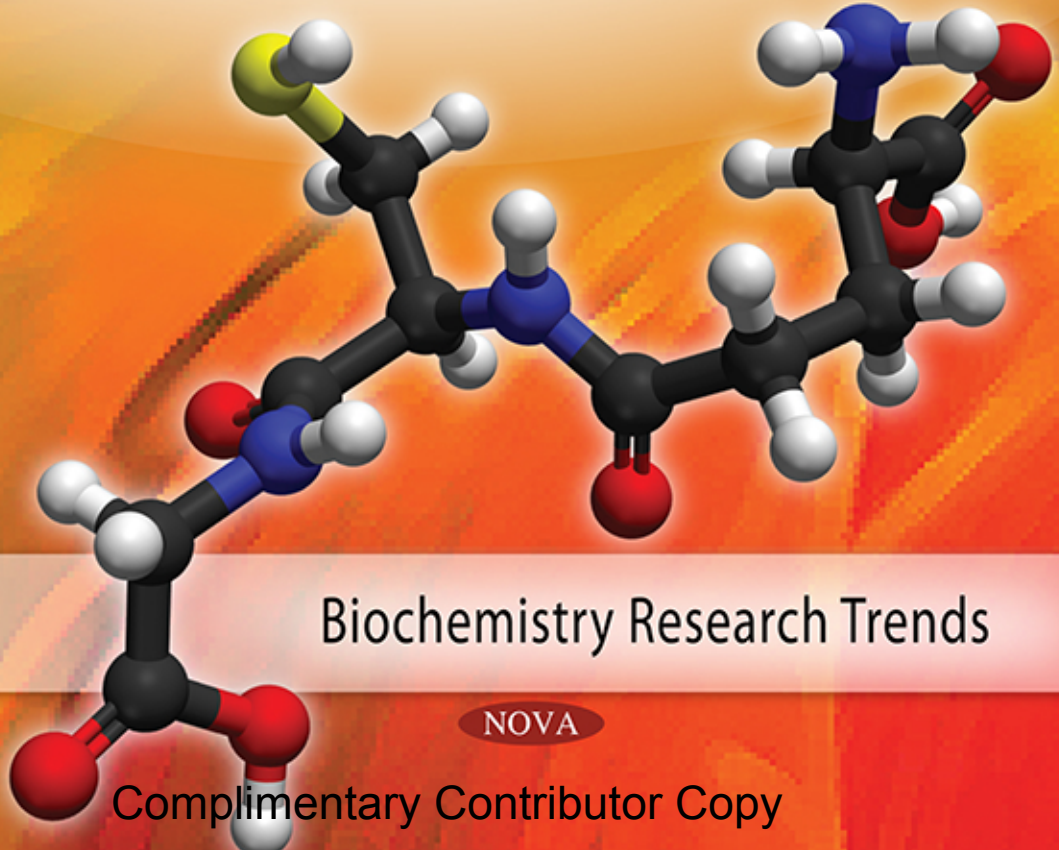
Ashley Wilber
Editor

Glutathione

Dietary Sources, Role in Cellular Functions and Therapeutic Effects



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DIETARY SOURCES, ROLE IN CELLULAR FUNCTIONS AND THERAPEUTIC EFFECTS

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GLUTATHIONE

DIETARY SOURCES, ROLE IN CELLULAR FUNCTIONS AND THERAPEUTIC EFFECTS

ASHLEY WILBER
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Contents

Preface

Glutathione is a widely mentioned biochemical molecule in present clinical biochemistry. There is no doubt that glutathione has several advantages to human bodies. Hence, there are many applications of glutathione products in present biomedicine. Glutathione plays a critical role in cell signalling and antioxidant defences. It plays a significant role in subjects with periodontitis and associated systemic comorbidities. Its depletion leads to oxidative damage. This book discusses dietary sources of glutathione as well as the role it plays in cellular functions, and it's therapeutic effects.

Chapter 1 – Tripeptide glutathione (L-γ-Glutamyl-L-Cysteinyl-Glycine) is a wide spread physiologically active compound in plants and animals, where it stands second to none in terms of its role as a significant redox buffer in oxidative stressed cells. GSH plays various roles as reductant in ascorbate preroxidase cycle, as a building block for the synthesis of higher thiols such as phytochelatins by phytochelatin synthase and/or as agent for conjugation of xenobiotics by glutathione sulfo-transferase. Though a tremendous progress has been made in GSH focused research, there seems information paucity in context with cross-talks on GSH role in plants and humans, and its significance and prospects in analytical chemistry and nanobiotechnology. Given the above, this chapter aims to present first, a basic overview about the main roles of GSH in plants and mammals (particularly in environmental stress context) followed by a short review about analytical methods for GSH analysis, and a detailed discussion on nanobiotechnology perspectives for GSH.

Chapter 2 – Glutathione (GSH) plays a critical role in cell signalling and antioxidant defences. It plays a significant role in subjects with periodontitis and associated systemic comorbidities. Its depletion leads to oxidative damage. Some of the prevalent redox reactions and interactions with dietary agonists are addressed. Glutathione may interact directly with ROS / reactive nitrogen species (RNS); or act as an essential cofactor for GSH S-transferases and glutathione peroxidases. Coordinated actions of GSH and its dependent enzymes which constitute the glutathione system, lead to detoxification of reactive oxygen and nitrogen species (ROS/RNS). Therapeutic interventions aimed at enhancing GSH concentrations *in vivo* include N-acetyl cysteine; activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) by folate supplementation and phytochemicals such as curcumin and resveratrol. An antioxidant defence system comprising a range of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) is active in removing ROS accumulating in cells, in addition to vitamin A, vitamin C, α-tocopherol and plant flavonoids which are available as dietary antioxidants.

Oxidative stress plays an important role in chronic periodontitis (CP), the metabolic syndrome (MetS) and associated conditions. There is a significant correlation between SOD activity, triglycerides, high-density lipoprotein and sVCAM-1 levels. The association between SOD activity and MetS components could be the most significant variable parameter in subjects with MetS; it has potential as a predictive tool to determine the degree of oxidative stress in these subjects. The impact of diabetes mellitus (DM) as a risk factor for CP in the context of antioxidant enzyme activity, SOD, glutathione reductase (GR), catalase and the marker of free radical damage, malondialdehyde, favours the role of oxidative stress in both DM and CP.

Dietary non-enzymic antioxidants play an important role in interacting with oxidative stress-inducing mechanisms. Their targeted interaction with the glutathione network results in an enhanced antioxidant profile in chronic inflammatory diseases associated with excessive pro-oxidant activities. Dietary agonists are able to overcome proxidant profiles associated with decreased Nrf2 linked to reduced CAT and GPx m RNA expression. Their administration contribute to coordinated cytoprotective responses in tissues.

Chapter 3 – Azathioprine is a purine antimetabolite drug commonly used as immunomodulator in the treatment of various chronic inflammatory diseases, such as inflammatory bowel disease (IBD). Azathioprine is activated *in vivo* after reaction with reduced glutathione (GSH) and conversion to mercaptopurine. Although this reaction may occur spontaneously, the presence of the enzyme glutathione-S-transferase (GST), in particular of isoforms GST-A1/GST-A2 and GST-M1, can increase its speed, leading to a faster activation of azathioprine to active thioguanine nucleotides. Moreover, GSTs may contribute to azathioprine effects by modulating GSH consumption, oxidative stress and apoptosis. Indeed, in young patients with IBD, deletion of GST-M1, which determines reduced enzymatic activity, was recently associated with reduced sensitivity to azathioprine and reduced production of its active metabolites. Therefore, genetic polymorphisms in genes for GSTs may be useful to predict response to azathioprine even if more *in vitro* and clinical validation studies are needed.

Chapter 4 – The authors described previously in PC 12 cells that inhibition of glutathione biosynthesis induced by high concentrations of glutamate or addition of buthionine sulfoximine led to a rapid oxidative shock followed by a slowly progressing apoptosis. On the other hand, the effects of glucocorticoids on brain cell survival or death remain a matter of controversy since both neuroprotective and neurotoxic actions have yet been described by different groups in various experimental models.

In the present paper the authors have thus studied the effect of dexamethasone, a potent synthetic glucocorticoid, on the toxicity induced by glutathione depletion in PC 12, a neuronal-like cell line. They first observed that Dex was able to markedly decrease the deleterious effect of buthionine sulfoximine addition on cell survival but failed to protect PC 12 cells from an oxidative shock induced by either cumene hydroperoxide or 6-hydroxy-dopamine. The characteristics of Dexamethasone action, i.e. time-course, dose-response curve and antagonism by mifepristone, strongly suggested that it represents a typical receptor-mediated event. The authors also demonstrated that PC 12 cells contained immunoreactive glucocorticoid receptors. Dexamethasone failed however to alter intracellular glutathione content and did not significantly influence two of the major cellular defences against oxidative shock: glutathione peroxidase and superoxide dismutase. Although the precise mechanism of the protection provided by dexamethasone remains to be defined, they consider

that their model may represent an useful tool to investigate the effects of glucocorticoids on brain cells survival.

Chapter 5 – *Rationale:* Reduction-oxidation reactions determine cell homeostasis and free-radicals productions are invariable components of the aerobic metabolism processes. The cells have an elaborate defense against free-radicals and the imbalance resulting in excessive accumulation of free-radicals, defined as oxidative stress which plays a key role in promotion of pathological processes including cancer. Hence physiological levels of free-radicals mediate crucial intracellular signaling pathways and are essential for cell survival whereas excess generates cell damage and death. Thereby, "hormetic" responses to free-radicals are resulting from the constant ongoing battle between the production of oxidants and the antioxidants defenses. Among the oxidative stress-dependent compounds are the thiol-antioxidants having glutathione (GSH) as its major representative intracellularly. Alterations in GSH levels are associated with human diseases including cancer where it has "double-edge sword" actions by protecting non-tumor cells against oxidative stress and by removal and detoxification of carcinogens.

