hypertension, preeclampsia, eclampsia, low birth weight, and low Apgar scores increase rapidly when Hgb levels surpass 130 g/l (Casanueva and Viteri 2003). Another group of investigators who report a connection between higher serum ferritin and spontaneous pre-term birth explain the phenomenon with ferritin being a marker of inflammation (Paternoster et al. 2002).

The World Health Organization (WHO) has recommended universal supplementation with iron from the second trimester onwards with 60 mg/d until delivery (and 3 months postpartum in the regions where the prevalence of anaemia is > 40%)(Casanueva and Viteri 2003). Ferrous iron is the form that is mostly used for correction of iron deficiency and iron medical preparations (also those exceeding RDA 10 times or more) are sold over the counter.

At the same time there is no scientifically proven and ultimate consensus about using the iron therapy (the criteria for its use as well as the dosage) during pregnancy.

The amount of iron that should be absorbed to satisfy the gestational needs has been estimated to be 4–5 mg/d during the second trimester and 6–7 mg/d during the third trimester (Hallberg et al. 1992). The need may be met in account of pre-pregnancy iron reserves, the percentage of absorption is higher as pregnancy progresses (Hahn et al. 1951) and there is no physiological loss of menstrual blood during pregnancy. Women with prepregnancy ferritin levels > 20 μg/l are reported not to have a marked decline in serum ferritin throughout the course of pregnancy (Kaufer and Casanueva 1990). During pregnancy, iron deficiency anaemia of uncomplicated nutritional origin is most often mild (Hgb usually 95–110 g/l) (Casanueva and Viteri 2003).

The maternal surface of the trophoblastic microvilli of the human placenta is very rich in transferrin receptors. As pregnancy progresses, different mechanisms (thinning of the syncytiotrophoblast, increments in placental blood flow and in transferrin receptors) enhance the transfer of iron to the placenta and the foetus (Parkkila et al. 1997). Non-transferrin bound iron may be involved in placental transfer of iron (Wessling-Resnick 2000). Elevated non-transferrin-bound iron has been detected in plasma and in umbilical cord blood shortly after the ingestion of iron supplements (Breuer et al. 2000).

A phenomenon, reported in subjects receiving daily iron supplementation, is a rapid decline of plasma ferritin after stopping the iron supplementation. Possible explanations for these findings are that the high plasma ferritins after daily iron supplementation inhibit food iron absorption or that they reflect, at least partially, an inflammatory process secondary to excess iron and oxidative stress that recedes with time (Casanueva and Viteri 2003).

The whole issue becomes more intricate as pregnancy itself induces oxidative stress (Morris et al. 1998, Geisser 1998) and as preeclampsia is proved to be (at least partially) mediated by oxidative stress (Hubel 1998, Roberts and Cooper 2000, Vaughan and Walsh 2002).

In gestational diabetes, both BMI and serum ferritin levels have been found to be independent predictors of 2-h glucose during an oral glucose tolerance test (Lao and Tam 2001).

Iron and neurodegeneration

Iron accumulation in the brain has been associated with Parkinson's disease, Alzheimer's disease, Huntington's chorea and HIV encephalopathy, basal ganglia disease and Hallervorden Spatz syndrome, while in Friedrich ataxia, excessive mitochondrial iron accumulation occurs particularly in brain and cardiac tissue (Crichton et al. 2002).

Brain cells, including neurons, astrocytes and microglia, show a decreased ability to respond to oxidative stress, particularly with respect to their levels of glutathione and glutathione peroxidase (Crichton et al. 2002). With ageing there is a significant increase in iron stores in the brain (Crichton et al. 2002).

Dopamine-rich regions of the brain may be especially sensitive to altered iron metabolism, first because monoamine oxidase produces hydrogen peroxide, and second because dopamine firmly binds iron and as a catechol can undergo redox cycling, thereby generating reduced oxygen species (Galey 1997).

Smith et al. (1997) examined the distribution of iron in the brain of cases of Alzheimer's disease (AD) employing a histochemical stain binding of iron (II) cyanide (ferrocyanide) to iron (III) in tissue to give a complex that can be observed. They found striking accumulations of ferric iron closely associated with senile plaques, neurofibrillary tangles, and neutrophil threads (i.e. the pathological lesions) in cases of AD, but not in control cases. As iron was specifically localized to lesions of AD and not the glial cells which contain abundant iron binding proteins, the authors suggested that the lesion-associated iron was distinct from that sequestered by normal storage proteins. The authors also noted that the pattern of redox-available iron in neurons was strikingly similar to the localisation of HO-1 (Smith et al. 1997).

Iron and skin

Chronic exposure to ultraviolet light has been shown to result in an increased skin level of nonhaem iron. This iron accumulation is likely to be the consequence of a UVB radiation-induced capillary damage and leakage of protein-bound iron (Bissett et al. 1991). Apart from ferritin, another potentially significant source of free iron may be other iron-containing proteins e.g. enzymes that contain [4Fe-4S] clusters have been shown to oxidize by super oxide with a loss of ferrous iron from the cluster (Galey 1997). There have been published no human experiments concerning local changes of iron in skin diseases before our group of investigators. In 2003 the results were repeated with atomic absorption spectrometry and gas chromatography-mass spectrometry showing that the atopic dermatitis dermis contained higher iron concentrations compared to controls (Leveque et al. 2003).

Iron and allergy

One potential hazard concerning iron therapy during pregnancy could be a hypothesis that (alimentary) iron could be the root cause of autism and childhood allergies (Padhye 2003). This phenomenon logically links the problems of iron supplements during pregnancy and allergic diseases of the skin. The hypothesis is based on the chronological matching of the frequency of pervasive developmental disorders (including autism) and the increase in iron consumption and iron-fortification of infant food mixtures (in last 30years). This period has shown a substantial decrease in iron deficiency but most of other childhood diseases tend to increase their frequency. Iron is capable to modulate the activity of immune system, generating the hyper reactive immune response. There has been demonstrated a connection between autoimmunity and autism (Singh et al. 1998). The effect of iron on intestine and microflora may contribute to the entrance of peptidic antigens to bloodstream. Developing thymocytes acquire iron from transferrin by endocytosis of the transferrin receptor but how transferrin crosses the blood–thymus barrier is unknown. Iron deficiency causes a decrease in circulating T lymphocytes as a result of thymus atrophy and decreased thymocyte proliferation (Bowlus 2003). Iron overload as a result of transfusions in thalassemia is associated with decreases in circulating CD4+ T lymphocytes and the presence of immature CD1a+ T lymphocytes. Expansion of CD8+CD28– T lymphocytes has been associated with iron overload due to mutations in the MHC class I-like gene HFE (Bowlus 2003).

The association of the iron transporter NRAMP1 with autoimmune disorders and the finding that iron can catalyse the production of cryptic epitopes suggests a unique role that iron may play in the pathogenesis of many disorders of autoimmunity (Bowlus 2003).

Iron and other health hazards

The relationship between iron metabolism and type 2 diabetes is bi-directional—iron affects glucose metabolism, and glucose metabolism impinges on several iron metabolic pathways. Oxidative stress and inflammatory cytokines influence these relationships, amplifying and potentiating the initiated events (Fernandéz-Real et al. 2002).

Increased iron stores have been found to predict the development of type 2 diabetes while iron depletion has been shown to be protective. Iron-induced damage might also modulate the development of chronic diabetes complications. Iron influences glucose metabolism, even in the absence of significant iron overload. In the general population, body iron stores are positively associated with the development of glucose intolerance, type 2 diabetes (Salonen et al. 1999), and gestational diabetes (Lao and Tam 2001).

Iron appears to influence the development of diabetic nephropathy and vascular dysfunction. In the apparently healthy general population, serum levels of ferritin have been found to be positively correlated with baseline serum glucose and with the area under the curve for glucose during the glucose oral tolerance test (Tuomainen et al. 1997).

It has been suggested that regulation of iron uptake by insulin occurs in parallel with its effects on glucose transport (Tanner and Lienhard 1997). HOxS induces both insulin resistance by decreasing internalisation of insulin (Bertelsen et al. 2001) and increased ferritin synthesis. Glycation of transferrin decreases its ability to bind ferrous iron (Fujimoto et al. 1995) and, by increasing the pool of free iron, stimulates ferritin synthesis.

Facchini (1998) found significant reductions in insulin concentrations 1 month after performing a 550-ml phlebotomy in healthy volunteers. It has also been suggested that the increased insulin sensitivity observed in vegetarian subjects might be related to their low-iron diet (Fernandéz-Real et al. 2002).

Chronic hepatitis C virus (HCV) infection has been known to be associated with a high risk of hepatocellular carcinoma (HCC) due to iron overload. Excess iron tends to generate ROS within cells, which causes mutagenic lesions, such as 8-hydroxy-2'-deoxyguanosine (8-OHdG). It has been suggested that iron-lowering treatments not only could significantly decrease elevated levels of hepatic 8-OHdG but also could prevent the development of HCC in these patients (Kato et al. 2001).

Four epidemiological studies have found a higher cancer risk in patients with larger iron stores than in those with smaller ones. In addition to its effect on carcinogenesis, iron can also maintain the growth of malignant cells as well as growth of pathogens. Breast cancer cells, for instance, display 5–15 times more transferrin receptors than normal breast tissue. Iron-carrying transferrin is in fact a growth factor. Hyposideremia in patients with cancer or infection is claimed not to be a para-phenomenon but a functioning defence mechanism "nutritional immunity" (Gurzau et al. 2003).

Tumour cells in a highly proliferative state have a high density of transferrin receptors, and antisense cDNA for the transferrin receptor has been shown to reduce TfR mRNA and expression, resulting in more inhibition of growth of human breast carcinoma cells than normal breast cells. Monoclonal antibodies against TfR severely restricted the growth of lymphoma tumours in mice. The chelator currently used to treat iron overload disease, deferoxamine (DFO), has shown anti-proliferative activity against leukaemia and neuroblastoma cells in vitro, in vivo, and in clinical trials, suggesting that iron deprivation may be a useful anti-cancer strategy (Huang 2003).

Human body antioxidant defence system

Halliwell and Gutteridge have proposed a broad definition of antioxidants (as they state, antioxidant is a term widely used but rarely defined): antioxidant is any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell and Gutteridge 1999).

Antioxidant defences comprise:

- agents that catalytically remove free radicals and other "reactive species". Examples are the enzymes SOD, CAT, peroxidases and "thiol-specific antioxidants".
- proteins that minimize the availability of pro-oxidants such as iron ions, copper ions and haem. Examples are transferrins, haptoglobins, haemopexin and metallothionein. This category includes proteins that oxidize ferrous ions, such as coeruloplasmin.
- proteins that protect bio molecules against damage (including oxidative damage) by other mechanisms, e.g. heat shock proteins.
- low-molecular-mass agents scavenging ROS and RNS. Examples are glutathione, ascorbic acid, α-tocopherol and (possibly) bilirubin and uric acid. (Halliwell and Gutteridge 1999)

Other important antioxidants may include thioredoxin, and selenoproteins other than GPx. The composition of antioxidant defences differs from tissue to tissue and between cell types within a tissue. Nitric oxide may be an important antioxidant in the vascular system. Extracellular fluids have different protective mechanisms from the intracellular environment (Halliwell and Gutteridge 1999). By the place of action the components of the antioxidative defence system can be divided as follows (the list is not complete):

Human plasma antioxidants

- Water-soluble: vitamin C, albumin, uric acid, coeruplasmin, apotransferrin

- Lipid-soluble: vitamin E, carotenoids, ubiquinol
  Cellular antioxidants
- Water-soluble: Glutathione (GSH), vitamin C, SOD, CAT, GPx
- Lipid-soluble: vitamin E, carotenoids, ubiquinol (Zilmer et al. 1999)

Besides the bio molecules that comprise the antioxidative defence system in the classical concept there are many molecules present in an organism that have antioxidant properties but that fulfil other functions (being metabolites etc) – pyruvate, a natural energy-yielding fuel in myocardium, neutralizes peroxides by a direct decarboxylation reaction, and indirectly augments the glutathione (GSH) antioxidant system by generating NADPH reducing power via citrate formation (Mallet et al. 2002). Blood plasma albumin, urea and bilirubin have proved to have substantial antioxidative properties (Zilmer et al. 1999, Mates et al. 2000)

Diet-derived antioxidants are important in maintaining human health (Halliwell 1999). In a diseased organism also a medication may act as pro-oxidant or antioxidant.

Membrane and extra cellular antioxidants

Coenzyme Q10, an important molecule in cellular breathing, has considerable antioxidant properties, and as being lipid-soluble, its main place of action (as an antioxidant) is cellular membranes and lipoproteins.

Vitamin E, a lipid-soluble vitamin, has become a classical example of antioxidants as its main known function for a considerable period of time was to be an antioxidant in cellular membranes and lipoproteins. α-Tocopherol is concentrated inside the membranes, in blood lipoproteins and adrenal glands. It quenches and reacts with $^1O_2$ and is a scavenger of $^{\bullet}OH$, able to protect membranes from these extremely reactive species. However, its major antioxidant action in biological membranes is to act as a chain breaking antioxidant, donating labile hydrogen to peroxy and alkoxy radicals, thereby breaking the radical chain. It has been proposed that α-tocopherol$^{\bullet}$ may be reduced by ascorbic acid or reduced glutathione. These are scavengers of ROS and other reactive species.

Vitamin C is active extracellularily as well as inside cells, but as its concentration is times higher in several cell types, it will be discussed in next chapter.

Living organisms have evolved mechanisms to sequester transition metal ions into protein-bound (with transferring or coeruloplasmin) forms that cannot catalyse $OH^{\bullet}$ formation and other free radical reactions in vivo. These mechanisms are especially important in such extracellular fluids as the blood plasma (Halliwell et al. 2000).

Blood plasma albumin, among its many other functions, serves as an important extra cellular antioxidant. Of extra cellular, mainly blood plasma antioxidants, the role has been quite well described for bilirubin and urea. Urate binds iron and copper and scavenges $OH^{\bullet}$, $^1O_2$ and peroxy radicals (Mates et al. 2000).

GSH is used for detoxification of several xenobiotics by glutathione S-transferases (GST). Increases in serum gamma-glutamyl transferase (GGT) lead to an increase in the production of free radicals, particularly in the presence of iron (Jefferies et al. 2003).

Intracellular antioxidants

The most important intracellular antioxidant is reduced glutathione. Glutathione (L-γ-Glu-L-Cys-Gly) is a tripeptide synthesized from the precursor amino acids cysteine, glutamate, and glycine de novo within the liver and is released into blood and bile (Jefferies et al. 2003). GSH is present in millimolar range in mammalian cells (Anderson et al. 1989). Gamma-glutamylcysteine synthase, a rate-limiting enzyme for the synthesis of GSH, may play crucial role in antioxidant defence mechanisms. Oxidants can up-regulate the transcription of gamma-glutamylcysteine synthase genes (Jefferies et al. 2003). The isopeptide nature of the γ-glutamyl linkage renders GSH resistant to cleavage by most peptidases. GSH is capable of limiting the extent of mitochondrial damage as it inactivates reactive oxygen species (Jefferies et al. 2003).