However, at the other end of the scale GSH protects tumor cells from apoptosis by increasing the resistance to cancer chemotherapeutic agents. By its physiological importance GSH levels can be controlled endogenous and exogenously by changing its biosynthesis with nutrients such as amino acids and vitamins. Almost none has been found in the available literature about B vitamin related-GSH metabolism in esophageal cancer . Nutritional deficits in fresh fruits, vegetables and dietary fiber are commonly referred as associated with the presence of esophagus cancer (EC). Moreover heavy consumption of alcoholic beverages and tobacco might interfere with vitamins and dietary components with potential anti-carcinogenic effects. To their understanding the adequacy of B-vitamins would allow the full effects of the sulfur-containing antioxidative defenses.

Methods: Twenty-six patients with EC (58.4 ± 11.8 years) and a control group of 20 healthy subjects (27 ± 8.4 years) were assessed for nutritional and biochemical markers at baseline (Mo). The EC patients were distributed in two groups G1/G2 to be either supplemented with placebo or vitamins B_2, B_6, B_{12} and folate during 15 days (M1) followed by cross-over for the same period (M2). The results were statistically analyzed.

Results: The EC patients were predominantly males addict to smoke and alcoholism, diagnosed with squamous-cell carcinoma, stage IV. Their food intake was inappropriate, particularly energy resulting in 46% with Body Mass Index (BMI) <18 kg/m^2 (15.9 ± 1.7 kg/m^2) presenting a body-weight loss of 21.3 ± 13.4% during the last 6 months. However plasma albumin and glucose were similar to controls. No significant difference was also found for cholesterol, folate, and levels of Methionine (Met), Homocysteine (Hcy), Glutamic acid (Glu), and in the glutathione disulfide/glutathione ratio (GSSG/GSH). After the intervention it was observed an increasing of B_{12} vitamin and decreased levels of Hcy.

Conclusion: EC in its advanced stage has a different pattern of thiol pathways with the most preserved amino acids being methionine, homocysteine and glutamate. Met/Hcy (transmethylation/remethylation) cycle was maintained whereas Hcy/Cys (transsulfuration) was reduced therefore accumulating Hcy. However even in the presence of lower Cys it seems that there is an effort of the host to generate GSH by uptaking more GSH- precursors (Cys and Glu) from the GSH-gamma GT cycle for keeping controlled the GSSG/GSH ratio. Short-term B-vitamin supplementation led to increased plasma vitamin B12 which together with normal folate contributed effectively for reducing Hcy. By keeping controlled Hcy and

GSH/GSSG the cell would tend to reduce the oxidative stress and probably the tumor progression, what could be attributed presently to the supplemented vitamins.

Chapter 6 – Adult T-cell leukemia/lymphoma (ATL) is a fatal malignancy caused by infection with human T-cell leukemia virus type Ⅰ (HTLV-1). Worldwide, 10 to 20 million people are infected with HTLV-1 and a part of viral carriers (6-7% for men and 2-3% for women) develops ATL after a long latent period (at least 20-30 years). There is much room for improvement in accepted curative therapy for ATL and the development of new therapeutic and preventive strategies is necessary. Functional foods and their ingredients are focused as natural resources for the prevention and treatment of life style-related diseases. Considering that only a part of viral carriers develops ATL after the long latent period, it is speculated that ATL onset is influenced by a diet taken daily. Recently, they have reported that carnosol, which is an ingredient contained in rosemary (*Rosmarinus officinalis*), induces apoptosis in ATL cells via glutathione depletion. This suggests that glutathione depletion caused by functional food ingredients may be a possible new target for the prevention and therapy of ATL. In this review, the authors present an overview of the developmental mechanism of ATL, glutathione as new preventive and therapeutic target of ATL, and glutathione regulation by carnosol.

Chapter 7 – Glutathione is a widely mentioned biochemical molecule in present clinical biochemistry. It is no doubt that glutathione has several advantages to human bodies. Hence, there are many applications of glutathione products in present biomedicine. An interesting application is the glutathione food supplementation. In the present brief article, the authors will summarized and discussed on the present status of glutathione food supplementation in Thailand.

Chapter 8 – Diabetes mellitus, as a disease with dramatically increasing incidence, affects the quality of life especially due to the presence of chronic diabetic complications like neuropathy, retinopathy or nephropathy. One of the factors in complex and not fully understood etiology of diabetes and its chronic complications is oxidative stress, characterized as an imbalance between reactive oxygen species (ROS) production and the function of antioxidant mechanisms. Glutathione S-transferase (GST) represents a family of enzymes catalyzing the conjugation of glutathione with various electrophilic compounds to facilitate their excretion. GSTs detoxify some of the secondary ROS generated during oxidation of membranes or other cellular constituents, act in the detoxification of organic hydroperoxides and protect cells from peroxide-induced cell death. GST enzymes are involved in the synthesis of inflammatory mediators, leukotrienes and prostaglandins and act also in cell signalling pathway as potential regulators of apoptosis. The most researched GST enzymes are glutathione S-transferase mu 1 (GST M1) and glutathione S-transferase theta 1 (GST T1). It has been shown that individuals carrying the *null* genotype of GST have significantly reduced activity of this enzyme compared to *wild* genotype carriers. This chapter brings comprehensive review about the possible role of oxidative stress in etiopathogenesis of type 1 diabetes, type 2 diabetes and chronic diabetic complications. Recent studies also assume the role of GST gene polymorphisms while the information varies according to the authors, region and studied population. More knowledge about this predisposition factor may bring the basis for the possible therapeutic intervention in the future.

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Chapter 1

Glutathione: Stress-Alleviation Role in Plants and Human, and Prospects in Analytical Chemistry and Nanobiotechnology

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Abstract

Tripeptide glutathione (L-γ-Glutamyl-L-Cysteinyl-Glycine) is a wide spread physiologically active compound in plants and animals, where it stands second to none in terms of its role as a significant redox buffer in oxidative stressed cells. GSH plays various roles as reductant in ascorbate preroxidase cycle, as a building block for the synthesis of higher thiols such as phytochelatins by phytochelatin synthase and/or as agent for conjugation of xenobiotics by glutathione sulfo-transferase. Though a

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tremendous progress has been made in GSH focused research, there seems information paucity in context with cross-talks on GSH role in plants and humans, and its significance and prospects in analytical chemistry and nanobiotechnology. Given the above, this chapter aims to present first, a basic overview about the main roles of GSH in plants and mammals (particularly in environmental stress context) followed by a short review about analytical methods for GSH analysis, and a detailed discussion on nanobiotechnology perspectives for GSH.

Abbreviation

ABD-F	7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide
APX	ascorbate peroxidase
ARDS	acute respiratory distress syndrome
BCPB	1-benzyl-2-chloropyridinium bromide
CAT	catalase
CE	capillary electrophoresis
CMQT	2-Chloro-1-methylquinolinium tetrafluoroborate
Cys	cysteine
CySH	5'-adenosinephosphosulphate reductase
DHAR	dehydroascorbate reductase
DHR-123	dihydrorhodamine 123
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
ECD	electrochemical detection/detector
ECV304	human urinary bladder carcinoma cell line
ESI	electrospray ionization
FD	fluorescence detection/detector
FDNB	2,4-dinitrofluorobenzene
FIA	flow injection analysis
GCS	γ-glutamylcysteine synthetase
Gly	glycine
GPX	guaiacol peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSH-EE	glutathione ethyl ester
GSMB	glutathione-bimane adduct
GSSG	glutathione disulfide
GST	glutathione sulfo-transferase
HPLC	high-performance liquid chromatography
HUVEC	human umbilical vein endothelial cells
IAA	iodoacetic acid
5-IAF	5-iodoacetamidofluorescein
LDL	low-density lipoprotein
LIF	laser-induced fluorescence
LOD	limit of detection
mBB	monobromobimane

MDHAR	monodehydroascorbate reductase
MFNS	magnetic fluorescent nanosensor
mGSH	mitochondrial GSH
MPA	3-mercaptopropionic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NAC	N-acetylcysteine
$NADP^+$/ NADPH	nicotinamide adenine dinucleotide phosphate
NDA	2,3-naphthalenedialdehyde
NEM	N-ethylmaleimide
NBD-Cl	4-chloro-7-nitrobenzo-2-oxa-1,3-diazol
OPA	o-phthaladehyde
PCs	phytochelatins
ROS	reactive oxygen species
SAM	S-adenosyl-L-methionine
SBD-BF	ammonium 5-bromo-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate
SBD-F	ammonium 7-Fluoro-2,1,3-benzoxadiazole-4-sulfonate
SOD	superoxide dismutase
ThioGlo®3	9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-phenyl)-3-oxo-3H-naphto[2,1-b]pyran
TNF	tumor necrosis factor α
UV	ultraviolet

1. Introduction

Sulfur (S), one of chalcogens, is widely spread in the nature in a variety of forms and is one of the significant contitutents of numerous compounds. These S-containing molecules are important in the redox interactions have a huge impact in living organisms. One of most important thiol compounds, glutathione (GSH; L-γ-Glutamyl-L-Cysteinyl-Glycine) was first observed in 1888 by de Rey Pailhade and was named as "hydrogenant le soufre". At that time it was not identified as GSH that we know today. That was done over more than 30 years after by Frederick Gowland Hopkins in 1921 [1]. He also proved that GSH is a ubiquitous molecule distributed in cells of plant and animals. Hopkins firstly defined GSH at that time as a dipeptide which was controversial until 1929; when Hopkins with Edward Calvin Kendal, as an independent confirmer, proved that GSH is consisted of glutamic acid (Glu), cysteine (Cys) and glycine (Gly) [2]. Later, the tripeptide composition of GSH was confirmed by Harington and Mead [3, 4]. This controversy was not deleterious for Hopkins because he was awarded by Nobel Prize in Physiology or Medicine in 1929 with Christiaan Eijkman for the discovery of vitamins. GSH was then deeply studied through enzymology when enzymatic catalysis of GSH was described [5]; and the enzyme which was responsible for it (GSH sulfo-transferase, GST) was isolated and purified in 1974 [6]. Thereafter, in the last quarter of the 20^{th} century due to the intensive development of biochemical and enzymological methods, number of findings leading to our current knowledge of GSH was unveiled.

A range of studies have confirmed GSH as a ubiquitous and functionally significant compound in living organisms. It helps plants to cope with different kinds of stresses and it also relieves different kind of stresses in humans. GSH has been also been considered as one of "guardians" of homeostasis in the living organisms. Moreover, GSH could be successfully used in specific nanotechnology applications using the novel approaches.

The aim of this chapter is to briefly review GSH at three significant fronts such as environmental, human nutrition, analytical chemistry and nanobiotechnology.

2. Role of Glutathione (GSH) in Plants

Plants are a critical component of ecosystems. However, being stationary in nature, plants may significantly interact with and respond to their immediate environment. To the other, environmental stress factors are mounting and creating adverse conditions that in turn are negatively impacting plant growth, development and productivity [7]. In fact, oxidative stress as a result of enhanced production of reactive oxygen species (ROS) has been considered as one of major mechanisms of environmental stress factors-mediated consequences primarily in plants and secondarily in plant productivity [7, 8]. Oxidative stress is a physiological condition where occurs an imbalance between the pro-oxidants (ROS and its reaction products) generation and their antioxidants-mediated metabolism/scavenging. Thus, vital cellular organelles, lipids, proteins, nucleic acids and pigments can be oxidatively damaged that may lead ultimately to cellular metabolism arrest due to un-metabolized ROS and its reaction products [7-9].

ROS-scavenging system in plants comprises a number of enzymes (such as superoxide dismutase, SOD; catalase, CAT; guaiacol peroxidase, GPX; glutathione sulfo-transferase, GST; ascorbate peroxidase, APX; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione reductase, GR) and non-enzymatic antioxidants (such as ascorbate, AsA; glutathione, GSH; carotenoids; tocopherols; phenolics), where scavenging/metabolism of ROS and its reaction products is possible as a result of a synchronous action of previous enzymatic and non-enzymatic antioxidants [8-12].

GSH is a multifunctional water-soluble peptide with a sulfhydryl (-SH) group, a substrate for DHAR in the AsA-GSH pathway, a potent xenobiotics-detoxifier, and a key player in cellular redox homeostasis [8-13]. Plants tend to maintain an optimum level of GSH pool required for normal metabolic activities under non-stress conditions. However, a number of stress factors including metal/metalloids stress, salinity/drought stress and different temperature regimes (low/high), as well as GSH reaction with different ROS lead to GSH oxidation (producing GSSG) [14-17]. Apart from the significance of GSH in plant disease resistance, cell proliferation, root development, the transport and storage of S, the synthesis of proteins and DNA [18-20], GSH is also involved in redox homeostasis and signaling [16, 21-28], detoxification of a range of xenobiotics, herbicides and air pollutants (sulfur dioxide and ozone), heavy metals, temperature stress (high/low) [8, 11, 18, 29].

The significance of GSH and its redox couple (GSH/GSSG) in plant tolerance to a number of environmental stresses (including metal/metalloids stress, salinity/drought stress and different temperature regimes (low/high) has been credibly reported [8-12, 16] (Table 1; Figure 1). A brief description on the previous aspects is given hereunder.

2.1. GSH vs. Plant Metal/Metalloids Stress

The contamination of agricultural soils with metal/metalloids has become a major problem severely impacting both the productivity of plants and the safety of plant products as foods and feeds. Modulation of GSH and its redox couple has been reported in different plants under metal/metalloid exposure. Reduced GSH pool may be significantly decreased or increased in metal/metalloid exposed-plants (Table 1). Cd-accrued decrease in the GSH pool has widely been reported in different plant species by a number of workers including Balestrasse et al. [30], Schutzendubel et al. [31], Zhang et al. [32], Hsu and Kao [33], Wu et al. [34] and Anjum et al. [35-37]. Gupta et al. [38] and Piechalak et al. [39] are among those workers who reported Pb-mediated decrease in GSH pool; whereas, Cu and U mediated decrease in GSH pool was evidenced by Nagalakshmi and Prasad [40] and Vandenhove et al. [41], respectively. Elevated GSH pool has also been evidenced in plants under Cd [42-49]. GSH pool can be modulated by the levels of the metals supplied, the plant species, the age of the plant and duration of the treatment (reviewed by Anjum et al. [8]). Different genotypes of the same plant differing in metal tolerance may exhibit varied GSH and GSSG levels [34, 36, 37, 47, 50-52]. The total GSH level and GSSG/GSH ratio can also be modulated by the stress-exposure duration [53]. Compared to sensitive plants, metal/metalloid tolerant plants exhibit higher levels of GSH level and GSSG/GSH ratio (reviewed by Anjum et al. [8]).

Table 1. Examples of reports on the modulation of glutathione (GSH) pool in plants exposed to metal/metalloids, salinity and drought stresses[1]

Stress types/levels	Plant species/part studied	Response	Reference
Cadmium			
25, 50 and 100 mg kg^{-1} soil	*Brassica campestris* leaves	–	[36, 37]
25 and 50 µM	*B. juncea* leaves	+	[50]
50, 100 and 200 mg L^{-1}	*B. juncea* seedlings	+	[51]
0.5, 1.0, 1.5 and 2.0 mM	*B. juncea* seedlings of ten cultivars	+	[47]
100 mg L^{-1}	*Candida tropicalis*	+	[54]
10 µM	*Ceratophyllum demersum* seedlings	–	[55]
0 – 100 µM	*Cucumis chloroplast*	–	[32]
50 µM	*Cucurbita pepo* glandular trichomes	–	[56]
50 and 100 µM	*Glycine max roots*	–	[30]
100 µM	*Fontinalis antipyretica* Young apical tips and older basal segments	+	[57]
0.5 and 5 µM, After 5 days exposure	*Hordeum vulgare* roots, stem and leaf	+	[34]
0.5 and 5 µM, after 10 days exposure	*H. vulgare* roots, stem and leaf	–	[34]
10, 20 and 40 mg L^{-1}	*Oryza sativa* roots and shoots	+	[52]
1 and 5 µM	*O. sativa*	+	[43]
50 µM	*O. sativa roots*	+	[58]
50 µM	*O. sativa* roots	+	[58]

Table 1. (Continued)

Stress types/levels	Plant species/part studied	Response	Reference
50 or 100 µM	*Phragmites australis*	+	[46]
5 mg kg^{-1} sand	*Pisum sativum*	+	[45]
0 – 20 µM	*P. sativum*	+	[42]
5 or 50 µM	*Pinus sylvestris roots*	–	[59]
5 or 50 µM	*Populus canescens roots*	–	[31]
10, 20, 40, 80, 160 and 320 µM	*Sedum alfredii*	+	[49]
250 µM	*S. drummondii* callus	+	[44]
25, 50 and 100 mg kg^{-1} soil	*Vigna radiata* cultivar leaves	–	[35]
25, 50 and 100 mg kg^{-1} soil	*V. radiata* cultivar leaves	–	[60]
Chromium			
50, 100 and 200 µM	*B. juncea* seedlings	+	[61]
50, 100 and 200 µM	*V. radiata* seedlings	–	[61]
Copper			
100 µM	*B. nupus* roots	–	[62]
100 µM	*B. nupus* leaves	+	[62]
50, 100 and 200 µM	*Scenedesmus bijugatus* cells	–	[40]
Mercury			
10, 20, 30, 40, 50 and 100 mg L^{-1}	*Sesbania drummondii* seedlings	+	[44]
10 µM	*Medicago sativa* roots	–	[63]
0 and 40 µM	*M. sativa* roots	+	[64]
Zinc			
0–3200 µM	*S. alfredii* shoot	+	[65]
50 µM)	*Phaseolus vulgaris* seedlings	+	[53]
50, 100, 200, 400 and 800 µg mL^{-1}	*Avicennia marina* Seedlings	×	[66]
Salinity			
100 mM NaCl	*Lycopersicon pennelli* leaf	+	[67]
100 mM NaCl	*O. sativa* root	+	[68]
200 mM NaCl	*O. sativa* seedlings	+	[69]
0, 75, 150 mM NaCl	*Brassica napus* leaf	+	[70]
100 mM NaCl	*Lycopersicon esculentum* root	–	[19]
0, 50, 100, 150, and 200 mM NaCl	*Pyrus betulaefolia* leaves	+	[71]
75 mM NaCl	*Populus×canescens* leaves	+	[72]
Drought			
RWC from 55 to 28%	*Acer saccharinum* embryo	+	[73]
PEG solution 10% (w/v) for 3 days	*Cucumis sativus* leaves	+	[74]
-1.0 and -2.1 MPa for 24-72 h using PEG-6000	*O. sativa* seedlings	–	[75]
RWC from 90 to 40%	*Helianthus anuus* leaves	–	[76]
PEG-6000 solution 23% (w/v) for 7 days	*O. sativa 'Xiangnuo no. 1' and 'Zimanuo'* leaves	–	[77]
PEG-6000 solution 23% (w/v) for 7 days	*O. sativa 'Xiangzhongxian no. 2' and 'IR50'* leaves	+	[77]

(–), (+) and (×) signs indicate increase, decrease or unaltered/unaffected, respectively.