In short, the established biofunctions of GSH are the following ones:

- GSH prevents the denaturation of haemoglobin and reduces methaemoglobin back to haemoglobin in the RBCs (the needed high level of GSH is maintained by reduction of GSSG rapidly back to GSH by NADPH and flavoenzyme glutathione reductase (GRed);
- as the major cellular non-enzymatic antioxidant GSH eliminates reactive oxygen species (ROS), like hydroxyl radical, peroxynitrite, peroxides and N_2O_3 and plays a principal role in cellular defence against high-grade oxidative and nitrosative stress, mainly via co-operation with Se-containing glutathione peroxidase (GPx);
- GSH is necessary for the synthesis of proteins and nucleic acids, in protein folding and for the processing of leukotrienes and prostaglandins;
- it is used for the detoxification of several xenobiotics by glutathione S-transferases (GST);
- GSH (GSNO) is involved in transport and storage of nitric oxide;
- GSH is involved in transport of amino acids into the liver and kidney cells;
- GSH is involved in the transmembrane transport of organic solutes;
- GSH has a role in maintaining communication between cells, and preventing protein – SH groups from oxidizing and cross-linking;

- GSH is a cofactor for several enzymes like the glutathione peroxidase family enzymes;
- it is involved in the regulation (glutathionylation) of action of several key-enzymes and proteins (GADDH, phosphorylase, creatinine kinase, ras, etc) and in the restoration of sulfhydryl groups of proteins (maintenance of enzymes and proteins in active forms);
- GSH acts also as a molecular regulator of whole cellular physiology (it participates in BCL-2's ability to suppress apoptosis, in regulation of the hexose monophosphate shunt, has role in signal transduction, etc);
- GSH is involved in many metabolic events including ascorbic acid metabolism;
- GSH can chelate copper ions and diminish their ability to generate free radicals;
- GSH is required for stabilization of cell membranes and it may be important in the absorption of iron and selenium;
- GSH redox status is important in the regulation of most cellular metabolic processes including transcriptional activation (Anderson et al. 1989, Anderson 1997, Zilmer et al. 1999, Sies 1999, Halliwell and Gutteridge 1999, Valencia et al. 2001, Dickinson and Forman 2002, Filomeni et al. 2002, Jefferies et al. 2003).

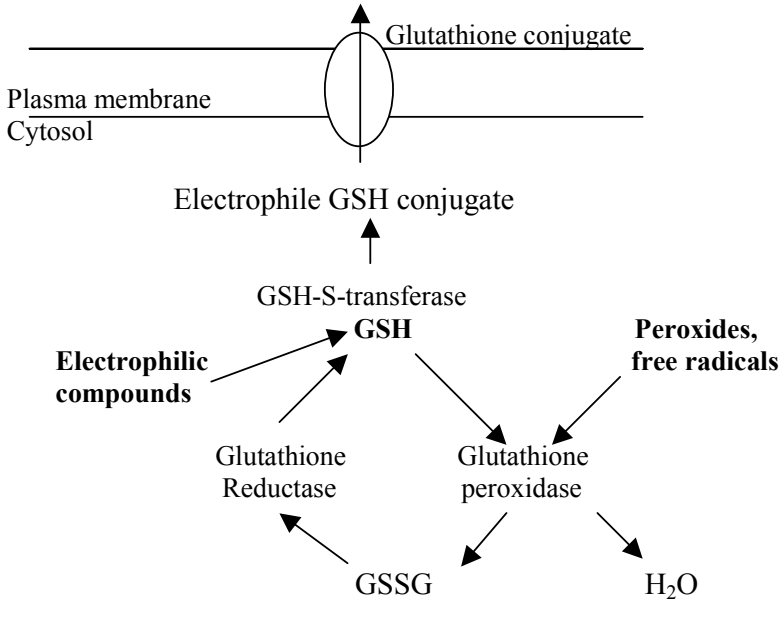

Figure 2: Some basic mechanisms of glutathione functions.

Many of the properties of GSH are linked to its thiol nature. Thiols, by virtue of their ability to be reversibly oxidized, are recognised as key components involved in the maintenance of redox balance. Additionally, increasing evidence suggests that thiol groups located on various molecules act as redox sensitive switches thereby providing a common trigger for a variety of ROS and RNS mediated signalling events (Moran et al. 2001).

Cellular thiols are critically important in maintaining the cellular antioxidant defence network. In addition, thiols play a key role in regulating redox-sensitive signal transduction process. Lipoic acid is a highly promising thiol antioxidant supplement (Sen 2001).

In addition to glutathione system (see below) the thioredoxin (TRX) system (TRX, TRX reductase, and NADPH) is a ubiquitous thiol oxidoreductase system that regulates cellular reduction/oxidation (redox) status. The oxidation mechanism for disease pathogenesis states that an imbalance in cell redox state alters function of multiple cell pathways (Yamawaki et al. 2003). The flavin containing thioredoxin reductase (EC 1.6.4.5) is an ubiquitous enzyme able to reduce $O_2^{\bullet-}$ and NO by using thioredoxin as a substrate.

Glutathione peroxidases (GPx) remove H_2O_2 by coupling its reduction to H_2O with reduced glutathione, GSH. GPx-s are present in human cells in concentrations often in the millimolar range and can act on peroxides other than $H_2O_2^{\bullet}$ They can catalyse GSH-dependent reduction of fatty acid hydro-peroxides, but cannot act upon fatty acid peroxides esterified to lipid molecules in liposomes or membranes: they have to be first released by lipases. GPx contains selenocysteine in the active site (Halliwell and Gutteridge 1999). Glutathione reductase enzymes reduce glutathione disulfide (GSSG) back to GSH, most of the NADPH needed for this reaction is generated in the oxidative pentose phosphate pathway (Halliwell and Gutterigde 1999). Molecules containing cysteine residues (the sulfhydryl groups) are easily participated in thiol-disulfide exchange. Therefore, oxidation of GSH to GSSG occurs. GSSG can exchange with protein sulfhydryls to produce protein-glutathione mixed disulfides (proteinSSG). Glutatathione peroxidases (GP) create a disulphur bond between two GSH molecules to form oxidized glutathione (GSSG). GSH reductase then catalyses the formation two molecules of GSH from one molecule of GSSG. The four GP enzymes are: (i) classical GPx-1; (ii) gastro-intestinal GPx-GI; (iii) plasma GPx-P; and (iv) phospholipid hydroperoxide PHGPx (Jefferies et al. 2003).

Superoxide dismutase (SOD) enzymes are important intracellular anti-oxidants that remove the superoxide radical catalytically (no other substrate has been detected) (Halliwell and Gutteridge 1999). Copper-zinc-containing superoxide dismutases (CuZnSODs) are present in virtually all eucayotic cells and in many procaryotes. In animal cells, most CuZnSOD is located in the cytosol, but some appears in lysosomes, peroxisomes, nucleus and the space between inner and outer mitochondrial membranes bound to inner membranes of mitochondria (Halliwell and Gutteridge 1999, Inoue et al. 2003).

Manganese SODs (MnSOD) catalyse essentially the same reaction as CuZnSODs, but in most animal tissues they are largely (if not entirely) located in the mitochondria (so erythrocytes don't contain it) (Halliwell and Gutteridge 1999).

Hydrogen peroxide is usually removed in aerobes by two types of enzyme. The catalases directly catalyse decomposition of H_2O_2 to O_2. Peroxidases remove H_2O_2 by using it to oxidize another substrate. In humans catalase is present in all organs, being especially concentrated in liver. In animal cells catalase mainly is located in peroxisomes (that contain many of the cellular enzymes that generate H_2O_2. Mitochondria contain little, if any, catalase (Halliwell and Gutteridge 1999). Because mitochondria are the major site of free radical generation, they are highly enriched with enzymes, such as Mn-type superoxide dismutase in matrix, and antioxidants including GSH on both sides of inner membranes, thus minimizing oxidative stress in and around this organelle (Inoue et al. 2003).

Vitamin C has considerable antioxidant activity in vitro, in part because of its ease of oxidation and because the semialdehyde ascorbate radical derived from it is of low reactivity. Ascorbate is a good reducing agent. Donation of one electron by it gives the semidehydroascorbate (or ascorbyl) radical. Loss of a second electron converts this radical to dehydroascorbate, an unstable molecule. Metabolic pathways exist that can recycle ascorbyl radical and dehydroascorbate back to ascorbate, using NADH or GSH as sources of reducing power. Dehydroascorbate can also decompose non-enzymically, yielding in a range of products. Ascorbyl radical is fairly unreactive, and this poor reactivity is the essence of ascorbate's antioxidant effects. Essentially, a highly reactive free radical (e.g. $RO^{\bullet}$, $RO_2^{\bullet}$, $OH^{\bullet}$, $NO_2^{\bullet}$) is reduced by ascorbate, and the new (ascorbyl) radical so generated is poorly reactive. Ascorbate can also scavenge several non-radical reactive species, such as hypochlorous acid, ozone and nitrating agents derived from peroxynitrite. Ascorbate is present in human plasma at levels usually in the range 20–90 μM. Higher levels have been reported in cerebrospinal fluid and gastric juice. Intracellular ascorbate levels may be in the mM range, at least in some cell types. One largely unexplored area is the sub-cellular distribution of ascorbate: since it can directly reduce several cytochromes and other biomolecules in vitro, ascorbate might have to be excluded from certain sub-cellular compartments. The ranges of ascorbate levels in vivo, both extracellularly and intracellularly, are within the concentration ranges that are capable of exerting powerful antioxidant effects in vitro (Halliwell 2001). Consumption of foods rich in vitamin C (fruits and vegetables) is associated with decreased risk of cardiovascular disease, of many types of cancer and possibly of neurodegenerative disease, but the extent of which vitamin C contributes to these effects is uncertain (Halliwell 2001).

Ferritin sequesters iron ions, while ceruloplasmin sequesters copper ions so that these ions are not available to catalyse the Haber–Weiss reaction generating $^{\bullet}OH$ or to perform the decomposition of hydroperoxides. Ceruloplasmin has

also ferroxidase activity: it oxidizes Fe^{2+} to Fe^{3+} and so inhibits $^{\bullet}OH$ formation from H_2O_2 and iron dependent lipoperoxidation (Mates et al. 2000).

Animal studies have shown that circulating cells exhibit membrane alterations when there is a lack of vitamin E in the diet. The mitochondrial membranes of the reticulocytes and lymphocytes appear bloated and disintegrated; the number of platelets, which are more adhesive, increases and produces more thromboxanes in comparison with those of normal rats. The response of T and B lymphocytes to mitogenic stimuli, the mixed lymphocyte reaction and the number of cells forming plates are considerably depressed. On the contrary, carotenoids and in particular beta-carotene either directly or indirectly as precursor of vitamin A, enhance T cell proliferation and cytotoxicity, macrophage killing, and TNF-α secretion. β-carotene is a powerful scavenger of $^1O_2$ (Mates et al. 2000).

Glutathione system in different conditions

One of the major mechanisms of protection against carcinogenesis, mutagenesis, and other forms of toxicity mediated by carcinogens is the induction of enzymes involved in their metabolism, particularly glutathione-S-transferase (GST), uridine diphosphate-glucuronosyl transferases, and quinone reductases. 40–60% of the human population are homozygous for deletion of the entire gene (GSTM1*0). The GSTM1-null phenotype has been associated with an increased risk for lung cancer in heavy smokers and for colorectal cancer. The GSH system provides a link between environmental insults and cancer. Diet can influence the level of expression GSH related isoenzymes and thereby affect the risk of cancer (Jefferies et al. 2003).

The main biochemical steps during inflammation, the production of cytokines and acute phase proteins, strongly modify the GSH pool. The GSH participates in many important physiological processes controlling the homeostasis of the cells. A higher demand of cysteine (Cys) supply causes difficulties in maintaining a constant GSH level (Santangelo 2003). The concentrations of whole blood glutathione and total thiols have been found to be significantly lower in patients of RA, as compared to healthy controls (Jaswal et al. 2003).

There has been found an inverse association between GSH and systolic blood pressure in untreated hypertensive subjects (Muda et al. 2003).

The correlation between MDA and GSH has been established in a small survey on acute myocardial infarction patients but no correlation with cardiac enzyme concentrations was detected in these settings. The persons with acute myocardial infarction had GSH levels significantly lower than age- matched controls (Kharb 2003).

Polymorphism has been observed in key enzymes of GSH metabolism and some alleles have been associated with an impaired redox buffer system downsteam diseases, and susceptibility to ischaemia (Jefferies et al. 2003).

Members of the glutathione-S-transferase (GST) super family catalyse the conjugation of GSH with target compounds to form GSH conjugates. These GSH conjugates are converted to mercapturic acids for excretion. These structurally related molecules are involved in diverse functions that relate to the heat-shock response, the detoxification of electrophiles, drug resistance, carcinogenesis, and immunomodulatory functions (Jefferies et al. 2003).

Serum gamma-glutamyl transferase (GGT) that normally catalyses the first step in the degradation of extracellular GSH is used as an index of cholestatic liver disease, high alcohol consumption, and the use of enzyme-inducing drugs. Increases in serum GGT lead to an increase in the production of free radicals, particularly in the presence of iron. It has been proposed that the catabolism of GSH can play a prooxidant role in selected conditions. Paolicchi et al. have suggested that the GGT-mediated cleavage of GSH—allegedly through the generation of the more reactive thiol cysteinyl-glycine—could cause the reduction of ferric iron Fe(III) to ferrous Fe(II), thus starting an iron redox-cycling process liable to result in the production of ROS and stimulation of oxidative reactions. GGT has been shown to stimulate a GSH-dependent lipid peroxidation in model systems involving Fe(III) complexes as redox catalysts and purified linoleic acid peroxidizable substrate (Paolicchi et al. 2002). People with high serum GGT levels have an increased risk of death because there is an association between GGT and other risk factors, and GGT itself is an independent predictor of risk (Jefferies et al. 2003).

So it can be concluded that alterations in the functioning of the central intracellular antioxidant glutathione and glutathione-linked systems have been described in pathological conditions of different pathogenetic mechanisms and organ systems.

AIMS OF THE PRESENT STUDY

Oxidative stress is a double-edged sword: at low-grade it is necessary in human organism, but it becomes excessively activated in many pathological conditions. As literature (and previous investigation projects of Tartu University Department of Biochemistry) have shown the evaluation of oxidative-stress-linked events and situations has to be multicomponent and systemic as there is no universal parameter that can be interpreted without appropriate context.

The work consisted of several studies to assess systemically the OxS-associated impact of alimentary iron overload – the two model systems were selected as no investigations alike have been published but the situations described in these model systems are quite common in today's medical routine praxis. The other branch of investigations was aimed on finding out if OxS-related parameters (antioxidative or pro-oxidative) have any predictive value in the process of post-angioplasty restenosis. So the aims were defined as follows:

1. To measure the affect of chronic alimentary iron overload on the markers of OxS in a model system of a village population.
2. To assess the influence of supplementary peroral ferrous iron on the parameters of OxS during uncomplicated pregnancy in the situation of borderline anaemia with no additional risk factors for profound OxS.
3. To assess if any of the parameters of OxS or their combination measured preprocedurally is informative in predicting restenosis after coronary angioplasty.
4. To summarize information gathered on the basis of aims 1 to 3 via comparision of the expression and level of OxS in other quite different clinical groups (atopic and contact dermatitis, Alzheimer's disease) to extract iron- and glutathione-targeted outcomes for routine clinical medicine.

MATERIALS AND METHODS

In different sets of investigation the subjects were 35 clinically healthy adults from a village in Southern Estonia whose everyday tap water contained iron up to 9 times more than the maximum allowed content; 13 clinically healthy pregnant nonsmokers with a borderline anaemia who had been prescribed oral ferrous supplements and 6 matched women as their control; 54 patients of coronary angioplasty in whom we evaluated the prognostic value of markers of OxS towards restenosis; in 6 subjects with allergic or irritant contact dermatitis and in 5 subjects with Ni-positive patch test sites in lesional area and contralateral control area the parameters of iron metabolism and oxidative stress were measured; in 12 chronic hand dermatitis patients (and 4 control subjects) the same parameters were determined in evaluation of the treatment effect of pure emollient crème and its combination with a glucocorticoid cream; in the postmortem brains of 13 sporadic Alzheimer's disease (AD) patients and 3 familial AD patients in comparison of 8 control subjects the parameters of OxS were quantitated.

The methods used in these sets of investigation are components of the OxS-describing complex investigation in the Department of Biochemistry.

To be more specific, lipid peroxidation is being evaluated by the following parameters (that are combined in different studies based on the specific conditions of the evaluated process): thiobarbituric acid reactive substances – malondialdehyde and others (TBARS), conjugated dienes (CD), low-density-lipoprotein baseline oxidation (LDL baseline) and LDL resistance to oxidation lag phase (LDL lag phase).

To quantitate the oxidative damage to protein fraction, protein carbonyl groups (presented to mg protein, PC) are measured.

From antioxidative enzymes catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GRed) and glutathione peroxidase (GPx) are measured.

The overall antioxidativity is described by two methods: total antioxidative status (TAS) and total antioxidative activity (TAA). The concentration of vitamin C in fluids can be measured.

Intracellular OxS is described by glutathione redox ratio (oxidized/reduced glutathione mg/mg) and the absolute amounts of glutathione fractions (GSSG, GSH, tGSH).