[1]Modified after Anjum et al. [10] and Gill et al. [12].

2.2. GSH vs. Salinity and Drought Stresses

Soil-salinity and drought are among the most important environmental factors limiting plant growth, development and productivity. In particular, a major part (> 22%) of agricultural soils on the globe is affected by salinity [78]. Both salinity and drought stresses significantly elevate ROS production. Plants differing in salinity tolerance may exhibit different levels of reduced GSH pool [19, 79-81]. In general, salt tolerant plants exhibit a higher GSH pool [19, 79-81]. However, this is not the case always. A salt tolerant plant genotype may also exhibit a decreased level of GSH [59, 82-84]. Drought-mediated increase in GSH pool has been evidenced including *Boea hygroscopica* [85], sunflower seedlings [86], wheat leaves [87] and rice genotypes [88]. In tomato, Mittova et al. [19] suggested the role of a coordinate upregulation of synthesis and metabolism of GSH for salt tolerance. It is argued that plant capacity to manage a fine tuning between GSH regenerating and utilizing/metabolizing systems control the GSH level in plants.

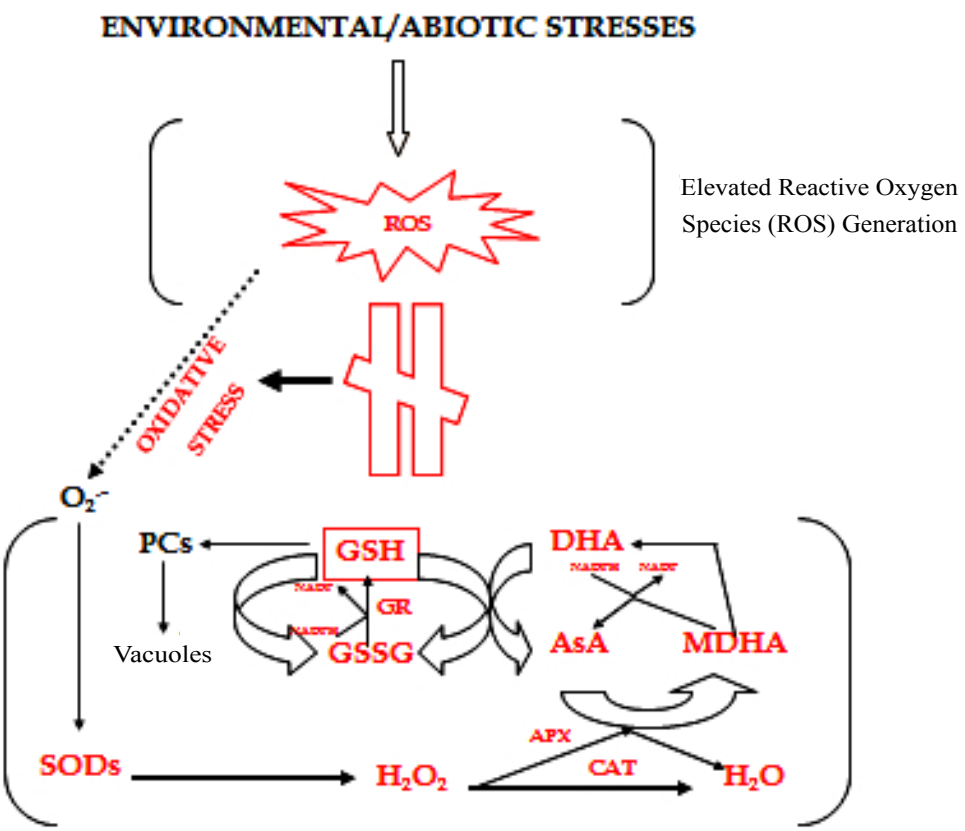

Figure 1. Simplified scheme highlighting the central role of glutathione (GSH) in plants. Modified after Gill and Tuteja [9], Anjum et al. [10], and Gill et al. [12].

2.3. GSH vs. Other Important Abiotic Stresses

The list of other abiotic stresses significantly impacting plant physiology and the GSH pool includes the low and high temperatures, ultra violet (UV)-B radiation and tropospheric ozone (O_3) [43, 89-92]. Up-regulation of GSH synthesis and subsequent enhanced GSH pool were evidenced to provide tolerance to heat stress in plants [93-95]. Elevated pool of GSH also provides chilling tolerance [96-98]. In fact, GSH-mediated cold stress acclimation was argued due to the up-regulation of Cys synthase, enzyme is responsible for the final step in Cys biosynthesis, a key limiting step in GSH production [99]. Increased GSH pool may also protect plants against UV-B radiation [100], light stress [101] and acute O_3 [102]. Mineral nutrients deficiency or access can also modulate plant GSH pool [103, 104].

3. Relevant Aspects of Glutathione (GSH) in Human Nutrition

Amino acids, once released after absorption of dietary protein, are intended for different missions: get energy after oxidation, incorporation into protein molecules, or form other molecules containing nitrogen. Several amino acids have their metabolic pathways linked to the metabolism of other amino acids, forming peptides for example. These co-dependencies become important when nutrient intake is limited or metabolic requirements are increased. For example, Cys, either formed from serine by the methionine and transsulfuration, or supplied in the diet as such, is a precursor for the synthesis of proteins and the tripeptide GSH (L-γ-Glutamyl-Cysteinyl-Glycine), plus several other essential molecules. If ingested by requirement, a large proportion of available Cys is used for protein synthesis and GSH. Protein turnover and hydrolysis of GSH by the γ-glutamyl transpeptidase and dipeptidase enzyme will result in the release of new Cys at amino acid pool. Cys is also a precursor for the synthesis of coenzyme A, and for the production of taurine and inorganic-S, which cooperate in the loss of the Cys residue as such [105].

GSH is the major intracellular thiol, and the intracellular ratio of reduced (GSH) to oxidized (GSSG) is greater than 10 [106, 107]. GSH serves as a supply of reducing equivalents or electrons and is involved in the protection of cells against oxidative damage through reduction of hydrogen peroxide and organic peroxides by GSH-peroxidases and non-enzymatic inactivation of free radicals with the donation of hydrogen to radical. GSH is an important source of the reducing equivalents for the intracellular reduction of Cys to Cys-SH. This process can occur by thiol-disulfide or enzymatically by thiol-transferase, with GSH providing the reducing equivalents. The previous processes result in oxidation of GSH to GSSG. Because GSSG can be reduced back to GSH through the GSH-reductase reaction, which uses oxidized nicotinamide adenine dinucleotide phosphate/reduced nicotinamide adenine dinucleotide phosphate ($NADP^+$/NADPH) as the oxidant/reductant, GSH plays a role to maintenance of the cellular redox state.

GSH may participate in the transport of amino acids through the membrane-bound enzyme γ-glutamyl transpeptidase, the same enzyme responsible for extracellular hydrolysis of GSH, catalyzes the transfer of the enzyme $^{\gamma}$ -glutamyl group of GSH to the α-amino group of an acceptor amino acid such as Cys or Glu. The -Glu amino acid is transported into the

cell, where the amino acid is released and the Glu moiety cyclizes to 5-oxoproline, which is subsequently hydrolyzed to regenerate glutamate. The Cys-Gly dipeptide that is the byproduct of -Glu transpeptidation can be hydrolyzed to Cys and Gly either extracellularly or intracellularly by dipeptidases hence, no net consumption of amino acids occurs as a result of the transport cycle.

GSH also serves as a co-substrate for several reactions including certain steps in leukotriene synthesis and melanin polymer synthesis. GSH is the substrate for a group of enzymes, GSTs that form GSH-conjugates from various acceptor compounds including various xenobiotics [108]. These conjugates are normally degraded by the enzymes of $^{\gamma}$-Glu cycle to yield the Cys-derivatives that may be acetylated using acetyl coenzyme A to become mercapturic acids, which are excreted in the urine. This process is usually a detoxification and excretion process.

3.1. The Effects of GSH

As in part we discussed earlier, the role of GSH stems from his involvement in numerous cellular reactions: 1) GSH effectively scavenges free radicals and other reactive oxygen species (e.g., hydroxyl radical, lipid peroxyl radical, peroxynitrite, and H_2O_2) directly and indirectly through enzymatic reactions [109]. In such reactions, GSH is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent GSH reductase. In addition, the selenium-containing enzyme GSH peroxidase catalyzes the GSH-dependent reduction of H_2O_2 and other peroxides [110]; 2) GSH reacts with various electrophiles, physiological metabolites (e.g., estrogen, melanins, prostaglandins, and leukotrienes), and xenobiotics (e.g., bromobenzene and acetaminophen) to form mercapturates [109]. These reactions are initiated by GST (a family of Phase II detoxification enzymes); 3) GSH conjugates with nitric oxide (NO) to form an *S*-nitrosoglutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO [109]. Recent evidence suggests that the targeting of endogenous NO is mediated by intracellular GSH [111]. In addition, both NO and GSH are necessary for the hepatic action of insulin-sensitizing agents [112], indicating their critical role in the regulation of lipid, glucose, as well as in amino acid utilization; 4) GSH serves as a substrate for formaldehyde dehydrogenase, which converts formaldehyde and GSH to *S*-formyl-glutathione [113]. The removal of formaldehyde (a carcinogen) is of physiological importance, because it is produced from the metabolism of methionine, choline, methanol (alcohol dehydrogenase), sarcosine (sarcosine oxidase), and xenobiotics (via the cytochrome P450–dependent monooxygenase system of the endoplasmic reticulum); 5) GSH is required for the conversion of prostaglandin H2 (a metabolite of arachidonic acid) into prostaglandins D2 and E2 by endoperoxide isomerase [114]; 6) GSH is involved in the glyoxalase system, which converts methylglyoxal to D-lactate, a pathway active in microorganisms. Finally, glutathionylation of proteins (e.g., thioredoxin, ubiquitin-conjugating enzyme, and cytochrome c oxidase) plays an important role in cell physiology [113].