Conjugated dienes (CD), LDL lag phase and thiobarbituric acid reactive substances (TBARS)

Conjugated dienes (CD), thiobarbituric acid reactive substances' content (TBARS) and LDL lag phase describe mainly the situation of OxS in the lipid fraction. CD are a form of compounds that are constructed from unsaturated fatty acids by free radical reactions. LDL lag phase describes the period during which the non-HDL-fraction of a sample/a subject is resistant to oxidation in the standard oxidation conditions.

CD were measured according to Recknagel and Glende (1984), described in detail in Kullisaar et al. (2003). Briefly freshly prepared serum was frozen at –80ºC with 16.8 nM BHT (butylated hydroxytoluene, final concentration) and analysed within a month. Samples (150µl) + 150µl 0.9% NaCl were incubated at 37º C for 30 min, 0.25% BHT (15µl) was added, the samples were extracted with heptane/isopropanol (1:1, whole volume 1800µl) and acidified by 500µl 5N HCl. After extraction with cold heptane (1600µl), samples were centrifuged (for 5 min at 3000 rpm) and absorbance of heptane fraction was measured at 234nm (spectrophotometer Jenway 6405).

LDL lag phase was calculated from the curve that is formed from the results of measuring the protein-adjusted quantity of the non-HDL fraction (prepared from EDTA-plasma) peroxidation in time spectrophotometrically at 234 nm (Esterbauer et al. 1992, Zhang et al. 1994). Briefly the lipoproteine fraction (non-HDL-fraction, LPF) was precipitated from 2 ml twice-diluted EDTA-plasma by adding 0.2 ml precipitation reagent (2% dextran sulfate: $MgCl_2$ (2M, pH 7.0) 1:1 v/v), vortexing for 1 min and centrifuging at 1500 g for 10 min. In order to remove EDTA from the LPF the pellet was suspended in 2 ml 0.9% PBS and reprecipitated by adding 0.1 ml precipitation reagent, vortexed and centrifuged. The precipitated LPF was dissolved in 2 ml 4% PBS and this solution was used immediately. The protein content in LPF was assayed by the method of Lowry et al. (Lowry et al. 1951). The protein concentration of EDTA-free LPF was adjusted to 2 mg protein/ml. The oxidation was initiated by the action of a freshly prepared aqueous solution of $CuSO_4 \bullet 5\ H_2O$ (final concentration 45 µM) to the LPF (2 mg protein/ml) and the oxidation of this fraction was evaluated by continuously monitoring the formation of conjugated dienes at a maximum absorbance at 234 nm with different intervals of incubation at 37ºC. The kinetics of the diene formation (the increase of the absorbance versus time) can be devided into three phases: lag phase (during which the diene absorption increases only weakly), propagation phase (rapid increase of the diene absorption) and decomposition phase. The resistance to oxidation was defined as the length of the lag phase, calculated from the interval between the intercept of the tangent of the slope of the curve with time-scale axis.

Lipid peroxidation can be also assessed via the basal level of thiobarbituric acid (TBA) reactive substances (TBARS).

TBARS was measured by method compiled on the ground of the method described by Ohkawa and our experiments (Ohkawa et al. 1979, Starkopf et al. 1995). Several methodological modifications were introduced, such as heating samples up to 80°C (instead generally used boiling), acidification, immediately adding an adequate concentration of an antioxidant (butylated hydroxytoluene, BHT) to the samples etc. to suppress artefactual changes during handling and assay procedures. All assays were triplicated. All samples were usually analysed the same day within 4 hours (samples are also stable at –18°C for 2–4 days).

Samples (250µl) plus 250µl 0.9% NaCl (reagent blank contains only isotonic saline) were incubated in small glass vials at 37º C for 30 min, 0.25% BHT (15µl) was added to interrupt the reaction and pH of the mixture was acidified with 500 µl acetate buffer (mixture of 0.2N acetic acid and 0.2 N CH_3COONa to get a buffer with pH 3.55–3.65 (at 35ºC). Then fresh 1% TBA (1000µl) solution was added, heated at 80º C for 70 min and after carefully cooling (in ice cold water for 5 min) the reaction mixture was acidified by cold 500µl 5N HCl. After extraction (5 min, shaking at minimum for 2 min) with ice-cold butanol (1700 µl), samples were centrifuged (for 10 min at 3000 rpm) and absorbance of the butanol fraction was measured at 534 nm (colour is stable during 30 min). In the case of Fe-TBARS 100µl pro-oxidant solution 2.374 mM $FeSO_4$ x $7H_2O$ (finally 475µM Fe^{2+}) was added to 250µl sample + 150µl 0.9% NaCl (reagent blank contains isotonic saline 400µl and 100µl pro-oxidant solution 2.374 mM $FeSO_4$ x $7H_2O$), incubated (37ºC, 30 min.) and after adding 0.25% BHT (15µl) assessment follows as for TBARS. Tetraetoxypropane has been used as internal standard for MDA.

Serum iron parameters (Fe-s, UIBC, TIBC and %sat; ferritin)

Serum iron (Fe), unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC) and saturation of iron-binding proteins (%sat) were measured with a manual spectrophotometrical assay (kit 565-A, Sigma St. Louis Mo) that is based on ferrozine, a sulfonated derivative of diphenyltriazine, forming a water-soluble magenta complex with iron. For the determination of the tissue iron content (Fe) and unsaturated iron binding capacity (UIBC) we also used the Sigma 565-A kit. We took 250 µl of the tissue homogenate, treated it as in the kit protocol, the only modification being centrifugation of the tubes for 10 min at 3000 RPM before every measurement.

Ferritin was analysed by the chemiluminescence method (the analysis was performed in Tartu University Clinics' joint laboratories on the Immulite analyser).

The interpretation of the parameters and indicativity please see in "The pro-oxidants, special emphasis on iron", 32–33.

Glutathione (GSH, GSSG, GSSG/GSH, tGSH)

Glutathione (GSH) and its fractions measured in whole blood indicate mainly the situation of oxidative stress within the cells (including RBCs and endothelial cells). Measurements of glutathione are actually measurements of thiols in whole blood (a modified method of Bhat) (Bhat et al. 1992). The method is based on the formation of a chromophoric product from the sulfhydryl reagent 5,5-dithiobis- (2-nitrobenzoic acid), Ellmann reagent, in the presence of GSH. The contents of total and oxidized glutathione are directly measured and the content of reduced glutathione is calculated from them.

Stock solutions of various regents were made in 0.1 M sodium phosphate/0.005 M EDTA buffer, pH 7.5. Solution of GSH was prepared before use in cold 0.01 N HCl.

Determination of total glutathione content (GSH plus GSSG) of whole blood was made as follows. 10µl whole blood (heparinized) was mixed with 0.99 ml of 0.2 M sodium phosphate buffer (pH 7.5), containing 0.01M EDTA for hemolyze, and let to stand for 10 min. To 50µl of hemolyzate 500µl 0.2 M sodium phosphate buffer, containing 0.01 M EDTA was added. The reaction was initiated with 0.3 mM NADPH and 0.5 U glutathione reductase (GSH-RED) and continued for 6 min. After that there were added 1ml 0.2 M sodium phosphate buffer, containing 0.01 M EDTA and 100µl 1mM 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.2 M sodium phosphate buffer. After 3 min the extinction was recorded spectrophotometrically at 412 nm (spectrophotometer Jenway 6300). The glutathione content was calculated on the basis of the standard curve obtained with known amounts of glutathione (GSH). Glutathione was expressed as µg/ml.

To assay for GSSG, 10µl whole blood (heparinized) was mixed with 2µl 4-vinylpyridine and kept at room temperature for 1 h. Then 0.99 ml of 0.2 M sodium phosphate buffer (pH 7.5) was added. To hemolyzate (50µl) there was added 500µl 0.2 M sodium phosphate buffer, containing 0.01 M EDTA. The reaction was initiated with 0.3 mM NADPH and 0.5 U glutathione reductase (GSH-RED) and continued for 6 min. After that there was added 1ml 0.2 M sodium phosphate buffer, containing 0.01 M EDTA and 100µl 1mM 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.2 M sodium phosphate buffer. After 3 min

the extinction was recorded spectrophotometrically at 412 nm. GSH concentration was calculated as the difference between total glutathione and GSSG.

Reduced glutathione (GSH) is a tripeptide (glycine-cysteine-glutamate) and oxidized glutathione (GSSG) consists of two tripeptides connected with a disulfide bond. Thus they form a redox-pair. As the molecule of GSSG is only two H-atoms smaller than two molecules of GSH, we believe tat when describing the redox ratio of glutathione, the formula μg of GSSG/μg GSH is more representative than mmol GSSG/mmol GSH.

Another mehod to measure red blood cells glutathione (RBC-GSH, tGSH) was used in some situations and mainly in the earlier years. The method does not give an opportunity to quantitate the fractions and redox ratio.

RBC-GSH was measured with Ellman reagent (Ellman 1959) as described in literature (Beutler et al. 1963). Briefly, tridistilled water (1800μl) and 1000μl of 500 mM perchloric acid were added to 200μl blood to hemolyse RBCs and remove protein. After 5 minutes procedure protein sediment was eliminated by centrifugation for 10 min at 3000 rpm. Then 600μl of supernatant was taken, 700μl Na_2HPO_4 (300mM) and 150μl Ellman reagent were added. This mixture was incubated for 10 min at 37°C. The absorbance of yellow dye was measured at 412 nm. The content of GSH was calculated on the ground of standard plot (1–20mg of GSH/dL).

GSH mg/dl RBCs = GSH conc. (from standard plot)/ hematocrit.
The reference value is 57–80 mg GSH per deciliter RBCs.

Catalase (CAT)

Catalase (CAT) is one of the most important intracellular antioxidant enzymes that together with superoxide dismutase control the level of intracellular free radicals – the role of catalase being neutralizing hydrogen peroxide.

CAT was measured by a method of Góth (1991a), the method being spectrophotometrical based on hydrogen peroxide forming a stable complex with ammonium molybdate.

Heparin serum was used. In the case of hemolysis it is necessary to determine serum (10μl of serum is necessary) hemoglobin concentration with the bensidine method. If haemoglobin concentration is more than 100mg/l the sample must be discarded (significantly haemolyzed sample gives an artificial increase of catalase activity).

200μl of serum was incubated in 1000μl substrate (65 μmol/ ml H_2O_2 in 60 mmol/l sodium-potassium phosphate buffer, pH 7.4) at 37°C for 60 s. The enzymatic reaction was stopped by adding 1000μl of 32.4 mmol/l ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) and the yellow complex of molybdate and

H_2O_2 was measured at 405 nm against control blank (1200µl buffer, 1000µl molybdate; control of enzyme: molybdate was added prior to the enzyme; control of substrate: 0,2 ml of buffer was added instead of the enzyme). Catalase activity kU/l was expressed as:

kU/l = (control of enzyme preparation- sample) / control of substrate) • 271

Superoxide dismutase (SOD)

A special kit RANSOD (Randox Laboratories LtD) was used. RANSOD allows to analyse the RBCs levels of SOD. RANSOD employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with special reagent (tetrazolium salt, called as I.N.T.) to produce red formazan dye. As SOD, present in sample, competes with I.N.T. for superoxide radicals it is possible to assess SOD by the degree of inhibition of formazan dye formation.

For preparation of RBC's lysate 0.5 ml of whole blood (taken with heparin) was centrifuged for 10 minutes at 3000 rpm and the serum was aspirated off. The RBCs were washed four times with 3 ml of 0.9 % saline with centrifugation for 10 minutes at 3000 rpm after each wash. The washed centrifuged RBCs were frozen (–18°C for 24 hours). After that RBCs were haemolyzed by 2.0 ml cold milliQ water, mixed and left to stand at +4°C for 15 minutes. The lysate was diluted with 0.01M pH 7.0 phosphate buffer, so that the inhibition was between 30% and 60% (a 25 fold dilution of lysate for human samples, dilution factor = 100).

0.05 ml of diluted sample and 1.7 ml of mixed substrate were pipetted into a cuvette (at 37°C) and mixed. Phosphate buffer instead of the sample was used as reagent blank. After mixing 0.25 ml of xanthine oxidase was added.

After mixing the initial absorbance A_1 (at 505nm) was read after 30 seconds and the timer started simultaneously. Final absorbance A_2 was read after 3 minutes. SOD activity was calculated and given as SOD U/g haemoglobin.

Glutathione peroxidase (GPx)

A special kit RANSEL (Randox Laboratories LtD) was used. RANSEL allows to analyse the levels of GPx in whole blood, RBCs and platelets.

RANSEL is based on the reaction of GSH with Cumene hydroperoxide (ROOH) catalysed by GPx and yielding in GSSG. The latter is converted by glutathione reductase (GSHRed) and NADPH to GSH and NADP. The concentration of GPx is assessed from the decrease in absorption at 340nm due to the oxidation of NADPH to NADP.

0.05 ml heparinized whole blood was diluted by 1 ml diluting agent, incubated for 5 minutes and 1 ml of double strength Drabkin's reagent (Drabkin's reagent: 0.0016 M KCN; 0.0012 M $K_2Fe(CN)_6$; 0.0238 M $NaHCO_3$) was added.

0.02 ml of diluted sample, 1.00 ml of the reagent and 0.04 ml of Cumene were pipetted into a cuvette (at 37°C). MilliQ water was used instead of a sample as reagent blank.

After mixing the initial absorbance was read at 340nm against air after one minute and the timer was started simultaneously. After 1 and 2 minutes the absorbance was read again. Reagent blank value was substracted from that of the sample. GPx activity was expressed as U/g Hgb.

Total antioxidant activity (TAA) and status (TAS)

Total antioxidant activity (TAA) describes the overall antioxidant potency of a medium. In case of measuring it in serum in a situation of no remarkable tissue damage it mostly reflects the extracellular/intravasal antioxidant activity. TAA (an original method of our Dept. of Biochemistry, Starkopf et al. 1995) is the percentage a sample inhibits a standard linolenic acid peroxidation.

For LA-standard stock LA (alfa-linolenic acid, from Sigma, code L2376) was dissolved (10 µl of linolenic acid was added to 1000µl 96% ethanol drop by drop (permanent mixing). This solution was kept for 2 days at –18°C in dark in a carefully closed glass vial. From this solution 400 µl was dissolved (drop by drop, permanent mixing) in 50 ml warm 40°C isotonic saline. Suitable portions of this solution (LA-standard stock) were frozen in carefully closed glass vials and the content of one vial was used as a standard in one experiment.

0.15µl 0.35% SDS was measured into a vial and 400 µl of heated (up to 40°C) LA-standard stock was added to get a homogenous solution of LA-standard. Then to a sample (30µl of serum diluted 1:3.3 in isotonic saline) 100 µl (final concentration 200 µM) $FeSO_4$ was added and incubated in the presence of LA-standard for 60 min at 37°C. Reagent blank contains only LA-standard and isotonic saline. Then 0.25% 35µl BHT was added and the mixture was treated with 500µl acetate buffer to acidified mixture (pH 3.55–3.60) and heated with fresh 1% TBA solution (1000µl) at 80°C for 40 min. After cooling for 5 min in ice cold water the reaction mixture was acidified by cold 500 µl 5 N HCl, extracted with cold butanol (1700µl), centrifuged (for 10 min at 3000 rpm) and absorbance of the butanol fraction (amount of TBARS) was measured at 534 nm. The TAA of a sample was expressed (%) as inhibition by the sample of LA-standard peroxidation as follows:

$$[1 - (A_{534}\ (\text{sample}) / A_{534}\ (\text{LA as control})] \bullet 100$$

In the earlier years this parameter was termed antioxidative status (AOS).

For determination of total antioxidant status (TAS) we used a kit (Randox Laboratories LtD, Cat. No. NX2332). A specific agent ABTS (2,2'-azino-di-benzthiazoline sulphonate) is incubated with a peroxidase (metmyoglobin) and hydrogen peroxide to produce the radical cation $ABTS^{\bullet+}$. This has a relative stable blue-green colour, and can be detected at 660 nm. Antioxidants in the added sample cause suppression of this colour production to a degree that is proportional to their concentration. The values are expressed as Trolox units (0–2.5 mmol/l). Trolox is a water-soluble vitamin E analogue.