Adequate GSH concentrations are necessary for the proliferation of cells, including lymphocytes and intestinal epithelial cells [115]. GSH also plays an important role in spermatogenesis and sperm maturation [4]. In addition, GSH is essential for the activation of T-lymphocytes and polymorphonuclear leukocytes as well as for cytokine production, and

therefore for mounting successful immune responses when the host is immunologically challenged [113].

Further, both *in vitro* and *in vivo* evidence show that GSH inhibits infection by the influenza virus [115]. It is important to note that shifting the GSH/GSSG redox toward the oxidizing state activates several signaling, thereby reducing cell proliferation and increasing apoptosis [116]. Thus, oxidative stress (a deleterious imbalance between the production and removal of reactive oxygen/nitrogen species) plays a key role in the pathogenesis of many diseases, including cancer, inflammation, kwashiorkor (predominantly protein deficiency), seizure, Alzheimer's disease, Parkinson's disease, sickle cell anemia, liver disease, cystic fibrosis, HIV, AIDS, infection, heart attack, stroke, and diabetes [113, 117, 118].

Functions of GSH can be in general explained by its role in detoxification, redox reactions, and the storage and transport of Cys. Because a major physiologic function of GSH is to provide cells with a reducing environment and to destroy the ROS formed in metabolism, organs that have low concentrations of other antioxidants (such as catalase and superoxide dismutase) are thought to be more dependent on GSH for ROS-detoxification than are organs that have alternative antioxidants [119].

GSH is an important endogenous antioxidant that participates directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms [120, 121].

The metabolism of GSH and S-adenosyl-L-methionine (SAM) are closely linked, as the liver forms GSH as a product of SAM metabolism. The liver uses as much as 70% of dietary methionine, and most is converted to SAM, an important metabolic substrate and is involved in the initiation of 3 major pathways: 1) trans-methylation for the synthesis of various proteins and lipids, among them phospholipids for cell membranes; 2) trans-sulfuration to form GSH and sulfated compounds via homo-Cys and Cys; and 3) aminopropylation for polyamine synthesis [122-124].

GSH is used in a range of metabolic and biochemical reactions such as DNA synthesis and repair, protein synthesis, prostaglandin synthesis, amino acid transport, and enzyme activation. Thus, every system in the body can be affected by the state of the glutathione system, especially the immune system, the nervous system, the gastrointestinal system and the lungs. GSH has also a vital function in iron metabolism. Yeast cells depleted of or containing toxic levels of GSH show an intense iron starvation-like response and impairment of the activity of extra-mitochondrial ISC enzymes, followed by death [125]. In fact, major metabolic functions of GSH are DNA synthesis and repair, protein synthesis, prostaglandin synthesis, amino acid transport, metabolism of toxins and carcinogens, enhancement of immune system function, prevention of oxidative cell damage and enzyme activation [126, 127].

3.2. Dietary Sources and Supplementation

GSH is present to the greatest extent in fruits, vegetables, and meat [128]. Asparagus are especially rich source of GSH, containing 26 mg of GSH per 100 g. Most raw vegetables provide this nutrient. Pumpkin with 14 mg, broccoli with 8 mg, 13 mg the potato, and tomatoes with 11mg, all for 100 g of edible portion. Among the major fruits, watermelon is one of the best sources of GSH. It has 28 mg per 100 g of edible portion. Strawberries have

12 mg, 15 mg grapefruit, oranges 11 mg and peaches 7 mg per 100 g. Avocados also provide 31 mg per 100 g. Nuts provide 15 mg per 100 g of edible portion. Fish, poultry or red meat provide about 10 mg per 100 g of edible portion. Pomegranate juice consumption increases GSH levels and reduces lipid and protein oxidation in human blood [129]. Raising GSH levels through direct supplementation of GSH is difficult. Research suggests that GSH taken orally is not well absorbed across the gastrointestinal tract [130]. In a study of acute oral administration of a very large dose (3 grams) of oral GSH, Witschi et al. [131] found that it is not possible to increase circulating GSH to a clinically beneficial extent by the oral administration of a single dose of 3 g of GSH. However, it is possible to increase and maintain appropriate GSH levels by increasing the daily consumption of Cys-rich foods and/or supplements.

Agents such as 1-cyano-2-hydroxy-3-butene, present in cabbage, brussels sprouts, broccoli, and cauliflower, have raised GSH concentrations several fold in animal models. The results of studies to date of GSH absorption have been conflicting. Several investigators have shown that orally administered GSH increases plasma concentrations of reduced and protein-bound GSH through intestinal absorption in animal models and human [132].

GSH is not commercially available as an oral or injectable product because of pharmaceutical problems, including poor oral bioavailability and a short half-life (2 min) with intravenous administration. Investigators have used bulk quantities of GSH purchased from various chemical companies. For these reasons, precursors of GSH have been investigated. In humans, oral doses of GSH 15 mg/kg increased plasma GSH 1.5- to 10-fold over the basal concentration in 4 of 5 volunteers tested [133]. The maximum concentration of plasma GSH generally occurred 1 hour after GSH administration. Equivalent amounts of amino acid constituents of GSH failed to increase plasma GSH concentrations. These data suggest that oral GSH can replete GSH concentrations in several tissues following GSH depletion, such as after toxicological or pathologic conditions that alter GSH homeostasis [134]. It has been speculated that because oral administration of GSH leads to its inactivation by peptidases, it should not be possible to significantly increase plasma GSH concentrations with oral administration. Richie et al. [135] determined the long-term effectiveness of oral GSH supplementation on body stores of GSH in healthy adults by a 6-month randomized, double-blinded, placebo-controlled trial of oral GSH (250 or 1,000 mg/day) on GSH levels in blood, erythrocytes, plasma, lymphocytes and exfoliated buccal mucosal cells was conducted in 54 non-smoking adults. They observed that GSH levels in blood increased after 1, 3 and 6 months versus baseline at both doses. At 6 months, mean GSH levels increased 30-35% in erythrocytes, plasma and lymphocytes and 260% in buccal cells in the high-dose group ($P < 0.05$). GSH levels increased 17 and 29% in blood and erythrocytes, respectively, in the low-dose group ($P < 0.05$). In most cases, the increases were dose and time dependent, and levels returned to baseline after a 1-month washout period. A reduction in oxidative stress in both GSH dose groups was indicated by decreases in the oxidized to reduced glutathione ratio in whole blood after 6 months. These findings show, for the first time, that daily consumption of GSH supplements was effective at increasing body compartment stores of GSH.

Cys is usually non-essential in the diet because it can be synthesized endogenously from methionine and phenylalanine. Impaired synthesis of Cys from methionine may necessitate the provision of a source of Cys to some patients with cirrhosis; however, supplementation with L-Cys could lead to hypercysteinemia and potential toxicity. Administration of oral L-Cys to patients with cirrhosis has been noted to cause a twofold greater maximal plasma Cys

concentration and plasma elimination half-life, and a delayed excretion of metabolic end products when compared with those of controls. However, an impaired cysteine uptake from the plasma has been proposed secondary to a decrease in plasma GSH. Another example of precursor use involves L-2-oxothiazofidine-4-carboxylate, which is converted to S-carboxyl-L-Cys and undergoes spontaneous decarboxylation to liberate L-Cys, thereby supporting GSH synthesis [105, 136]. N-acetylcysteine (NAC) may be a direct source of Cys following hydrolysis or may reduce plasma-Cys through thiol- disulfide exchange, liberating endogenous Cys. Under no GSH-deficiency, NAC does not increase total GSH, since the intracellular concentration is under feedback control.

The amino acids Gly or Cys are also suitable for the assessment of *in vivo* rate of synthesis of GSH. In pediatric patients hospitalized with sepsis receiving parenteral nutrition, it was determined by using ^{13}C-Cys, the rate of synthesis of GSH was much lower than that of controls. It was observed that supplementation with NAC led to a significant increase in only 9 days of treatment, the concentration of GSH, Cys and the rate of synthesis of the tripeptide. An antiinflammatory effect was also found as a result of the decrease in plasma concentration of interleukins. It could then verify that the Cys-supplementation therapy to restore GSH during early treatment of malnutrition decreased infant morbidity and mortality [136], and subsequently demonstrated that children with edematous severe acute malnutrition [137] may have a greater requirement for Cys during early and mid-nutritional rehabilitation because they used dietary-Cys more efficiently than did their nonedematous counterparts and because the splanchnic tissues of all children with this malnutrition have a relatively high requirement for Cys.