Protein carbonyl content (PC)

Protein carbonyl (PC) measurement is the most widely accepted possibility to evaluate the oxidative damage to the protein fraction as the content of carbonyl groups increases in free radical induced reactions. The information identifies oxidative damage to proteins that may preceed the clinical symptoms of a disease (Perry et al. 2003, Dalle-Donne et al. 2003b). The usage of protein CO groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins. Most of the assays for detection of protein CO groups involve derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl (DNP) hydrazone product. This then can be detected by various means, such as spectrophotometric assay, enzyme-linked immunosorbent assay (ELISA), and one-dimensional or two-dimensional electrophoresis followed by Western blot immunoassay (Dalle-Donne et al. 2003a).

PC were quantified by a compiled method based on the methods of Levine et al. (1990) and Grattagliano et al. (1996) with our little modification. 0.050 ml freshly prepared plasma (approximately 3 mg of protein) was precipitated with 0.200 ml of 10% TCA and after centrifugation for 10 min at 3500p the pellet was treated with 0.5 ml 10 mM 2,4 DNPH in 2M HCl or only with 0.5 ml 2M HCl as a control blank. Samples were incubated for 60 min at room temperature with continuous mixing. Next, 0.5 ml of 20% TCA was added and proteins were precipitated by centrifugation for 10 min at 3500p and the supernatant was discarded. The pellet was washed 3 times with 1ml 1:1 ethanol/ethyl acetate (centrifugation was followed after 10 min standing of the mixture). The final precipitate of protein was dissolved in 0.6 ml 6M guanidine/20mM K-phosphate buffer, pH 2.3 for 30 min at 37°C with vortexing. Any insoluble materials were removed by centrifugation. The different spectrum of the DNPH-derivates versus HCl controls was followed spectrophotometrically at 350–380nm with a scan program. The concentration of carbonyl groups (CC) was calculated from

the spectrum maximum, using a molar absorption coefficient (e) of 22 mM – 1cm–1 as the extinction coefficient for aliphatic hydrazones.

CC (nmol/ml) = Abs365(max) (Test-Blank) x 10^6/ e

Protein content (mg/ml) was determined in each sample (parallel) versus guanidine and calculated from a bovine serum albumin standard curve dissolved in guanidine-HCl and read at 280 nm (Lyras et al. 1996). The carbonyl content was expressed as nmol carbonyl/ mg protein (Cao and Cutler 1995). Reactive carbonyls (nmol/ mg protein) were in the range 0.3…2.50.

When analyzing tissue samples the difference is in the initial stages: tissue samples were homogenized 1:10 in Hepes buffer with aprotinin (freshly prepared), centrifuged after 15 minutes standing at 4000p at 4^0C for 10 minutes, the supernatant was mixed with streptomycine sulphate (final concentration 1%), allowed to stand at room temperature with vortexing every 3 minutes for 15 minutes and precipitated by centrifugation at 4^0C for 10 minutes at 4000p. 0.100 ml of supernatant was mixed either with 0.400 ml 10mM DNPH in 2M HCl or 0.400 ml 2M HCl alone as control blank. The following steps were identical to the procedure with plasma.

Biochemical studies have demonstrated increased DNPH-derived adducts by assay of tissue homogenates from normal ageing and Alzheimer disease (Smith 1991). However, in light of the large number of free carbonyls in the vasculature of all samples, particularly of the aged, the specificity and sensitivity of whole-tissue analysis to detect disease related increases, particularly for an age-related disease, are limited. Therefore, disease-related neuronal damage could be masked by damage to vessels when whole-tissue homogenates are used (Smith et al. 1998). Human diseases associated with protein carbonylation include Alzheimer's disease, chronic lung disease, chronic renal failure, diabetes and sepsis (Dalle-Donne et al. 2003).

Vitamin C in serum (VitC)

The content of vitamin C in serum was measured with a ferrozine method based on Butts and Mulvihill's method (1975) modified by Miller et al. (unpublished material, acquiered by personal contacts).

Ferrous chromogenic methods depend on the reduction of Fe^{3+} to Fe^{2+} ions by ascorbate, which is simultaneously converted to dehydroascorbate. The formed Fe^{2+} ions are subsequently reacted with a chelator (such as dipyridyl or Ferrozine) and the resulting complex quantitated spectrophotometrically. Other slower-acting reducing substances may also generate Fe^{2+} from Fe^{3+}, but not to a significant extent during a 4.5–5 second period.

The ferrozine method measures only reduced ascorbic acid. The method can be applied to investigate plasma or serum and urine.

The needed reagents were: 1) Ascorbic acid standard (10, 25, 50, 75 and 100μmol/l for standard curve and 50 or 100μmol/l for every set, (2) Ferric ammonium sulphate dodecahydrate, 8.3 mmol/l in 20 mmol/l sulphuric acid, (3) Ferrozine [3-(2pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine] 2.23 mmol/l in acetate buffer 367 mmol/l, pH 4.0. Working colour reagent was prepared 1:9 from (2) and (3) *ex tempore*.

For sample preparation 200 μl of 10%TCA was taken and 50μl of plasma was added with vortexing and centrifuged 10 min at 3000 rpm.

To 100 μl of sample, standard or blank, 300 μl of diluent (Aq dest) was added, incubated 60 seconds at 37°C, 1000 μl of working colour reagent (37°C) was added and the absorbance was measured at 550 nm at exactly 5 seconds.

A standard curve was drawn once for every set of reagents and two standards (50 and 100 μmol/l) in every set of samples was included to be sure that the standard curve fitted.

The concentration of ascorbic acid was read from the standard curve. The result was multiplied 5 times (dilution coefficient) to get the actual concentration in the sample. Reference range of this method is 75–150 μmol/l for plasma.

Dissertant performed self-handidly the approbation and compilation of the methods of measuring carbonyl groups in serum and homogenates and measuring iron and UIBC in homogenates. All the measurements of PC, Fe, UIBC and vitamin C described in this dissertation are performed by the dissetant.

Statistical analysis

Statistical analysis was performed with Microsoft Excel 2000 (average, standard deviations, Student's t-test for unpaired data between groups) and the indices that showed significant difference with this test were controlled with one-way ANOVA test by the help of Microcal Origin 3.5 programme (Microcal Software Inc.) in one set of experiments.

RESULTS

A long-term presence of excess iron in drinking water may lead to positive iron balance in an organism that becomes apparent in the measurable impairment of redox balance towards oxidative stress. In our investigation the subjects with alimentary iron overload had higher iron content of serum than endemic average (22.8±7.3 compared to 16.1±1.8μmol/l in women and 25.8±7.3 compared to 20.2±1.9 μmol/l in men), at the same time total iron binding capacity tended to be lowered (meaning on the account of unsaturated iron binding capacity) and the transferrin content was unaltered. CD and TBARS levels were significantly higher in subjects with alimentary iron overload than endemic normal (age-matched subjects of healthy citizens of Tartu) (Publication I). The RBC glutathione was significantly lowered in the iron-overloaded group (50.4±13.4 mg/dl compared to endemic normal 64.8±9.4 mg/dl, $p<0.001$).

The effect of peroral iron supplement preparation (to be precise the preparation was Spartocine from USB Pharma, Belgium containing ferrous aspartate) on the markers of oxidative stress measurable in venous blood was evaluated in a group of healthy pregnant women with borderline anaemia in cooperation with Tartu University Clinic of Obstetrics and Gynaecology (Publication II).

In agreement with previously published data (Raijmakers et al. 2001, Little and Gladen 1999) we found that the content of conjugated dienes and glutathione redox ratio tend to increase as pregnancy progresses. The content of carbonyl groups and TAA stayed on the same high level (an adult normal range for TAA is 38±3.7%). As for CAT there was much diversity but the means did not differ from each other and from the endemic normal. Compared to endemic normal CD were relatively high (45.4 to 53.9 in different groups versus 38.4±7.5μM). LDL lag phases in turn were relatively short when compared to endemic normal (30.0–38.5 versus 49±10 min). No statistically significant correlations between individual parameters were found – including the tested correlations between glutathione redox ratio and any of the measured iron parameters.

No significant differences between groups were seen before the test period except the difference in haemoglobin content – although all the subjects included had haemoglobin values between 97 and 111 g/l the doctors seem not to resist the urge to treat a woman with haemoglobin smaller than 105 g/l. The only parameter that gave $p<0.05$ after 4 weeks was GSSG – but both groups had a roughly 2-fold increase in GSSG content that affected tGSH value also. Altogether, the GSSG/GSH ratio was high (0.76±0.28) from the beginning in both groups, the endemic normal being 0.17. Mostly the value could be attributed to relatively low content of GSH (the endemic normal being 195±17μg/ml).

Table 1. The overall dynamics of parameters during the investigation period in the study group of healthy pregnant nonsmokers with borderline anaemia.

Parameter	Before	After	p
Hgb, g/l	106.1±6.4	107.8±7.1	0.30
Serum Fe, mmol/l	14.7±9.6	14.1±10.1	0.31
UIBC, mmol/l	50.4±17.4	50.7±17.4	0.27
%sat	24.2±17.4	21.9±14.3	0.22
Ferritin ng/ml	13.1±13.1	10.0±8.3	0.08
CD, μM	47.4±13.5	53.8±17.5	0.06
TAA, %	46.1±5.0	44.5±5.5	0.15
CAT, kU/l	50.4±38.1	46.2±26.9	0.28
PC, nmol/mg prot	2.02±0.42	1.91±0.37	0.18
Glutathione, μg/ml	174.9±64.1	292.4±74.7	0.0002 **
GSSG, μg/ml	71.9±30.3	132.2±41.6	0.000015 **
GSH, μg/ml	102.9±43.0	160.2±65.1	0.01*
GSSG/GSH	0.76±0.28	1.03±0.64	0.03 *
LDL lag phase, min	33.4±10.3	34.2±5.8	0.44

Serum Fe in both groups was about 14 mmol/l after the investigation period, but the "starting points" appeared to be slightly (though not significantly) different. Ferritin was about at the level of anaemia criteria (that is 12 ng/ml). There were no changes in catalase activity.

To evaluate the value of the gathered information (also including the studies done in cooperation with The Department of Dermatology and Venearology) in prognosing a clinical situation we performed a prospective investigation where we linked the preprocedural values of complex OxS parameters with the possibility of postangioplasty restenosis.

The results (Publication III) revealed that glutathione redox-ratio was the most informative parameter (again) that implies on paramount importance of endothelial factors in the process of restenosis.

As it was predicted there could be found numerous correlations between parameters when subjects were analysed from the point of view of classical risk factors (hypertension, smoking status, diabetes etc).

When analyzing the enrolled diabetic patients compared to non-diabetic patients we found differences in glutathione redox-status (GSSG/GSH 1.92± 2.19 in diabetics versus 0.90± 0.76 in non-diabetics, p=0.03) and LDL-lag phase (64.17±18.58 min in diabetics and 53.89±13.65min in non-diabetics, p=0.03). Smokers were significantly younger (50.7±9.4 versus 59.5±8.1 years, p=0.005) and current non-smokers had significantly higher BMI (28.6± 3.3versus 25.5±2.9, p=0.006). The persons with chronic (extracardial) illnesses had higher TAA (37.7±6.0mmol/l versus 33.8±5.3mmol/l, p=0.03) and they had

been smoking longer (23.3±12.9 versus 13.4± 14.9 years, p=0.03). Hypertensive patients had LDL lag phase shorter than normotensive patients (51.3±15.2min versus 61.2±14.2 min, p=0.04). The activity of GPx in high blood pressure group was lower than in the subjects with normal blood pressure (9.64±7.0 U/g Hgb versus 24.83±18.1 U/g Hgb, p=0.01). Patients with previous myocardial infarction had less CD (41.6±12.6μM versus 54.04±21.5μM, p=0.02) but no difference in LDL lag phase. All subjects but one of those who developed restenosis had stent(s) that represented well the overall use of stents in that period. Two women out of 8 female subjects developed restenosis. The finding that LDL oxidation lag phase is longer (though not significantly) in the restenosis group cannot be explained by the fact that diabetic patients had the same tendencies as only one diabetic patient had confirmed restenosis.

When restricting the analysis to just comparing the persons who had developed restenosis with subjects who had not had aggravation of symptoms in a year the only oxidative stress-related parameters that had significant differences with both statistical methods was the glutathione redox ratio and the activity of GPx. All subjects who had confirmed restenosis had had previously myocardial infarction but not all subjects who had had previous myocardial infarction developed restenosis. Nevertheless having a myocardial infarction in anamnesis was a significant predictor for restenosis (p=0.01). When comparing the restenosis group with clinically stable group we found no difference in cholesterol or HDL-cholesterol. Although we measured also the concentration of vitamin C in blood plasma it proved not to be informative in our settings (the values rangeing from 20–250 μM in the restenosis group and from 50–180 μM in the no-further-complaints group, the averages±SD being correspondinly 125.2±82.7 μM and 104.1±30.7 μM, p=0.16).

The glutathione redox-ratio was significantly higher (that means more oxidised) in the restenosis group (2.21±2.36 versus 0.88±0.74, p=0.002). SOD activity in both groups was similar to endemic normal (that is 722±86 U/g Hgb, unpublished data) so the change of this enzyme's activity would not likely be a common reason for impaired endothelial defence in angioplasty patients. The activity of GPx in the restenosis group was 40.36 ± 35.16 U/g Hgb and 15.01 ± 12.29 U/g Hgb in the clinically stable group (the endemic normal being 45±11 U/g Hgb (unpublished data)) – so the change of the activity of GPx might also not serve as an indicative predictive parameter for restenosis as the restenosis subjects resemble the endemic normal more than the subjects not developing complaints in a year.

The results are associated with another investigation where the dissertant also participated in: the investigation that compared the Swedish genetic and sporadic forms of Alzheimer's disease (AD) (Publication IV). The investigation revealed statistically significantly higher level of OxS in the lesional area of familial (genetically determined) AD. A conclusion was drawn that the higher level of OxS measured by more oxidated glutathione redox ratio and greater

oxidative damage of protein fraction (measured by protein carbonyl groups in tissue homogenates) may have connection with the higher speed of progression of the genetically determined AD.

The cooperation projects with the Department of Dermatology and Venearology gave the following major results: (i) contact dermatitis caused an at least 4-fold increase in the iron level in the positive patch test sites and the indices of iron, TIBC and CD were high in the apparently healthy skin of the patients with persistent hand dermatitis (Publication V), (ii) The treatment of chronic hand dermatitis patients with emollient cream alone or with its combination with glucocorticoids improved the glutathione redox ratio but did not alter the iron parameters (Publication VI), (iii) allergic contact dermatitis increased the blood glutathione redox ratio that could be normalized with an "antioxidant cocktail", (iv) different allergens have differences in the biochemical OxS markers in positive patch-test sites: nickel sulphate rises the skin iron content and glutathione redox ratio but epoxy resin does not (Publication VIII).

When comparing different investigated groups with each other we could see a shift towards OxS in several clinical situations and an enhancement of both antioxidative defence and OxS in the investigated group of pregnant women (Figure 3).

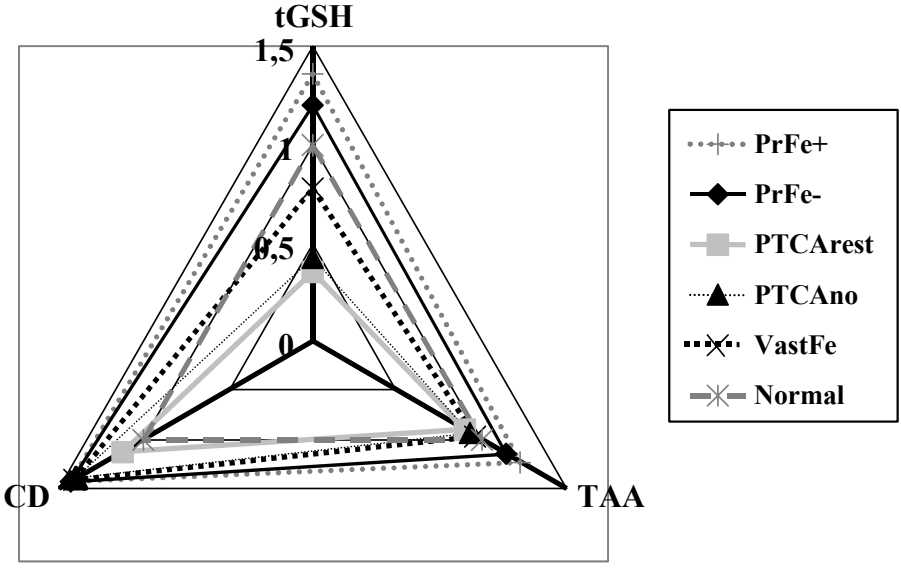

Figure 3. The parameters of different study groups, compared to average of normal (markes as X).
Abrreviations: PrFe+ – pregnant women with iron supplementation; PrFe– – pregnant women without iron supplementation; PTCArest – group of post-angioplasty restenosis; PTCAno – group of no post-angioplasty progression of disease; VastFe – drinking water iron overload group; Normal – endemic average.

DISCUSSION

Oxidative stress (OxS) status has become a matter of growing interest and numerous fundamental and clinical investigations. Oxidation and reduction modulate all cellular events, including modification of signalling molecules in intra- and intercellular communication (Pompella et al. 2003, Halliwell and Gutteridge 1999). HOxS as an important factor in the pathogenesis of numerous pathological conditions should not be unnoticed in clinically healthy populations and subject groups with minimal clinical problems (as borderline anemia in pregnancy) both in clinical medicine and organization of medical care.

Any chronic iron overload is a major cause of organ/tissue failure (including liver, kidney, pancreas, endothelium) worldwide, but its pathogenesis remains to be elucidated, state Bartfay et al. (2003). Even without being so categorical iron, especially ferrous iron, has an important role being present in apoptosis and infection (Boelaert et al. 1996), inflammation (Karatas et al. 2003), carcinogenesis and atherosclerosis (Jefferies et al. 2003). In 1981 Sullivan first proposed the iron hypothesis of atherosclerotic heart disease and Salonen et al. were the first to report the connection between ishaemic heart disease and body iron stores (Salonen et al. 1992). From that point on there have been implications on possible link between the physiological blood loss (meaning also the loss of iron as 1 ml of RBC contains ca 1 mg of iron) of premenopausal women and their lower risk for coronary heart disease when compared to age-matched men (Sullivan 1981, Berge et al. 1994).

The term" successful aging" that comprises maintaining both mental and physical health until the end of life has become of major importance in the aging society – both on the level of an individual and on the level of the whole society. As an undermining factor for successful aging the contribution of HOxS has been described in several processes (from neurodegenerative problems to atherosclerosis, carcinogenesis and skin processes) (Polla et al. 2003). Thus the conclusion drawn by Polla et al. (2003) that iron should be left out from all vitamin- and mineral supplements designed for the elderly – may be the minimum that should be bared in mind in clinical practice.

The chronic alimentary iron overload has not been conclusively described from the point of view of development of OxS (including cellular one). The associations of African iron overload with up to 100 mg of iron from a local beer, associations between alcochol intake and iron and the problems of haemochromatosis (e.g. mutation C282Y) are the main areas of sparse investigation (Crichton and Ward 2003, Heath and Fairweather-Tait 2003). A recent publication revealed that elevated (defined as over 200 ng/ml of serum ferritin) levels of body iron combined with serum elevations of LDL or reduced levels of HDL are associated with significantly higher levels of C-reactive

protein – that has been proved to be an independent risk factor for cardio-vascular disease (Mainous et al. 2004). No publications on possible iron over-load of humans due to drinking water iron content can be found in Medline. In 2004 an article has been published linking myocardial infarction and chemistry of ground water – one mg/l increment in iron of ground water increased the risk of first-time acute myocardial infarction for 10%, but the differences were not statistically significant (Kousa et al. 2004).

The results of our investigation raise the concern of drinking water being a real additional source of pro-oxidant ferrous iron (Publication I) as a chronic iron overload with tap water resulted in positive iron balance and an increase in RBC total glutathione content in addition to a measurable increase in lipid peroxidation.

Pregnancy is associated with an alteration in expression of OxS (both parameters of oxidation and antioxidative defence are elevated). These changes may serve an adaptational role and as long as the importance of them is not clearly understood no attempts to correct these changes is advisable. But preeclampsia is associated to an even more serious dysbalance towards oxidative stress and development of this pathological condition is definitely undesirable. In addition to this speculations have been made (Gratacós 2000) that an uncompensated oxidative stress experienced during pregnancy could predict or affect some pathological conditions of the mother in the future. The effect on the developing fetus cannot be excluded either (e.g. from the point of view of future diabetes or atherosclerosis).

When the best results for the baby and the mother are achieved at haemo-globin levels classically considered to be an anaemia requiring therapeutic control (Rasmussen 2001, Casanueva and Viteri 2003), maybe the time has come to trust the nowadays small-blood-losing operating techniques and the physiological changes in the organism of a pregnant woman – and give up the supplementation of (at least ferrous) iron in case of borderline anemia. At least until the actual need and possible damage from treating and not treating to the mother and the baby has been elucidated.

In our observational investigation we could not detect any deleterious impairments of parameters measuring different sides of OxS in pregnant women with borderline anaemia and no additional risk factors for developing profound OxS. We found however a significant difference between iron-supplemented and non-iron-supplemented groups in the content of RBC GSSG that at least should drive the attention to the glutathione system during iron supplementation (Publication II). Unfortunately no-one has established the normatives of reduced and oxidised glutathione or their ratio for different stages of pregnancy – it has been only shown that the redox ratio increases till the end of second trimester (Casanueva and Viteri 2003). So our study – although quite limited in terms of enrolement and sample size – clearly shows that extensive investigations have to be conducted until a single number or difference in time

regarding the glutathione system may be rendered as pathological in pregnant women.

For many years numerous investigators have implied on ferrous iron supplementation as a potential hazard to health but the recommendations of the WHO and some national suggestions still recommend high doses of ferrous iron to pregnant women. As in this particular situation not only the health of young women but also of their offspring may be negatively influenced there remains to be conducted extensive scientific investigation of potential effects of this action. Even a short-term use of iron medical preparations should be monitored from the point of view of OxS to avoid damages in a situation influencing two generations at a time – pregnancy. Sub clinical tortuous damages during pregnancy might increase the risk for the mother or the baby as the OxS-mediated free radical processes may initiate the pathogenesis of diseases e.g. diabetes.

The issue of iron and glutathione during pregnancy may lead to health hazards to the offspring. One study implying on this possibility indicates the preventive effect of Lactobacillus GG against atopic eczaema in infants (Kalliomäki et al. 2001) – as the microbe is efficient in sequestering iron.

Our study on atopic dermatitis confirms the concern as both lesional areas and apparently healthy skin had high redox ratio of glutathione and nonhaem iron content. In cooperation with the Tartu University Department of Dermatology and Venearology we performed an investigation of subjects with allergic or irritant contact dermatitis (Publication V, VII), reactive patch test reaction sites (Publication VIII) and chronic hand dermatitis with different schemes of treatment (Publication VI). In those projects the dissertant performed the measurements of iron, UIBC, TIBC and %saturation of iron-binding proteins in the biopsy samples and participated in interpretation of the results from the point of view of iron metabolism and interactions. The work also demonstrated the striking importance of glutathione redox ratio in evaluation of tissue (intracellular) OxS.

We could assume that more intensive lipid peroxidation (LPO) in the lesions of chronic hand dermatitis is due to “free” iron as in patients’ skin the iron level was 2.5 times higher than in healthy control subjects. Furthermore, the iron level in normal skin was significantly associated with disease duration. Redox active iron could trigger additional LP. Skin barrier function is intimately associated with lipid metabolism. Intensive LP, besides damaging cell membranes may affect skin barrier lipids that might lead to further damage of the skin barrier function. Glutathione has been postulated to play an important role in inhibiting contact dermatitis. Increase of GSSG/GSH before treatment and decrease after it confirms direct connection between skin inflammation and glutathione redox status (Publication VI).

It seems that the glutathione system, particularly the levels of both GSH and glutathione redox status, may have prognostic value for predicting restenosis after coronary angioplasty. The speculation is in consistency with the results of

Pollman et al. (1999) who registered a decline of GSH content after balloon inflation. The previously comprised glutathione redox ratio may thus be an important cofactor for unfavourable outcome. Indeed, glutathione has become a matter of growing biomedical and clinical interest (Dickinson and Forman 2002, Filomeni et al. 2002, Jefferies et al. 2003, Townsend et al. 2003, Pompella et al. 2003). Clinical data (Kugiyama et al. 2001) and a wide spectrum of GSH biofunctionality (a principal antioxidative compound in cellular defence against high-grade oxidative and nitrosative stress; participation in the metabolism of nitric oxide, leukotrienes and prostaglandins; involvement in the regulation (glutathionylation) of key-enzymes like phosphorylase, creatinine kinase, etc.; restoration of activity of proteins and enzymes; stabilization of biomembranes and a modificator of intracellular signals including those of inflammation, gene expression and cell proliferation) indicate the importance of glutatione. Hence, maintenance of physiological glutathione redox ratio has high impact as the response of cells to any stress involves changes in thiol content as they are consumed to protect cells by different actions like antioxidation, detoxification, signalling, and the direct modification/regulation of biomolecules.

Any interpretation of data about GPx remains speculative due to the diverse nature of restenosis, existence of isoforms of GPx and multifaced biofunctionality of GSH (there a lot of GPx- nonrelated pathways for GSH). However, one possible explanation of the lower activity of GPx in the subjects who did not develop restenosis compared to the endemic normal and restenosis group may be that the failure to regulate the activity of GPx may serve as one of the mechanisms of restenosis-prone endothelial response.

Another finding that can not be overlooked is that our endemic normal for serum catalase activity is 55±23 kU/l and the average of subjects with both favorable outcome and restenosis was 2–3 times higher. When looking into the results in detail we found one subject with endemic normal values in the restenosis group and 17 in the clinically stable group. What is more interesting is that the correlation coefficient between GPx and CAT values was –0.25 in the stable group and –0.88 in the restenosis group.

The publications (Boullier et al. 2000, Igarashi et al. 2003, Cipollone et al. 2003, Mazeika et al. 2003) report somewhat conflicting results concerning the predictive value of lipoproteins towards restenosis. We did not find any significant differences between groups in the content of total cholesterol or HDL-fraction. The classical risk factors of atherosclerosis as hypertension, diabetes and hypercholesterolaemia were not predictive for restenosis in our group. The results of the effect of PTCA on the “classical” parameter of MDA are conflicting. In 1997 Oosenbrug stated a temporary increase in MDA (Oostenbrug et al. 1997) but in 2002 Olsson et al. (2002) questioned the effect as they could not measure any increase in MDA after 30 to 90 minutes and Cedro et al. (2002) had results consistent with the latter in 1 min after deflation. As angioplasty temporarily increases at least F2-isoprostane production (Iuliano

et al. 2001) the pre-existing protective potential of the local endothelium becomes of major importance. So it may be speculated that the predictive factors should be searched for from the list of endothelial parameters rather than from those of LDL or other circulating compounds. The results on an experimental model (Janiszewski et al. 1998) and a link between tissue factor activity and restenosis (Tutar et al. 2003) have supported this concern as tissue factor is normally of perivascular origin and its pathological expression of endothelial cells may contribute to thrombosis and atherosclerosis progression.

As a result of our investigation we would suggest that the troubled redox status of glutathione as a member of complex analysis for predictive factors should be an alarm for possibly increased risk of restenosis.

The content of glutathione and its fractions as well as redox-ratio are important parameters of cellular life as the glutathione system is a modulator of gene expression, antitoxic, antioxidative – and pro-oxidative in some special conditions (as an intracellular signalling system) (Pompella et al. 2003).

All our investigations showed that evaluation of the glutathione system may be an indicative parameter in a complex investigation of patients e.g. for predicting post-angioplasty restenosis (Publication III) or determining the degree of damage in diseased skin (Publication V, VI, VIII) or the status of oxidative stress during pregnancy (Publication II) or in settings of potentially advanced contact with pro-oxidants (Publication I). The central role of ferrous iron as a pro-oxidant was also proved in the clinical situations tested by us (alimentary iron overload, dermatitis, Alzheimer's disease, pregnancy).

Any piece of information gathered in the complicated network of the functioning of human organism in conditions of excess oxidative stress contributes to the better understanding of medical as well as biological puzzles of human disease. From the point of view of OxS many scientific findings have not reached the bedside of a patient: for many years numerous investigators have implied on ferrous iron supplementation as a potential hazard to health but the recommendations of the WHO and some national suggestions still recommend high doses of ferrous iron to pregnant women. As in this particular situation not only the health of young women but also of their offspring may be negatively influenced there remains to be conducted extensive scientific investigation of potential effects of this action. In our observational investigation we found a significant difference between iron-supplemented and non-iron-supplemented groups in the content of RBC GSSG that at least should drive the attention to the glutathione system in iron supplementation.

When trying to search a (group of) parameter(s) that would be helpful in predicting restenosis after coronary angioplasty we found that – again – the glutathione system was the most informative.

The important outcome is that in all of these investigated situations the most indicative parameter was some component of the glutathione system. And this is the outcome for clinical medicine – both the system of glutathione and the availability of ferrous iron in organism can be modified to a certain extent.

Iron and glutathione – two powerful but modifiable factors in the process of oxidative stress

The effect of iron chelation and deprivation

The importance of iron in clinical medicine has been established centuries ago, but mostly in connection with the problems of iron deficiency. Recent decades have brought an additional accent to the story of iron and health. The central role of iron in oxidative stress and the possibility to reduce the generation of $OH^{\bullet}$ with deprivatisation of iron in the environment has lead to a therapeutic approach in managing diseases that are mediated by profound OxS. The positive effect has been shown in ishaemia-reperfusion injury, eye-, brain- and muscular diseases as well as in slowing down the aging processes in the skin (Polla et al. 2003). The reduction of ishaemia-reperfusion injury by chelation of iron with desferroxamine (DFO) has been demonstrated by Qayumi et al. (1992) on a swine model and Nakamura et al. (1992) on the isoleted rabbit heart. But all is not clear in that matter: in 1997 Jensen et al. (1997) did not find statistically significant improvement of myocardial function after iron chelation in patients with transfusional iron overload – although the impairment of myocardial function was in tight correlation with the degree of iron overload.

In a mouse model of Duchenne' muscular dystrophy Bornman et al. (1999) have demonstrated the reduction of necrotic fibers in iron-deprived mice.

In Alzheimer's disease it has been demonstrated the increase of iron content in lesions (Smith 1997) and the increase of the concentration of an iron-binding protein p97 in serum (Kennard et al. 1996) as well as the potentiating influence of iron to the effect of amyloid to neurons (Goodman and Mattson 1994) There are no conclusive results of clinical intervention trials of iron clelation or deprivation in that disease, but the reports of ongoing trials are promising (Ritchie et al. 2003, Regland et al. 2001). Recent findings show that iron chelation may prevent the reductions in dopamine and motor disturbances associated with Parkinson's disease (Levenson 2003, Kaur et al. 2003).

Some (but moderate to this time) effect of iron chelation has been described in the treatment of tumours both in animal models and human investigations (Buss et al. 2003) – and the key to only moderate efficacy in human investigation has been proposed to be not the marginal significance of the process but the weak power of the used chelator (DFO) in tested circumstances.

Both animal- and human studies have proved the efficiency of iron chelator 2-furildioxime (FDO) in prevention of skin photodamage (Bissett et al. 1994) and the same was observed on hairless mice with another iron chelator – kojic acid (Mitani et al. 2001).

The possible modulation of the GSH balance: N-acetylcysteine (NAC), glutamine, the potential role of synthetic analogues of glutathione

The GSH participates in many important physiological processes controlling the homeostasis of the cells. A higher demand of Cysteine (Cys) supply causes difficulties in maintaining a constant GSH level. The role of GSH as a key regulator of thiol redox intracellular balance is established. This reveals that GSH is essential in regulating the cell's life cycle and that the reduction of intracellular GSH contributes to chronic inflammation. The fact that Cys availability is generally a limiting factor for the GSH synthesis has stimulated the development of a pharmacologically useful Cys pro-drug. The simplest derivative and analogue of reduced glutathione is N-acetylcysteine (NAC), which appears to be the prototype of all Cys suppliers (Santangelo 2003).