Other authors [138] demonstrated that the administration of high doses of NAC added to GSH significantly decreased the peroxidative stress of patients with septic shock. McPherson and Harding [139] described the clinical and nutritional beneficial effects of Cys-rich proteins, affirming that may have advantages over the simple amino acid or its derivatives, as nutraceuticals, to safely and beneficially improve antioxidant status in health and disease. Hepatic GSH concentrations have been restored to nearly normal in liver biopsies of patients with cirrhosis following long-term oral SAM administration. Studies have documented improvement in pruritus, jaundice, and biochemical parameters in patients with intrahepatic cholestasis of pregnancy treated with SAM. The clinical efficacy of SAM in the treatment of cholestasis associated with hepatic diseases has been reviewed. For the treatment of liver disorders, such as intrahepatic cholestasis, the recommended dose of SAM is 800 mg parentally or 1600 mg/day orally. The cysteine groups of SAM synthetase might be protected from oxidation by a normal concentration of GSH. When there is a reduction in liver GSH or increased concentrations of GSSG by toxin or disease, a vicious cycle might start. Depletion of GSH could lead to inactivation of SAM synthetase, with further decrease in GSH concentrations, worsening the deficiency in SAM synthetase. In this context, SAM administration may act as a precursor for GSH synthesis and also bypass the deficiency in SAM synthetase [140, 141]. Some studies have shown that dietary supplementation with a whey-based product can increase glutathione levels in cystic fibrosis [142]. This nutritional approach may be useful in maintaining optimal levels of GSH and counteract the deleterious effects of oxidative stress in the lung in cystic fibrosis. Glu-supplementation to total parenteral nutrition maintains tissue GSH levels and improves survival after reperfusion injury, ischemia, acetaminophen toxicity, chemotherapy, inflammatory stress, and bone marrow transplantation [143].

3.3. Factors Affecting GSH Bioavailability

Dietary GSH is not a major determinant of circulating GSH, because of its hydrolysis by intestinal and hepatic ν-Glu-transferase, being, for example, not possible, as previously described, to increase circulating GSH to a clinically beneficial extent by the oral administration of a single dose of 3 g of GSH [131].

A number of factors such as dietary protein deficiency, dexamethasone, erythropoietin, tumor growth factor, hyperglycemia, and γ-glutamylcysteine synthetase (GCS) phosphorylation decrease GCS transcription or activity. Glucosamine, taurine, n-3 PUFAs, phytoestrogens, polyphenols, carotenoids, and zinc, which inhibit the expression of inducible NO synthase and NO-production, may prevent or attenuate GSH-depletion in cells. Conversely, high-fat diet, saturated long-chain fatty acids, low-density lipoproteins, linoleic acid, and iron, which enhance the expression of inducible NO synthase and NO-production, may exacerbate the loss of GSH from cells [144].

Recent studies provide convincing data to support the view that Cys is generally the limiting amino acid for GSH synthesis in humans, as in rats, pigs, and chickens. Thus, factors (e.g., insulin and growth factors) that stimulate Cys (cystine) uptake by cells generally increase intracellular-GSH concentrations. In addition, increasing the supply of Cys or its precursors (e.g., cystine, *N*-acetylcysteine, and L-2-oxothiazolidine-4-carboxylate) via oral or intravenous administration enhances GSH synthesis and prevents GSH deficiency in humans and animals under various nutritional and pathological conditions (including protein malnutrition, adult respiratory distress syndrome, HIV, and AIDS). Because Cys generated from methionine catabolism via the trans-sulfuration pathway (primarily in hepatocytes) serves as a substrate for GCS, dietary methionine can replace Cys to support GSH synthesis in vivo [113]. The evidence also indicates that the dietary amino acid balance has an important effect on protein nutrition and therefore on GSH homeostasis [114]. In particular, the adequate provision of S-containing amino acids as well as Glu and Gly (or serine) is critical for the maximization of GSH synthesis.

γ-glutamylcysteine (GGC) is a dipeptide and substrate for synthesis of the antioxidant GSH, whose health promoting properties include reducing risks of oxidative stress-related injuries and diseases. Nakamura et al. [145] investigated the efficacy of GGC on GSH synthesis and oxidative stress in human endothelial cells, showing that, besides its substrate role in GSH synthesis, GGC may play a role in protection against oxidative stress by serving as an antioxidant and modulating the expression of protein(s) related to antioxidant defense, speculating that GGC may serve as a novel intra- and intercellular therapeutic dipeptide for oxidative stress-related injuries and diseases. Johnson et al. [146] found that blood-GSH levels rose nearly 50% in healthy individuals taking 500 mg of vitamin C daily. Vitamin C facilitates an increase in blood-GSH level by supplying the body with a nutrient that is critical for its synthesis.

In addition, to vitamin C, dietary sources of GSH and several other nutritional compounds can help increase GSH levels include NAC, alpha-lipoic acid, glutamine, methionine, and non-denatured whey protein [147]. NAC has been used in combination with glutamic acid (or glutamine) and glycine and in addition, NAC has been studied in combination with dietary proteins [148]. Omata et al. [149] proposed that Zn-deficient - induced neuronal oxidative stress and the increased susceptibility of Zn-deficient neuronal cells to oxidative stressors could be in part due to an impaired GSH synthesis when neuronal

Zn decreases, obtaining that low Zn availability affects the GSH synthetic pathway in neuronal cells and fetal brain both at transcriptional and posttranslational levels. This can in part underlie the GSH depletion associated with Zn-deficiency and the high sensitivity of Zn-deficient neurons to pro-oxidative stressors.

3.4. Clinical Outreach, Enteral and Parenteral Support

Low concentration GSH is commonly observed in wasting and negative nitrogen balance, as seen in critically ill, cancer, HIV/AIDS, sepsis, lung diseases, trauma, burns and athletic overtraining.

In the critically ill patient, there is a continuous production of ROS that need to be neutralized to prevent oxidative stress. Quantitatively speaking, the GSH system is the most important antioxidant endogenous defense and it is reduced in critical illness [150]. To increase it, glutamine supplementation has been shown to be effective by protecting against the oxidative damage and reducing the morbimortality. For optimal maintenance of the redox state in such patients is important that the supply of substrates to normalize the antioxidant capacity of the body, for which an appropriate nutritional therapy is needed. Previous studies [151] show that glutamine intake in critically ill patients (supplement formulas parenteral nutrition with glutamine dipeptide (*Dipeptiven* ®) improves the antioxidant defenses, which leads to lower lipid peroxidation and lower morbidity during admission at the ICU. Spies et al. [152] reported a 2-year investigation of 58 patients with septic shock randomized to receive NAC 150 mg/kg iv over 15 minutes followed by 12.5 mg/h over 90 minutes. Using a 10% or more increase in whole body oxygen consumption as their endpoint, 13 patients who received NAC were considered responders, and 16 were considered non-responders. None of the patients who received placebo (n = 29) exhibited a 10% increase in whole body oxygen consumption. In summary, NAC transiently improved tissue oxygenation in about half of the patients with septic shock to whom it was administered. Chawla et al. [153] measured the concentrations of cysteine, GSH, and taurine in healthy subjects and patients with cirrhosis fed either mixed food, nasogastric hyperalimentation with *Vivonex* (Sandoz Nutrition, Minneapolis, MN), or *FreAmine III* (McGraw, Irvine, CA) intravenous hyper-alimentation. *Vivonex* and *FreAmine III* do not contain cysteine. Subnormal plasma concentrations of GSH and cysteine were observed in patients with cirrhosis independent of their diet. The data support the hypothesis of an acquired dysfunction in the hepatic trans-sulfuration pathway, rather than a change in bioavailability. Because most plasma-GSH originates in hepatocytes, the authors hypothesized that decreased plasma GSH also could signify intracellular depletion. This would potentially impair the ability of the hepatocyte to maintain normal redox potential, destroy peroxides and free radicals, and detoxify drugs.

Intracellular GSH has been shown to play an important role in aspects of T-cell function, including the binding, internalization, and degradation of interleukin-2, as well as DNA synthesis. In particular, GSH and sulfhydryl compounds are known to augment the activation of cytotoxic T-cells in mixed lymphocyte cultures, T-cell proliferation in response to mitogens, and the differentiation of T- and B-lymphocytes. *In vivo* administration of GSH has been demonstrated to enhance the activation of cytotoxic T-cells, but depletion of GSH intracellularly inhibited the activation of lymphocytes, increased susceptibilities of human lymphoid cells to radiation, and suppressed cell-mediated cytotoxic functions, suggesting that

intracellular GSH can modulate the function of immune cells. The current hypothesis is that because adequate concentrations of GSH are required for proper lymphocyte function, a deficiency in GSH may contribute to the immunodeficiency seen in the later stages of HIV infection. In addition, the action of inflammatory cytokines may mediate cachexia and the wasting that accompanies late stages of AIDS, which also may be alleviated by GSH replacement [154-157].

GSH-deficiency has been proposed to have a role in the pathophysiology of a number of lung diseases, including chronic obstructive pulmonary disease [158], acute respiratory distress syndrome (ARDS) [159, 160], neonatal lung damage [161] and asthma [162, 163]. The lung is particularly at risk from oxidative damage as it is exposed to oxygen, oxygen radicals produced by alveolar macrophages, inhaled environmental and blood-borne toxins, including cigarette smoke and atmospheric pollutants. Free radical induced toxicity is worsened by concomitant GSH deficiency, and free radical production further depletes GSH through use. GSH present in the epithelial lining fluid of the lower respiratory tract may be the first line of defense against oxidant stress. In idiopathic pulmonary fibrosis, for example, GSH concentrations are only 25% of normal values in the epithelial lining fluid and may be involved in the underlying pathophysiology of the disease [106, 134, 164, 165]. Preliminary results indicate that GSH changes the ROS-level in isolated cells grown in a laboratory, which may reduce cancer development. None of these tests were performed on humans. However, once a cancer has already developed, by conferring resistance to a number of chemotherapeutic drugs, elevated levels of GSH in tumor cells protect cancerous cells in bone marrow, breast, colon, larynx and lung cancers [89, 166-170].

By acting as an antioxidant and by virtue of binding to cellular mutagens, GSH has the ability to react with peroxides and several electrophiles, including carcinogenic epoxide metabolites. GSH has been shown to directly modulate proliferation of highly purified T-cells, suggesting that GSH is essential for steps closely involved with DNA synthesis. Depressed intracellular GSH in the liver and in mammary tissue has been shown to promote carcinogen binding to DNA. Oral glutamine, for example, has been proposed to have a useful role in increasing host GSH concentrations in the gut, liver, lung, kidneys, heart, and muscle after exposure to radiation or chemotherapy without enhancing tumor growth. Flagg et al. [171] investigated the association between dietary GSH intake and the risk of oral and pharyngeal cancer in an epidemiologic study. In this case-control study, the investigators noted an inverse relationship between dietary intake of GSH and the risk of oral cancer, but only in a cohort consuming GSH mostly from raw fruit and vegetables (rather than meat or cooked vegetables, for example). However, the possibility that GSH intake from fruit and vegetables might be protective against oral cancer risk could not be distinguished from the more general benefit of consuming raw fruits and vegetables, such as increased ingestion of fiber. The investigators hypothesized anti-carcinogenic protective mechanisms of GSH to include its direct antioxidant function, indirect maintenance of other antioxidants, possible mediation of DNA synthesis and repair, and the ability to bind with cellular mutagens.

GSH-deficiency may also contribute to the nephrotoxicity of ischemic events and drug toxicity. This type of toxicity may be exhibited by cyclosporine. Although the exact mechanism of cyclosporine nephrotoxicity remains unknown, its administration has been associated with in vivo reduction of GSH concentrations in the livers and kidneys of rats, which may be related to adverse effects of this immunosuppressive agent. Cyclosporine has peroxidative properties, induces lipid peroxidation in renal microsomes, and may lead to

inactivation of microsomal glucose-6-phosphate activity and toxicity. Therefore, contribution to cyclosporine nephro- and hepatotoxicity has been postulated to be caused by its generation of free radicals and depletion of GSH. Investigators also have hypothesized that cyclosporine can modify resistance to chemotherapy by augmenting the cytotoxic effect of drugs through inducing a GSH deficiency. Administration of exogenous GSH had a beneficial effect on renal function by virtue of its antioxidant properties, and possibly by a vasodilator action to increase the glomerular filtration rate as well [172].

The brains of patients with Parkinson's disease exhibited a reduction of GSH, selective to the substantia nigra. This did not appear to be related to drug therapy. It was postulated to be of significance in the pathogenesis of this disease via production of oxidative damage. These patients had an increased concentration of the GSH degradative enzyme γ-Gly-transpeptidase in the substantia nigra, and a normal concentration of the synthetic enzyme γ-Glu-Cys synthetase. GSH-depletion occurred without a change in GSSG, suggesting efflux of GSH out of the glia, perhaps with additional increased conversion of GSH to GSSG in response to increased hydrogen peroxide formation [173, 174].

Oxidation of low density lipoprotein, LDL, is the first step in the development of atherosclerosis. The oxidized LDL cytotoxicity also manifested in the activation of apoptotic mechanisms. The oxidized LDL acts as pro-oxidant increases ROS levels and stimulates expression of the critical enzyme for the synthesis of GSH, and protects cells against oxidative stress induced by oxidized LDL. The increased level of GSH dependent oxidation of LDL [175]. Enzyme GSH peroxidase and GSH protect against oxidative stress induced by oxidized LDL. In cultured endothelial cells, it was observed that pretreatment with an agent that decreases cellular GSH deposits, the butilsulfoxide, causes an increase in cytotoxicity caused by oxidized LDL. The situation is reversed if used antioxidants.

A study of malnourished children yielded information on the decrease in GSH levels and the rate of synthesis, also have low levels of extra-and intra-cellular Cys and elevated levels of markers of induced lipid peroxidation with also a smaller contribution of Cys from protein metabolism. The features of *kwashiorkor* syndrome, in which the rate of protein breakdown is decreased by an enzyme deficiency, are associated with oxidative damage due to the imbalance between the generation of free radicals and antioxidant capacity; oxidative damage occurs with consequent liver and immune subfunctions [176].

At cellular level, given the role of mitochondria in oxygen consumption, metabolism and cell death regulation, alterations in mitochondrial function or dysregulation in cell death pathways contribute to many diseases such as cancer, SH, or neurodegeneration. Consistent with its role in regulating mitochondrial GSH (mGSH), mitochondrial cholesterol accumulation emerges as a key factor regulating ROS and electrophile detoxification, and hence disease progression by sensitizing to secondary hits such as TNF, hypoxia or toxic amyloid peptides. Diseases where mitochondria cholesterol, oxidative stress, and mGSH depletion have been shown to play a role, such as cancer, fatty liver disease, and Alzheimer disease, is important to evaluate. However, a challenge to counteract mitochondrial oxidative stress is to recover mGSH pool when GSH transport is defective due to alterations in membrane dynamics triggered by increased mitochondrial cholesterol accumulation. In addition to the ability of mitochondrial-permeable GSH-EE to directly increase mGSH levels bypassing the mitochondrial transport defect, it has been recently described additional strategies that supply mitochondria with GSH, including parental molecules that generate GSH once inside the mitochondrial matrix. This approach has been recently illustrated with

the use of *S*-D-lactoyl-GSH [177, 178]. This compound is an intermediate of the glyoxalase system, which is hydrolyzed in the mitochondrial matrix yielding lactate and GSH; hence showing the ability to replenish mGSH resulting in recovery of mitochondrial function and antioxidant defense.

4. Glutathione (GSH) in Analytical Biotechnology

4.1. Analytical Methods for GSH Determinations

As the GSH molecule is ubiquitous across the biological species, the need for its quantification for purposes of subsequent study is raised in the time. The following sub-sections briefly discuss the methods/analytical techniques to date developed for GSH-quantification/determination.

4.1.1. GSH Derivatization

There are no choromophores and fluorophores in the chemical structure of GSH and other thiol analogs and therefore their detection can be performed only at low wavelengths of the UV and usually with limited sensitivity and selectivity, compared to electrochemical or fluorometric detection [179, 180]. Therefore, it is advantageous to perform a derivatization step to enhance the detection. Also higher stability of product against oxidation is achieved by using derivatization agent [181]. Although there are three groups on GSH which are susceptible to derivatization (carboxylic, amino and thiol functionalities), labeling of the thiol moiety is preferred for its specificity and for its function as protective group. Oxidized form of glutathione (i.e., GSSG) can be derivatizated only on the amino and carboxylic groups. Derivatization agent can improve the limit of detection (LOD) of spectrophotometry and spectrofluorimetry methods, while assays based on electrochemical detection (ECD) are not dependent on derivatization [182]. Chromophores mostly used for derivatization are N-ethylmaleimide (NEM), iodoacetic acid (IAA), and iodoacetamide, forming thioesters. Better sensitivity can be achieved by fluorophores. Most of them are reacting selectively with thiol group, where only GSH can be detected. GSSG can be measured by fluorophores binding on amino groups, with major disadvantage of occurrence of other interfering peaks and lower sensitivity [180].

4.1.2. HPLC Analysis of GSH

Most of GSH in different samples have been detected using fluorescence detection methods. Recently, in contrast, only three HPLC methods have employed UV-absorbance. Human blood components are most often used matrix, as a GSH plays important role as a major endogenous antioxidant produced by the cells, participating in the neutralization of ROS. Human blood is followed by rat organs, cultured cells and wine. Couple of labels for fluorescence detection of GSH have been used, most often o-phthaladehyde (OPA), NEM, monobromobimane (mBB) and ammonium 7-Fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F). A summary of the HPLC methods for the determination of GSH is given in Table 2.

One of non-traditional but very selective, sensitive and robust method for GSH detection is ECD (amperometric detection) which is very suitable and sensitive [183-185][186]. Scheme of detector is shown in Figure 2.

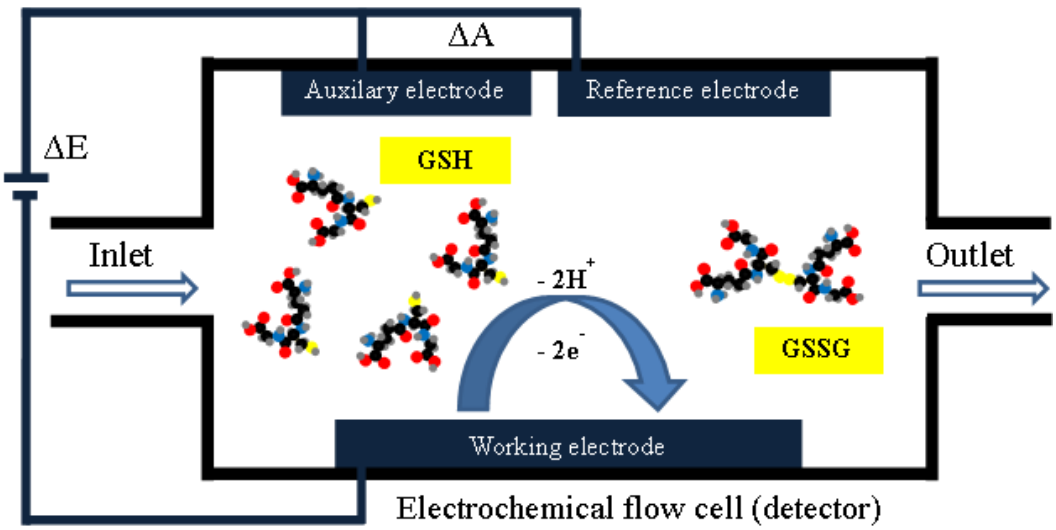

Figure 2. Scheme of basic principle of electrochemical (amperometric) detection of GSH in electrochemical flow cell for liquid chromatography.