Fischer et al. demonstrated on an animal model that administration of NAC (dogs, 100 mg/kg from 10 minutes before to 1 hour after bypass) preserved systolic function and enhanced myocardial edema resolution after cardio-pulmonary bypass/cardioplegic arrest. Furthermore, OxS was significantly reduced in the treated animals (Fischer et al. 2003). In a recently published artcle (Jeremias et al. 2004) where the authors could not show a prevention of vascular post-angioplasty-restenosis with NAC (rabbits got 150 mg/kg of NAC 7 days before and 21 days after balloon injury) they concluded that one potential explanation for the lack of benefit of NAC in their study was the relatively high glutathione levels at baseline (in the range of 30 μmol/l) as compared to ~10 μmol/l in humans. It is therefore conceivable that further elevation of glutathione beyond 30 μmol/l is of no clinical effect, while glutathione induction in humans may be more beneficial.

NAC has cancer chemopreventive properties attributable to its nucleo-philicity, antioxidant activity, and a variety of other mechanisms. Albini et al. have demonstrated that NAC has anti-invasive, antimetastatic, and antiangio-genic effects in *in vitro* and *in vivo* test systems. Additionally, in a mouse model of Kaposi's sarcoma it has been shown that the daily administration of NAC with drinking water, initiated after the tumour mass had become established and detectable, produced a sharp inhibition of tumour growth, with regression of tumours in half of the treated mice along with a prolonged median survival time (Albini et al. 2001).

Glutamine (GLN) is an important metabolic fuel for intestinal epithelial cells and a precursor to the antioxidant glutathione (GSH), which has antiapoptotic effects. In cultured intestinal epithelial cells, GLN depletion increases oxidant-induced apoptosis (Evans et al. 2003). GLN supplementation is established to reduce significantly tumour development in a breast cancer model and to restore the depressed serum levels of glutathione (Todorova et al. 2003). It is also shown that oral GLN supplementation significantly reduces GSH levels in the

cancer cells and stimulates apoptosis in these cells through this action (Todorova et al. 2003).

Apart from the effect of GLN on stimulating apoptosis in cancer cells it is beneficial to the normal cells of the organism. As concluded in the literature review, oral GLN supplementation may decrease the incidence and/or severity of chemotherapy-associated mucositis, irinotecan-associated diarrhea, paclitaxel-induced neuropathy, hepatic veno-occlusive disease in the setting of high dose chemotherapy and stem cell transplantation, and the cardiotoxicity that accompanies anthracycline use. It has been claimed that in the presence of oxidative stress, glutamine is rate limiting for GSH synthesis. Thus GLN supplementation may enhance the therapeutic index by protecting normal tissues from, and sensitizing tumour cells to chemotherapy and radiation-related injury (Savarese et al. 2003). All of this effect though may not account on the effect on GSH as GLN has numerous functions in human organism (Newsholme et al. 2003). Evans et al. have shown that GLN specifically protects intestinal epithelial cells against cytokine-induced apoptosis, and that this occurs by a mechanism that is distinct from the protection against oxidative stress mediated by cellular GSH (Evans et al. 2003). Recent findings show that the extracellular glutamine level affects the susceptibility of cells to different apoptosis triggers: whereas glutamine-starving cells are more sensitive to Fas ligand-mediated apoptosis, they are desensitized against the cytotoxic effects of TNF-alpha (Oehler and Roth 2003).

As intracellular glutathione content and redox ratio are important determinants of cellular metabolism including the process of apoptosis, it is pharmacologically tempting to influence the situation. It would be therapeutically beneficial to modify the drug resistance of cancer cells or viability of ischemic neuronal cells – which both have glutathione as a triggering mechanism. Several glutathione analogues have been synthesized and used experimentally and in clinical trials, targeted especially to cancer cells' chemotherapy resistance. These compound include glutathione – S-transferase inhibitors and prodrugs, glyoxalase I inhibitors, and S-nitroseglutathione (Hamilton and Batist 2004). A different approach is mimicking the glutathione effects in neuroprotection (Karelson et al. 2002).

Another possibility to modify the antioxidative defence system is systematic co-administratoion of antioxidant vitamins in adequate doses – like we have demonstrated in Publication VII, where 25-days administration of D-alpha-tocopherol 300–100 mg/die and vitamin C 200 mg/die was accompanied by normalisation of systemic glutathione redox ratio in an allergic contact dermatitis patient.

As it can be concluded from our investigation and the information summarised herein non-haem ferrous iron as a central pro-oxidant from one side and the critical balance of the glutathione system from the other side often

determine the faith of health and disease. The possibilities to modify these parameters are being only in the beginning of wider clinical use but as numerous investigations are being undertaken worldwide the future of manageing disease-associated profound OxS are promising.

CONCLUSIONS

Ferrous non-haem iron is one of the central pro-oxidants in human organism. On the other hand, reduced glutathione is one of the major intracellular antioxidants. These two powerful factors reciprocally influence the critical balance of pro-oxidants and antioxidants – that determines many processes of the cellular function including growth and apoptosis and is crutial in the pathogenesis of numerous diseases.

The current investigation evaluates the level of potentially harmful oxidative stress in several groups of healthy subjects and patients (healthy adults with alimentary iron overload, pregnant women with borderline anaemia, patients of coronary angioplasty, atopic and contact dermatitis, Alzheimer's disease). Special emphasis is drawn on non-haem iron content and RBC-s glutathione redox ratio as exceptionally indicative and – what is critically important for clinical medicine – modifiable parameters. Nevertheless can either of those or any other parameter in this context be analysed alone, but only in a complex of evaluating oxidative damage on lipids, proteins, and in some cases in nucleic acids and measuring the indices of systemic antioxidative defence, so a complex investigation of oxidative damage has been undertaken. The information summarised herein has both fundamental value and practical outcome in opening a window for targeted clinical evaluation and modulation of non-haem iron and glutathione system to predict, avoid and combat high-grade oxidative stress caused events.

Based on the investigation of different clinical situations the following conclusions may be drawn:

1. A chronic alimentary overload with iron in our test group indicated an increased risk of high-grade oxidative stress as measured by the indices of lipid peroxidation and total glutathione content.
2. Supplemental peroral ferrous iron administration during uncomplicated pregnancy was not conclusively proven to have adverse effects on OxS but the significant difference from the unsupplemented group in GSSG content indicates a possible increase in risk.
3. Of the preprocedural markers of OxS only glutathione redox ratio (and GPx) showed a significant correlation with postangioplasty restenosis. As glutathione balance (redox ratio) is to some extent a modifyible system it would be useful to include determinations of glutathione in routine management of angiplasty patients and in complex evaluation of restenosis risk in the future.
4. In various clinical situations (the mentioned above along with atopic and contact dermatitis) the markers of glutathione system proved to be one of the most sensitive to the changing situation of OxS during disease or treatment. On the other hand the amount of prooxidant nonhaem iron correlated with the OxS severity measured by other parameters and the clinical situation.

SUMMARY IN ESTONIA

Mitteheemse kahevalentse raua ja glutatiooni redoks-suhte hindamine oksüdatiivse stressi patogeneetilisuse näitajana erinevatel kliinilistel gruppidel

Käesolev uurimistöö hindab mitmetel tervete ja patsientide gruppidel (terved täiskasvanud kroonilise raua ületarbimise tingimustes, kerge aneemiaga rasedad, koronaarse angioplastika patsiendid, atoopilise ning kontaktdermatiidi põdejad, Alzheimeri tõve haiged) potentsiaalselt kahjuliku oksüdatiivse stressi taset, eriline tähelepanu on pööratud mitteheemse raua hulgale kui suhteliselt kergesti mõjutatavale ning oksüdeeritud/redutseeritud glutatiooni suhtele kui ülimalt informatiivsele ja samuti modifitseeritavale parameetrile. Siiski ei saa selles kontekstis kumbagi neist ega ka ühtki teist parameetrit käsitleda isoleerituna, vaid ainult kompleksis oksükahjustuse hindamisega nii lipiidide, valkude kui mõnel juhul ka nukleiinhapete tasandil ning süsteemse antioksüdantse kaitse näitajatega.

Raua kui ühe olulisima ning laialt levinud prooksüdandi toimet inimorganismi oksüdatiivse stressi mõjutatud protsesside kujundajana on raske üle hinnata. Kahes esmapilgul lihtsas situatsioonis (normiületav rauasisaldus tsentraalses veevarustussüsteemis ja poolprofülaktiline rauapreparaatide manustamine rasedatele) õnnestus näidata võimalikke riske edasisteks patogeneetilisteks arenguteks. Alimentaarne raud kui võimalik raua ülelaadumise allikas on kirjanduses suhteliselt vähe tähelepanu leidnud. Seost on leitud hemokromatoosiga, on näidatud pinnasvee rauasisalduse ja müokardi infarktide hulga võimalikku seost (Kousa et al. 2004).

Rauapreparaatide manustamisel rasedatele ei ole kliiniline praktika aga vastavuses teadusuuringute tulemustega: kui uuringud näitavad emale ja lapsele soodsaimaks ema hemoglobiiniväärtust 95–105 ning väidavad, et naisel, kes alustab rasedust ferritiiniväärtusega vähemalt 20 ng/l, ei kujune rauavaegust, siis klinitsistid üle maailma püüdlevad visalt hemoglobiiniväärtuseni vähemalt 120 g/l. Meie tervetest rasedatest koosnevas uuringugrupis ei kujunenud süsteemset sügavat oksüdatiivset stressi 36 mg ferroraua lisamanustamisel võrrelduna rauapreparaati mittesaanutega, kuid oksüdeeritud glutatiooni väärtused saavutasid neljanädalase jälgimisperioodi lõpuks statistiliselt tõenäose gruppidevahelise erinevuse.

Uuringu osas, kus hindasime oksüdatiivse stressi parameetrite võimalikku informatiivsust koronaarse angioplastika prognoosi suhtes, leidsime restenoosining stabiilse kliinilise seisundi gruppide vahelise erinevuse taas glutatiooni redoks-suhtes ning glutatiooni peroksüdaasi aktiivsuses. Klassikalistes koronaartõve riskitegurites gruppidevahelist erinevust ei täheldatud. Järeldasime, et

endoteeli kaitsevõime hindamine riski analüüsimisel, ning võimalusel ka modifitseerimine, võiks olla osa angioplastikapatsiendi käsitlusest.

Sarnaselt eelkirjeldatud kliinilistele situatsioonidele osutus glutatiooni redoks-suhe informatiivseks ka atoopilise ja allergilise kontaktdermatiidi patsientide puhul, kus nii glutatioonisüsteem kui nahas olev mitteheemse raua hulk olid sõltuvuses kliinilise seisundi raskusest (või vastupidi).

Mitteheemne kahevalentne raud on üks inimorganismi kesksetest pro-oksüdantidest. Teiselt poolt on redutseeritud glutatioon üks põhilisi rakusiseseid antioksüdante. Need kaks võimsat faktorit mõjustavad vastassuundadest organismi pro- ja antioksüdantide kriitilist tasakaalu – mis määrab rakufunktsiooni ja paljude metaboolsete radade – kaasa arvatud kasvu ja apoptoosi – kulgemise ning on määrav mitmete haiguste patogeneesis.

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Arch Dermatol Res 2004; 295: 517–520.

CURRICULUM VITAE

I. Üldandmed

Nimi:	Aune Rehema
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Kodakondsus.	Eesti Vabariigi kodanik
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Töökoht, amet:	TÜ Biokeemia Instituut, assistent; OÜ Dr Aune, juhataja
Haridus (kõrgkool, eriala):	Tartu Ülikool, ravi 1993, üldinternatuur 1995, peremeditsiini residentuur 2001
Keelteoskus:	inglise kõnes ja kirjas, vene

Teenistuskäik (asutuse nimetus, ametikoht, töötamise aeg – eeskätt teadusasutustes):

1995–2002	Vastseliina Ambulatooriumi juhataja
1996–2000	TÜ Biokeemia Instituudi doktorant
2001–2002	TÜ Biokeemia Instituudi vanemlaborant
2002–	TÜ Biokeemia Instituudi assistent
2003–	OÜ Dr Aune juhataja

II. Teaduslik ja arendustegevus

1. Peamised uurimisvaldkonnad. Lõpetatud on prognoosiuuring PTCA patsientidel koostöös TÜ Kardioloogia kliinikuga, saime kirjandusega haakuvad tulemused, kus sõltuvust klassikalistest ateroskleroosi riskiteguritest ei leitud, küll aga osutus oluliseks endoteliaalne kaitsepotentsiaal. Läbitud ning publitseeritud tervete täiskasvanute peroraalse raua ülelaadumisuuring (krooniline alimentaarne raua ülelaadumine põhjustab olulise oksüdatiivse stressi) Koostööprojektis TÜ Naistekliinikuga, kus uurisime tervetele madala hemoglobiiniväärtusega rasedatele rakendatud rauaravi (raud kui pro-oksüdant!) mõju oksüdatiivse stressi parameetritele, on tulemused vormistatud artikliks. Neerupuudulikkuse tõttu dialüüsravi ning i/v Fe-kelaati saavatel patsientidel on määratud valkudele tekitatud oksükahjustuse ulatust. Osaletud koostööprojektis TÜK Nahahaiguste kliinikuga oksüdatiivse stressi, eriti pro-oksüdantide osast atoopilise ja allergilise kontaktdermatiidi kliinilise pildi raskusastme kujunemisel. Koostööprojektis TÜK Spordimeditsiini kliinikuga mõõtsime kestvusalade füüsilise koormuse

korral kujunevat oksüdatiivset stressi ning selle taastumise dünaamikat. Valkude oksükahjutusi on uuritud ka sporaadilise ja geneetiliselt determineeritud Alzheimeri tõve patsientidel – koos muude parameetritega. Koostöös TÜ Mikrobioloogia Instituudiga on esimesed tulemused kokku võetud antioksüdantsete laktobatsillide omaduste uurimisel – konkreetselt nende rauasidumisvõime ning rauasisalduse määramine. Töö idee – samad OS parameetrid, mida jälgime eri riskigruppidel ning nende seos (koronaarse) ateroskleroosiga – on toonud välja huvitavad korrelatsioonid, mille praktiline kasu ateroskleroosi süsteemses profülaktikas (nii primaarses kui sekundaarses) on tõenäoline.

2. Teaduslikke publikatsioone: 15 (sh. seitse CC artiklit).
3. Uurimistoetused ja lepingud: doktorantide sihtfinantseerimine TARBK1128, sihtfinatseerimine TARBK0411; ETF grant 3358, ETF grant 5327 (toetus artikli publitseerimiseks).
4. Muu teaduslik organisatsiooniline ja erialane tegevus puudub.

III. Õppetöö

1. Loenguid 1998.a. 10 tundi, praktikume/seminare 80 tundi. Loenguid 1999.a. 10 tundi, praktikume/seminare 80 tundi. Loenguid 2000.a. 8 tundi, praktikume/seminare 90 tundi. Loenguid 2001.a. 6 tundi, praktikume/seminare 106 tundi. 2002. aastal loeng 2 tundi, seminare 4 tundi. 2003. aastal 6 t loenguid, 74 tundi seminare ja praktikume.
2. Õppevahendid:
   - Introductory Chapters of Medical Biochemistry (CD-ROM). Eds. M. Zilmer, E. Karelson. Tartu University, Dept of Biochemistry, Tartu 2000, Zilmer, M, Soomets, U, Karelson, E, Starkopf, J, Vihalemm, T, Zilmer, K, Rehema, A. (in Estonian).
   - Introductory Chapters of Medical Biochemistry (CD-ROM). Eds. M. Zilmer, E. Karelson. Tartu University, Dept of Biochemistry, Tartu 2001, Zilmer, M, Soomets, U, Karelson, E, Starkopf, J, Vihalemm, T, Zilmer, K, Rehema, A. (in English, in press).
   - Medical Biochemistry I. Biomolecules: biochemical and clinical aspects (CD-ROM). Ed. M. Zilmer, Tartu University, Biomedicum, Tartu, 2001, Zilmer, M, Karelson, E, Vihalemm, T, Soomets, U, Starkopf, J, Zilmer, K, Rehema, A (in English).
3. Juhendamine: –

IV. Administratiivtöö ja muud kohustused

–

Erialane enesetäiendus

1996 3 kuud Kopenhaagenis Bispebjergi Haiglas kliinilise biokeemia alal

V. Ühiskondlik ja publitsistlik tegevus

Vastseliina vallavolikogu liige 1996–1999, 1999–2002, 2002–...;
Vallavolikogu sotsiaalkomisjoni esimees 1999–...

–

CURRICULUM VITAE

I. General data

Name and surname:	Aune Rehema
Date and place of birth:	25th Jan 1970, Tartu, Estonia
Citizenship:	Estonian Republic
Marital status:	unmarried, 1 daughter born on 14.02.2002
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Position:	University of Tartu, Department of Biochemistry, assistent; Doctor-in -charge of Dr Aune LLC (family doctor praxis).
Educational history:	Miina Härma Tartu Secondary School No 2 1987, University of Tartu, Medical Faculty 1993, specializing on family medicine 2001.
Knowledge of languages:	English, Russian

Professional employment:

1995–2002	Principal of Vastseliina Dispensary
1996–2000	Tartu University Department of Biochemistry, PhD student.
2001–2002	Tartu University Department of Biochemistry senior laboratory assistent.
2002–...	Tartu University Department of Biochemistry assistent.
2003–...	LLC Dr Aune, doctor-in-charge.

II. Scientific experience

1. Main fields of investigation: Oxidative stress (main focus on prooxidant ferrous iron) in different clinical situations and experimental models.
2. The overall count of publications and publications in last five years: 15 (incl 7 CC articles), 14 of them in last 5 years.
3. Publications: the list is added.
4. Grant funding and support: PhD-student grant No TARBK 1128 (1998–2000), ESF grant 3358, support for publication from Estonian Science Foundation grant No 5327 in 2004.
5. Other scientific-organizational experience: –

III.Teaching experience

1. The hours of auditorial work in last five years include 32 h of lectures and 354 h of seminars/practical works.
2. Textbooks for students:
   - Introductory Chapters of Medical Biochemistry (CD-ROM). Eds. M. Zilmer, E. Karelson. Tartu University, Dept of Biochemistry, Tartu 2000, Zilmer, M, Soomets, U, Karelson, E, Starkopf, J, Vihalemm, T, Zilmer, K, Rehema, A. (in Estonian).
   - Introductory Chapters of Medical Biochemistry (CD-ROM). Eds. M. Zilmer, E. Karelson. Tartu University, Dept of Biochemistry, Tartu 2001, Zilmer, M, Soomets, U, Karelson, E, Starkopf, J, Vihalemm, T, Zilmer, K, Rehema, A. (in English, in press).
   - Medical Biochemistry I. Biomolecules: biochemical and clinical aspects (CD-ROM). Ed. M. Zilmer, Tartu University, Biomedicum, Tartu, 2001, Zilmer, M, Karelson, E, Vihalemm, T, Soomets, U, Starkopf, J, Zilmer, K, Rehema, A (in English).
3. Supervision –

IV. Administrative work and other obligations

–

Special courses

–

Society work and publicing activities

Member of Vastseliina Parish Council 1996–1999, 1999–2002, 2002–…;
Chairman of the Council's social Committee 1999–…

Glutathione redox ratio may have prognostic value for predicting restenosis after coronary angioplasty

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Abstract

Background

Excess oxidative stress is present both during vascular intervention and in restenosis following coronary angioplasty (PTCA). Restenosis is still a major drawback in the use of this procedure.

Methods

The aim of our study was to find any oxidative stress-related factors that might help to predict restenosis. We measured the level of both parameters of cellular OS (erythrocyte (RBC) glutathione peroxidase (GPx) and superoxide dismutase (SOD), blood concentrations of total, oxidized and reduced glutathione (tGSH, GSSG and GSH) and glutathione redox ratio (GSSG/GSH) and serum activity of catalase (CAT)) and parameters of systemic OS (serum concentration of conjugated dienes (CD) and lag phase of LDL oxidation (LDL lag phase) as well as plasma protein carbonyls (PC), total antioxidant activity (TAA) and total antioxidant status (TAS)) in 54 PTCA patients. 12-month event-free survival was taken for the end point for no restenosis (n=44) and angiographically confirmed narrowing of the treated vessel more than 50% of post-procedural was considered definite restenosis (n=7).

Results

The parameters showing difference for these groups were GSSG/GSH (2.21± 2.36 vs. 0.88± 0.74, p=0.002) and GPx (40.36±35.16 vs. 15.01±12.29 U/g Hb, p= 0.003). Differences in TAS and hematokrit were not confident.

Conclusions

Intracellular glutathione system is involved in maintenance of balance between pro-oxidants and antioxidants, modification of signals etc. Glutathione redox ratio seems to be a possible candidate to be included in complex investigation of angioplasty patients for predicting restenosis.

Key words: Angioplasty; Restenosis; Oxidative stress; Glutathione

Background

Coronary angioplasty with or without stenting has become a possibility of treating segmentary narrowings in coronary arteries. The high incidence of post-procedural restenosis remains one of the major drawbacks. Because of that the high hopes concerning the procedure have decreased to the point where the cost-effectiveness of angioplasty is worse than of bypass surgery in some specific patient groups [1]. It has been claimed that percutaneous coronary interventions and local approaches to prevent restenosis such as coated stents are not expected to prevent atherosclerosis progression, myocardial infarction and cardiovascular death [2]. Although extensive investigations have been undertaken and numerous parameters reported (including single nucleotide polymorphisms, vessel caliber and lesion length) no definite factors that might predict restenosis have been elucidated yet. Finally discovering them might be beneficial for selecting patient groups for this procedure or/and influencing these factors to decrease the incidence of restenosis by defining risk groups and individual managing of patients before and after coronary angioplasty.

The main problem is that traditional risk factors of atherosclerosis do not predict restenosis [3]. Even before the redox hypothesis of restenosis was formulated [4], the indices of the phenomenon – lipid peroxides, oxidized thiols etc. – have been measured in different modes trying to evaluate their dynamics during angioplasty [5–8], and even an antioxidant has been used in attempt of reducing the restenosis rate [9]. Papers report that angioplasty increases local F_2– isoprostane formation [10], in patients with angiographic restenosis plasma concentrations of a hydroperoxide increase and of a nitric oxide end product (NO(x)) decrease [11]. Several promising results have been reported, proposing

the role of TBARS, plasma homocysteine, C-reactive protein (baseline C-reactive protein levels did not correlate with restenosis, but were associated with almost a two-fold increase in the rate of death or myocardial infarction after coronary stenting) and non-HDL-cholesterol [12–16]. Despite of that we still lack indicative predictive parameters for restenosis.

The pathogenetic link between excess oxidative stress and restenosis is multicomponent. Mechanisms identified in restenosis include stent under-expansion, immediate recoil, formation of thrombus and the 'normal' healing in response to arterial injury involving proliferation of intimal smooth muscle cells and extra cellular matrix, as well as vascular remodeling and decreased apoptosis [17]. ROS can induce endothelial dysfunction and macrophage activation, resulting in the release of cytokines and growth factors that stimulate matrix remodeling and smooth muscle cell proliferation [2]. The role of redox processes as mediators of vascular repair and contributors to post-angioplasty restenosis is increasingly evident [18]. Oxidation and reduction influence many intra-and intercellular signaling systems, involving the signals leading to apoptosis, inflammation and neointimal proliferation, the different roles of glutathione being only an example [19].

We conducted an observational survey of 54 PTCA patients trying to find a link between the pre-procedural markers of oxidative stress and restenosis. Prior to the procedure we measured the content of intracellular antioxidant enzymes SOD, CAT and GPx, the more general parameters TAS and TAA and the relation GSSG/GSH in blood, the parameters of lipid peroxidation CD and LDL lag phase and a parameter of protein oxidation – carbonyl groups of protein. After a year the results were grouped and analyzed based on the clinical outcome.

Materials and methods

Subjects and procedure

We analyzed blood (fractionated immediately after withdrawal) from 54 patients who underwent PTCA in Tartu University Hospital and used data from their medical records as well as from a questionnaire concerning their lifestyle. Consecutive patients to whom the procedure was performed on weekdays were included (for technical reasons). All participants were asked to evaluate their quality of life a year later and post another small questionnaire. Tartu University ethics committee approved the survey (protocol 47/22 – 1997) and all participants gave their written informed consent. Restenosis was evaluated angiographically if one was necessary for reoccurring/worsening of clinical symptoms.

In our group we had 8 women, 6 diabetes patients, 19 subjects were hypertensive and 16 reported other chronic illnesses. We had 11 current smokers, but only 7 persons had no smoking in history. The average age of the subjects was 56.2±9.4 years.

The angioplasties were performed by one team with the same equipment. Stenting was used if sufficiently good antegral flow was not achieved by balloon affiliations only.

Conjugated dienes (CD) and LDL lag phase

Unless mentioned otherwise, all reagents were obtained from Sigma (Sigma Aldrich , St Louis, Mo), analytical grade.

Conjugated dienes (CD) and LDL lag phase describe mainly the situation of OS in the lipid fraction. CD are a form of compounds that are constructed from unsaturated fatty acids by free radical reactions. LDL lag phase describes the period during which the non-HDL-fraction of a sample/a subject is resistant to oxidation in the standard oxidation conditions. CD were measured according to Recknagel and Glende [20]. Briefly freshly prepared serum was frozen at –80^{o}C with 16.8 nM BHT (butylated hydroxytoluene, final concentration) until analyzed within a month. Samples (150µl) + 150µl 0.9% NaCl were incubated at 37º C for 30 min, 0.25% BHT (15µl) was added, the samples were extracted with heptane/ isopropanol (1:1, whole volume 1800µl) and acidified by 500µl 5N HCl. After extraction with cold heptane (1600µl), samples were centrifuged for 5 min at 3000 rpm and absorbance of the heptane fraction was measured at 234nm. LDL lag phase is calculated from the curve that is formed from the results of measuring the protein-adjusted quantity of the non-HDL fraction (prepared from EDTA-plasma) peroxidation in time spectrophotometrically at 234 nm [21, 22]. Briefly the lipoproteine fraction (non-HDL-fraction, LPF) was precipitated from 2 ml twice-diluted EDTA-plasma by adding 0.2 ml precipitation reagent (2% dextran sulfate : $MgCl_2$ (2M, pH 7.0) 1:1 v/v), vortexing for 1 min and centrifuging at 1500 g for 10 min. In order to remove EDTA from the LPF the pellet was suspended in 2 ml 0.9% PBS and reprecipitated by adding 0.1 ml precipitation reagent, vortexed and centrifuged. The precipitated LPF was dissolved in 2 ml 4% PBS and this solution was used immediately. The protein concentration of EDTA-free LPF was adjusted to 2 mg protein/ml. The oxidation was initiated by the action of a freshly prepared aqueous solution of $CuSO_4 \bullet 5\ H_2O$ (final concentration 45 µM) to the LPF (2 mg protein/ml) and the oxidation of this fraction was evaluated by continuously monitoring the formation of conjugated dienes at a maximum absorbance at 234 nm with different intervals of incubation at 37°C. The kinetics of the diene formation (the increase of the absorbance versus time) can be divided into three phases: lag phase (during which the diene absorption

increases only weakly), propagation phase (rapid increase of the diene absorption) and decomposition phase. The resistance to oxidation was defined as the length of the lag phase, calculated from the interval between the intercept of the tangent of the slope of the curve with time-scale axis.

Glutathione (tGSH) and fractions

Glutathione (tGSH) and its fractions – reduced glutathione (GSH) and glutathione disulphide (GSSG) – measured in whole blood indicate mainly the situation of oxidative stress within the cells (including RBCs and endothelial cells). Measurements of glutathione are actually measurements of thiols. We used a modified method of Bhat [23]. The method is based on the formation of a chromophoric product from the sulfhydryl reagent 5,5-dithiobis-(2-nitrobenzoic acid), Ellmann reagent, in the presence of GSH. The contents of total and oxidized glutathione are directly measured and the content of reduced glutathione is calculated from them.

Stock solutions of various regents were made in 0.1 M sodium phosphate/0.005 M EDTA buffer, pH 7.5. Solution of GSH was prepared before use in cold 0.01 N HCl.

For determination of total GSH 10μL whole blood (heparinized) was mixed with 0.99 ml of 0.2M sodium phosphate buffer (pH 7.5), containing 0.01M EDTA for hemolyze, and let to stand for 10 min. To 50μl of hemolysate 500μl 0.2 M sodium phosphate buffer, containing 0.01 M EDTA was added. The reaction was initiated with 0.3 mM NADPH and 0.5 U glutathione reductase (GSH-RED) and continued for 6 min. After that there were added 1ml 0.2 M sodium phosphate buffer, containing 0.01 M EDTA and 100μl 1mM 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.2 M sodium phosphate buffer. After 3 min the extinction was recorded spectrophotometrically at 412 nm (Spectrophotometer Jenway 6300). The glutathione content was calculated on the basis of the standard curve obtained with known amounts of glutathione (GSH). Glutathione was expressed as μg/ml.

To assay for GSSG (μg/ml), 10μl whole blood (heparinized) was mixed with 2μl 4-vinylpyridine and kept at room temperature for 1 h. Then 0.99 ml of 0.2M sodium phosphate buffer (pH 7.5) was added. The hemolysate (50μl) was treated as described in the previous section for tGSH. GSH concentration was calculated as the difference between total glutathione and GSSG.

The GSSG/GSH ratio was calculated as μg GSSG/ μg GSH.

Catalase (CAT)

Catalase is one of the most important intracellular antioxidant enzymes that together with superoxide dismutase control the level of intracellular free radicals. Located in the cytosol and peroxisomes, it reduces H_2O_2 to water. CAT was measured in serum by a method of Góth [24], the method being spectrophotometrical based on hydrogen peroxide forming a stable complex with ammonium molybdate. Serum CAT is a charge isoform of RBC CAT and the formation of charge isoforms of catalase is caused by a reversible, conformational modification due to matrix effect of serum [25].

In the case of hemolytic serum hemoglobin concentration was determined with the bensidine method. If hemoglobin concentration was more than 100mg/l the sample was discarded (significantly hemolyzed sample gives an artificial increase of catalase activity) – in two cases we had to do this.

200µl of serum was incubated in 1000µl substrate (65 mmol/l H_2O_2 in 60 mmol/l sodium-potassium phosphate buffer, pH 7.4) at 37° for 60 s. The enzymatic reaction was stopped with adding 1000µl of 32.4 mmol/l ammonium molybdate ($(NH_4)_6Mo_7O_{24} \bullet 4H_2O$) and the yellow complex of molybdate and H_2O_2 was measured at 405 nm against control blank (1200µl buffer, 1000µl molybdate; control of serum: molybdate was added prior to serum; control of substrate: 0.2 ml of buffer was added instead of serum). Catalase activity in kU/l was expressed as:

CAT kU/l= (control of enzyme preparation- sample) / control of substrate) • 271. (1)

Total antioxidant activity (TAA) and total antioxidant status (TAS)

Total antioxidant activity (TAA) describes the overall antioxidant potency of a medium. In the case of measuring it in serum in a situation of no remarkable tissue damage it mostly reflects the extracellular/ intravasal antioxidant activity. TAA (an original method of our Dept. of Biochemistry [26] is the percentage a sample inhibits a standard linolenic acid peroxidation.

For LA-standard LA (alpha-linolenic acid, from Sigma, code L2376) was dissolved (10 µl of linolenic acid was added to 1000µl 96% ethanol drop by drop (permanent mixing). This solution was kept for 2 days at –18°C in dark in a carefully closed glass vial. From this solution 400 µl was dissolved (drop by drop, permanent mixing) in 50 ml warm 40°C isotonic saline. Suitable portions of this solution (LA-standard) were frozen in carefully closed glass vials and the content of one vial was used as a standard in one experiment.

0.15µl 0.35% SDS was measured into a vial and 400 µl of heated (up to 40°C) LA-standard was added to get a homogenous solution of LA-standard. Then to a sample (30µl of serum diluted 1:3.3 in isotonic saline) 100 µl (final

concentration 200 μM) $FeSO_4$ was added and incubated in the presence of LA-standard for 60 min at 37°C. Reagent blank contains only LA-standard and isotonic saline. Then 0.25% 35μl BHT was added and the mixture was treated with 500μl acetate buffer to acidified mixture (pH 3.55–3.60) and heated with fresh 1% TBA solution (1000μl) at 80°C for 40 min. After cooling for 5 min in ice cold water the reaction mixture was acidified by cold 500 μl 5 N HCl, extracted with cold butanol (1700μl), centrifuged (for 10 min at 3000 rpm) and absorbance of the butanol fraction (amount of TBARS) was measured at 534 nm. The TAA of a sample was expressed (%) as inhibition by the sample of LA-standard peroxidation as follows:

$$[1 - (A_{534} \text{ (sample)} / A_{534} \text{ (LA as control)}] \bullet 100. \quad (2)$$

We used a kit (Randox Laboratories LtD, Cat. No. NX2332) for an assay of total antioxidant status (TAS). The values are expressed as Trolox units (0–2.5 mmol/l). Trolox is a water-soluble vitamin E analogue.

Protein carbonyls (PC)

Protein carbonyls (PC) were quantified by a slightly modified method of Levine et al. [27]. 0.050 ml freshly prepared plasma (approximately 3 mg of protein) was precipitated with 0.200 ml of 10% TCA and after centrifugation for 10 min at 3500p the pellet was treated with 0.5 ml 10 mM 2,4 DNPH in 2M HCl or only with 0.5 ml 2M HCl as a control blank. Samples were incubated for 60 min at room temperature with continuous mixing. Next, 0.5 ml of 20% TCA was added and proteins were precipitated by centrifugation for 10 min at 3500p and the supernatant was discarded. The pellet was washed 3 times with 1ml 1:1 ethanol/ethyl acetate (centrifugation was followed after 10 min standing of the mixture). The final precipitate of protein was dissolved in 0.6 ml 6M guanidine/20mM K-phosphate buffer, pH 2.3 for 30 min at 37°C with vortexing. Any insoluble materials were removed by centrifugation. The different spectrum of the DNPH-derivates versus HCl controls was followed spectrophotometrically at 350–380nm with a scan program. The concentration of carbonyl groups (CC) was calculated from the spectrum maximum, using a molar absorption coefficient of 22 mM –1cm–1 as the extinction coefficient for aliphatic hydrazones.

$$CC \text{ (nmol/ml)} = Abs365(max) \text{ (Test-Blank)} \times 10^3 / 22 \quad (3)$$

Protein content (mg/ml) was determined in each sample (parallel) versus guanidine and calculated from a bovine serum albumin standard curve dissolved in guanidine-HCl and read at 280 nm [28]. The carbonyl content was expressed as nmol carbonyl/ mg protein.

Superoxide dismutase (SOD)

A special kit RANSOD (Randox Laboratories LtD) was used. RANSOD allows analyzing the RBCs levels of SOD. RANSOD employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with special reagent (tetrazolium salt, called as I.N.T.) to produce red formazan dye. As SOD, present in sample, competes with I.N.T. for superoxide radicals it is possible to assess SOD by the degree of inhibition of formazan dye formation.
SOD activity was calculated and given as SOD U/g hemoglobin.

Glutathione peroxidase (GPx)

For determination of GPx activity a special kit RANSEL (Randox Laboratories LtD) was used. RANSEL allows to analyze the levels of GPx in whole blood, RBCs and platelets.

RANSEL is based on the reaction of GSH with Cumene hydroperoxide (ROOH) catalyzed by GPx and yielding in GSSG. The latter is converted by glutathione reductase (GSHRed) and NADPH to GSH and NADP. The concentration of GPx is assessed from the decrease in absorption at 340nm due to the oxidation of NADPH to NADP. GPx activity was expressed as U/g Hb.

Statistical analysis

Statistical analysis was performed with Microsoft Excel 2000 (average, standard deviations, Student's t-test for unpaired data between groups) and the indices that showed significant difference ($p<0.05$) with this test were controlled with one-way ANOVA test by the help of Microcal Origin 3.5 programme (Microcal Software Inc.).

Results

All subjects were alive a year later and no non-fatal myocardial infarctions were recorded in them.

Out of 54 procedures 3 were unsuccessful and the patients were assigned to reconstructive surgery. The data of these patients (all men) were not used in further analysis.

In the group of reoccurring symptoms (n=9) there were angiographically proven restenoses defined as narrowing of a vessel by >50% in 7 patients (in 2 women and 5 men) and one patient (man) had developed stenosis in another branch. The anamnestic parameters of the subjects having restenoses and symptom-free/ stable subjects (stable group) are presented in Table1.

All subjects who had confirmed restenosis had had previously myocardial infarction but not all subjects who had had previous myocardial infarction developed restenosis. Nevertheless having a myocardial infarction in anamnesis was a significant predictor for restenosis (p=0.01, Table1).

When comparing the restenosis group with clinically stable group we found no difference in cholesterol or HDL-cholesterol (Table 2). The glutathione redox-ratio was significantly higher (that means more oxidized) in the restenosis group. SOD activity in both groups was similar to endemic normal (that is 722±86 U/g Hb, unpublished data) so the change of this enzyme's activity would not likely be a common reason for impaired endothelial defense in angioplasty patients. The activity of GPx in the restenosis group was 40.36 ± 35.16 U/g Hb and 15.01 ± 12.29 U/g Hb in the clinically stable group (the endemic normal being 45±11 U/g Hb (unpublished data)) – so the change of the activity of GPx might also not serve as an indicative predictive parameter for restenosis as the restenosis subjects resemble the endemic normal more than the subjects not developing complaints in a year.

There also appeared a significant difference between groups in hematokrit values (38.33±3.72% vs. 41.25±3.60%, p= 0.04) and TAS (0.98±0.16mmol/l vs. 0.84±0.15mmol/l, p= 0.014) with Student's t-test but the results were not confined by 1-way ANOVA.

Discussion

The novelty of our study stands in the complex approach to the problem of post angioplasty restenosis from the point of view of systemic and cellular oxidative stress. Intracellular factors (GSH, GPx, SOD, CAT) and circulating lipids' and proteins' oxidative state (LDL lag phase, CD, PC) as well as more general parameters TAA and TAS were quantitated prior to the procedure. The endpoints were event-free survival and restenosis within a year. Although most restenoses develop within six months post procedurally [29] we selected longer

period as the indication for repeating angiography in our settings depended on the patients' subjective assessment of his symptoms. Like clinicians in their treatment decisions we relied on the subjective criteria, as, though not done in the PTCA group but in coronary artery bypass grafting, the SHOT study clearly related the presence of angina pectoris and occurrence of myocardial infarction in 1 year to occlusion rates [30].

As it was predicted there could be found numerous correlations between parameters when subjects were analyzed from the point of view of classical risk factors (hypertension, smoking status, overweight, diabetes etc).

When analyzing the enrolled diabetic patients compared to non-diabetic patients we found differences in glutathione redox-status (GSSG/GSH 1.92± 2.19 in diabetics versus 0.90± 0.76 in non-diabetics, p=0.03) and LDL-lag phase (64.17±18.58 min in diabetics and 53.89±13.65min in non-diabetics, p=0.03). Smokers were significantly younger (50.7±9.4 versus 59.5±8.1 years, p=0.005) and current non-smokers had significantly higher BMI (28.6± 3.3versus 25.5±2.9, p=0.006). The persons with chronic (extracardial) illnesses had higher TAA (37.7±6.0mmol/l versus 33.8±5.3mmol/l, p=0.03) and they had been smoking longer (23.3±12.9 versus 13.4± 14.9 years, p=0.03). Hypertensive patients had LDL lag phase shorter than normotensive patients (51.3±15.2min versus 61.2±14.2 min, p=0.04). The activity of GPx in high blood pressure group was lower than in the subjects with normal blood pressure (9.64±7.0 U/g Hb versus 24.83±18.1 U/g Hb, p=0.01). Patients with previous myocardial infarction had less CD (41.6±12.6μM versus 54.04±21.5μM, p=0.02) but no difference in LDL lag phase. All subjects but one of those who developed restenosis had stent(s) that represented well the overall use of stents in that period. Two women out of 8 female subjects developed restenosis. The finding that LDL oxidation lag phase is longer (though not significantly) in the restenosis group cannot be explained by the fact that diabetic patients had the same tendencies as only one diabetic patient had confirmed restenosis.

When restricting the analysis to just comparing the persons who had developed restenosis with subjects who had not had aggravation of symptoms in a year the only anamnestic difference was in having previous myocardial infarction(s) and the only oxidative stress-related parameters that had significant differences with both statistical methods was the glutathione redox ratio and the activity of GPx. It seems that the glutathione system, particularly the levels of glutathione redox status, may have prognostic value for predicting restenosis after coronary angioplasty.

Indeed, glutathione has become a matter of growing biomedical and clinical interest [30–33, 19]. In balloon-catheter injury models, it has been documented that mechanical injury promotes an initial loss of cellularity, and also that programmed cell death or apoptosis is observed early after balloon distention injury and may contribute to the early medial smooth muscle cell loss. ROS may mediate the cell suicide program as well as cell growth. Pollman

et al documented that balloon injury was associated with a marked reduction in glutathione levels and demonstrated that treatment with antioxidants such as N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) inhibited the acute induction of cell death. They concluded that balloon injury induces acute cell death via a redox-sensitive mechanism [35]. Souza et al (2000) demonstrated a massive occurrence of electron transfer reactions during or shortly after vascular injury. They also observed a profound decrease of the GSH/GSSG ratio and glutathione pool showing that intracellular thiols were major targets for the electron transfer reactions [36]. Clinical data [37] and a wide spectrum of GSH biofunctionality (a principal antioxidative compound in cellular defense against high-grade oxidative and nitrosative stress; participation in the metabolism of nitric oxide, leukotrienes and prostaglandins; involvement in the regulation (glutathionylation) of key-enzymes like phosphorylase, creatinine kinase, etc.; restoration of activity of proteins and enzymes; stabilization of biomembranes and a modification of intracellular signals including those of inflammation, gene expression and cell proliferation) indicate the importance of glutatione. Already in 1998 it has been shown that the RBCs afforded some protection against oxidative damage to the endothelial cells by taking up and deactivating the superoxide ions. As demonstrated, this protection depends upon intact RBC glutathione system [38]. Hence, maintenance of physiological glutathione redox ratio has high impact as the response of cells to any stress involves changes in thiol content as they are consumed to protect cells by different actions like antioxidation, detoxification, signaling, and the direct modification/regulation of biomolecules.

Any interpretation of data about GPx remains speculative due to the multifaceted nature of restenosis, existence of isoforms of GPx and multifaceted biofunctionality of GSH (there a lot of GPx- nonrelated pathways for GSH). However, one possible explanation of the lower activity of GPx in the subjects who did not develop restenosis compared to the endemic normal and restenosis group may be that the failure to regulate the activity of GPx may serve as one of the mechanisms of restenosis-prone endothelial response. Already in 1993 the activity of GPx was measured during PTCA in humans – and the results indicated the fall of GPx activity that was maximal 10 min after balloon deflation and lasted at least 60 min [39].

Another finding that cannot be overlooked is that our endemic normal for serum catalase activity is 55±23 kU/l and the average of subjects with both favorable outcome and restenosis was 2–3 times higher. This might be of the set implied by Cedro as “enhanced lipid peroxidation and inefficient antioxidant defense mechanisms are likely to be present in an atherosclerosis-related disease “ [8]. When looking into the results in detail we found one subject with endemic normal values in the restenosis group and 17 in the clinically stable group. What is more interesting is that the correlation coefficient between GPx and CAT values was –0.25 in the stable group and –0.88 in the restenosis group.

The publications [3, 16, 40] report somewhat conflicting results concerning the predictive value of lipoproteins towards restenosis. We did not find any significant differences between groups in the content of total cholesterol or HDL-fraction. Of the last-mentioned HDL-content at least showed a trend that might transform in statistical difference in other subject groups. Based on our results it may be speculated that the most indicative predictive factors should be searched for from the list of endothelial parameters rather than from those of LDL or other circulating compounds. The results of the effect of PTCA on the "classical" parameter of MDA are conflicting. In 1997 Oosenbrug stated a temporary increase in MDA [6] but in 2002 Olsson et al questioned the effect as they could not measure any increase in MDA after 30 to 90 minutes [41] and Cedro et al [8] had results consistent with the latter in 1 min after deflation. As angioplasty temporarily increases at least F2-isoprostane production [10] the pre-existing protective potential of the local endothelium becomes of major importance. The results on an experimental model [7] and a link between tissue factor activity and restenosis [42] have supported this concern as tissue factor is normally of perivascular origin and its pathological expression of endothelial cells may contribute to thrombosis and atherosclerosis progression.

As a result of our investigation we would suggest that the troubled redox status of glutathione as a member of complex analysis for predictive factors should be an alarm for possibly increased risk of restenosis.

Conclusions

Intracellular glutathione system is involved in maintenance of balance between pro-oxidants and antioxidants, modification of signals etc. Glutathione redox ratio seems to be a possible candidate to be included in complex investigation of angioplasty patients for predicting restenosis.

Competing interests

There are no competing interests to declare.

Authors' contributions

AR principally designed the investigation and coordinated the process, instructed the participants, interviewed the subjects, gathered the data from clinical protocols, measured the PC and drafted the manuscript. MU participated in the clinical design and coordination of the project. CK carried out the assays of SOD and GPx as well as interpreted the results. TK carried out the TAS and TAA assays. KZ carried out the assays of CD and LDL lag phase. TV measured glutathione and fractions and interpreted the results. KT carried out the CAT assays and helped with the statistics. MZ conceived the study, and participated in its design and interpretation of the results as well as in preparation of the manuscript. All authors read and approved the final manuscript.

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Tables

Table1 – Anamnestic data of the subjects.

Parameter	Restenosis (n=7)	Stable (n=44)	p
Age, years	54.86 ± 10.59	57.00 ± 9.61	0.30
BMI, kg/m^2	26.53 ± 2.70	27.67 ± 3.70	0.23
Years of stenocardia	5.94 ± 8.95	7.37 ± 10.29	0.39
Years since diagnosis	2.60 ±3.81	2.16±3.88	0.08
Previous IM (n)	7	11	0.01*
Diabetes (n)	1	5	0.37
Hypertension (n)	3	16	0.16
Smoking (n)	3	7	0.23

The anamnestic and paraclinical findings of the subjects who had confirmed restenosis and who had no increase in stenocardia intensity /stage nor fatal/nonfatal myocardial infarction (termed “stable” in the table) a year after angioplasty. Data are presented mean ± SD. p is calculated with t-test. p is considered significant (*) when <0.05.

Table 2 – Comparison of preprocedural biochemical markers.

Parameter	Restenosis	Stable	p
Cholesterol, mmol/l	6.14 ± 2.39	6.22 ± 1.64	0.46
HDL cholesterol, mmol/l	0.94 ± 0.19	1.15 ± 0.29	0.09
Hb, g/l	134.70 ± 11.77	140.41 ± 10.18	0.09
Ht, %	38.33 ± 3.72	41.25 ± 3.60	0.04
LDL lag phase, min	62.86 ± 6.31	53.70 ± 14.33	0.05
CD μM	42.24 ± 14.99	52.11 ± 18.81	0.10
CAT kU/l	165.67 ± 80.54	134.93 ± 93.02	0.21
TAA %	34.57 ± 5.53	35.66 ± 6.16	0.33
TAS mmol/l	0.98 ±0.16	0.84 ± 0.15	0.014
PC nmol/mg prot	1.10 ± 0.41	1.09 ± 0.53	0.48
GSSG/GSH μg/μg	2.21 ± 2.36	0.88 ± 0.74	0.002*
GPx U/g Hb	40.36 ± 35.16	15.01 ± 12.29	0.003*
SOD U/g Hb	737.93 ± 192.18	709.44 ± 159.62	0.34

The biochemical findings of the subjects who had confirmed restenosis and who had no intensification of complaints (the "stable" group) a year after angioplasty. Data are presented mean ± SD. p is calculated with t-test. p is marked significant (*) when <0.05 also with one-way ANOVA.