The area of use of this method is in plant biology science [187, 188][187-193] and it is also widely useful in veterinary medicine [194] and human medicine research for direct GSH concentration determination as a marker for oxidative stress parameter in patients [195, 196]. But *in vitro* interaction of GSH with another molecules studied in past by stationary electrochemistry [197] also spread into the area of flow techniques to which HPLC and FIA (flow injection analysis) methods belong [198-200].

Table 2. Summary of detection methods connected to HPLC for the determination of glutathione (GSH)

Detector	Matrix	Label	LOD (GSH)	LOD (GSSG)	Ref.
UV 355 nm	Human plasma	CMQT	-	-	[201]
UV 355 nm	Human whole blood	FDNB	0.5 µM	0.5µM	[202]
UV 315 nm	Human plasma	BCPB	-	-	[203]
FD Ex/Em 467/525 nm	White wine	NDA	0.1 µM	4.0 µM	[204]
FD Ex/Em 340/450 nm	Grape juice/wine	OPA	0.06 mg L^{-1}	-	[205]
FD Ex/Em 350/420 nm	Rat Striatum	OPA	2.7 nM	5.4 nM	[206]
FD Ex/Em 350/420 nm	Plasma, whole blood, rat hepatocytes	OPA, NEM	14.0 fmol	5.6 fmol	[207]
FD Ex/Em 330/376 nm	Rat liver, lung, brain	NPM	-	2.5 nM	[208]

Detector	Matrix	Label	LOD (GSH)	LOD (GSSG)	Ref.
FD Ex/Em 385/515 nm	Human plasma	SBD-F	0.03 μM	-	[209]
FD Ex/Em 390/510 nm	Human astrocytoma	ABD-F	-	-	[210]
FD Ex/Em 390/478 nm (GSH) 340/425nm (GSSG)	Human plasma	mBB (GSH) OPA (GSSG)	0.5 pmol	0.040 pmol	[211]
FD Ex/Em 335/515 nm	Human plasma	dansyl chloride	-	-	[212]
FD Ex/Em 385/515 nm	Human plasma	SBD-BF	0.02 μM	-	[213]
FD Ex/Em 375/510 nm	Human serum	SBD-F	8.2 fmol	-	[214]
FD Ex/Em 340/420 nm	Human red blood cells, fibroblasts	OPA	50 fmol	-	[215]
FD Ex/Em 365/445 nm	Rat liver, lung, kidney and brain	ThioGlo3	50 fmol	-	[216]
FD Ex/Em 365/430nm	Rat liver	OPA	20 pmol	-	[217]
FD Ex/Em 370/ 470 nm	Human plasma	mBB	70 amol	-	[218]
FD Ex/Em 342/389 nm (NPM) 340/440 nm (OPA)	V79cl, V79HGGT	NPM, OPA	1.30 pmol	-	[219]
FD Ex/Em 390/478 nm	Human plasma	mBB GSMB	1.4 nM	8 nM	[220]
ECD	Human blood	-	0.011 μM	0.156 μM	[221]
ECD	HL-60 cells	-	2 fmol	-	[222]
ECD	Pig urinary bladder	-	-	-	[223]
ECD	HepG2 cells	-	15 fmol	-	[224]
ECD	DU145 and A549 cells	-	0.33 pmol	-	[225]
ECD	Rat hepatocytes	-	40 pg	300 pg	[226]
ECD	Human serum	-	19 fmol	80 fmol	[227]
ESI-MS/MS	Mouse liver	IAA	-	-	[228]
ESI-MS	Human peripheral blood mononuclear cells	NEM	0.01 μM	0.05 μM	[229]
ESI-MS	Rat lung, liver, heart, kidney, brain	DTNB	0.16 μM	0.16 μM	[230]
ESI-MS/MS	Human plasma	-	3.3 nM	-	[231]
MS/MS	Human whole blood	-	1.8 μM	-	[232]

4.1.3. Capillary Electrophoresis (CE) Analysis of GSH

For GSH, the most often used detection method connected to capillary electrophoresis (CE), is UV-detection. Wide range of samples such as human plasma, erythrocytes, heart tissue, carcinoma cells, rat airways and hepatocytes, yeasts, maize and bacteria, were used as

a matrix for detection of GSH. CE also can serve as support method for detection of QDs nanoparticles modified by GSH [233] such as for study of interaction between glutathione modified QDs and DNA [234].

A summary of the CE methods for the determination of GSH is given in Table 3.

Table 3. Summary of detection methods connected to capillary electrophoresis for the determination of glutathione (GSH)

Detector	Matrix	Label	LOD (GSH)	LOD (GSSG)	Ref.
UV 200 nm	Human plasma	-	0.5 μM	0.3 μM	[235]
UV 200 nm	Erythrocytes	DTNB	75 fmol	37.5 fmol	[236]
UV 200 nm	Human capillary blood	-	5.0 μM	0.9 μM	[237]
UV 285 nm	Yeasts	methyl propiolate	1.5 μM	-	[238]
UV 200 nm	*Zea mays*	-	0.65 ppm	0.50 ppm	[239]
UV 390 nm	Marine microalga	mBB	1.41 μM	-	[240]
UV 355 nm	Orange juice	CMQT	-	-	[241]
UV 185 nm	Heart tissue	-	0.4 nmol mg^{-1}	-	[242]
LIF Ex/Em 488/520 nm	Human plasma, Tobacco leaves	NBD-Cl	0.046 μM	0.012 μM	[243]
LIF Ex/Em 488/520 nm	HUVEC and ECV304	5-IAF	-	-	[244]
LIF Ex/Em 473/520 nm	Acute promyelocytic leukemia-derived NB4 cells	NDA, DHR-123	0.5 μmol	-	[245]
LIF Ex/Em 410/482 nm	Must /wine	mBB	65 nM	-	[246]
LIF Ex/Em 488 nm	Probiotic bacteria	5-IAF	0.5 ng mL^{-1}	-	[247]
ECD	Rat airways, plasma	NEM	11 μM	8 μM	[248]
ECD	Human hepatocarcinoma cells	-	2.2 fmol	-	[249]
ECD	Rat hepatocytes	-	3.2 fmol	-	[250]
ECD	Erythrocytes	-	-	-	[251]

4.2. Glutathione (GSH) as a Compound for Analytical Nanotechnology

GSH could be very versatile molecule even for nanobiotechnology and it is due to presence of cysteine as for applications shown in Figure 3. Using the thiol groups as capping agents, highly fluorescent GSH-capped quantum dots (GSH–QDs) can be synthesized [252]. GSH molecule also contains one amine and two carboxylate groups. These functional groups provide the possibility of coupling with various biomolecules and cross-linking and thus form a polymerized structure [252, 253]. Cross-linked GSH-capped QDs (cGSH–QDs) seems to be more stable than the GSH-QDs. Also GSH coating and crosslinking has only minor effect on the fluorescence in QDs, in contrast e.g. in silica coating, which can cause its reduction. Moreover cytotoxicity assay showed lower cytotoxicity of cGSH-QDs than other QDs modifications [254]. Also the chirality of GSH coating plays an important role in toxicity. D-GSH, the non-biologically active form of GSH, showed less cytotoxicity than L-GSH-coated QDs. This can result in target-coating QDs to adjust their biocompatibility [255]. The GSH-QDs show higher quantum yield than QDs derived from other water-soluble ligands. Furthermore, the fluorescence of the QDs is tunable in a large scale and QDs showed excellent photostability. The paramagnetic particles could be also covered with glutathione-modified QDs and can serve as a platform for fluorescent transporter [256]. High demand on new probes with good sensitivity, long stability, good biocompatibility, and low invasiveness make GSH-QDs an attractive candidate for biological applications [253].

Among others, also GSH-coated near-infrared quantum dots (GSH-NIR-QDs) can be used for *in vivo* imaging, instead of common NIR-dyes like Cy7 and oxazine 750, which has low solubility in aqueous solution, low quantum yield, and low photostability. QDs coated with GSH are more stable than other coating agents like mercaptoacetic acid (MAA) and mercaptoundecanoic acid (MUA) and also the cytotoxicity of GSH is reduced due its natural occurrence in tissues [257]. GSH provides more biocompatible capping of QDs than many other ligands [182] and was successfully capped on different nanoparticles [258]. It is also suggested, that GSH is better for surface stabilization, than other thiol ligands because of better surface passivation of QDs. GSH-capped CdSe and CdTe QDs has the highest quantum yields compared other thiol-compound ligands [259].

4.2.1. Neutralization of H_2O_2

GSH plays an important role in detoxification of hydrogen peroxide, other peroxides, and free radicals [260]. As a thiol-ligand GSH has been widely used in the aqueous synthesis of different semiconductor nanocrystals [259]. A novel chemosensor based on GSH-induced fluorescent enhancement of MPA-capped CdTe QDs was developed for hydrogen peroxide determination. [258]. H_2O_2 detection uses glutathione as a cofactor. This redox system is coupled to an electrode with electrochemical transduction of the signal obtained in the presence of H_2O_2. A gold electrode was coated by co-electrodeposition of a film of chitosangraphite nanoplatelets (CHI-GN) and used for the immobilization of molecules of the glutathione peroxidase enzyme mimetic. The peroxide-sensing mechanisms involve the reversible redox reaction of GSH using H_2O_2 as an electron acceptor, producing water and GSSG [261].

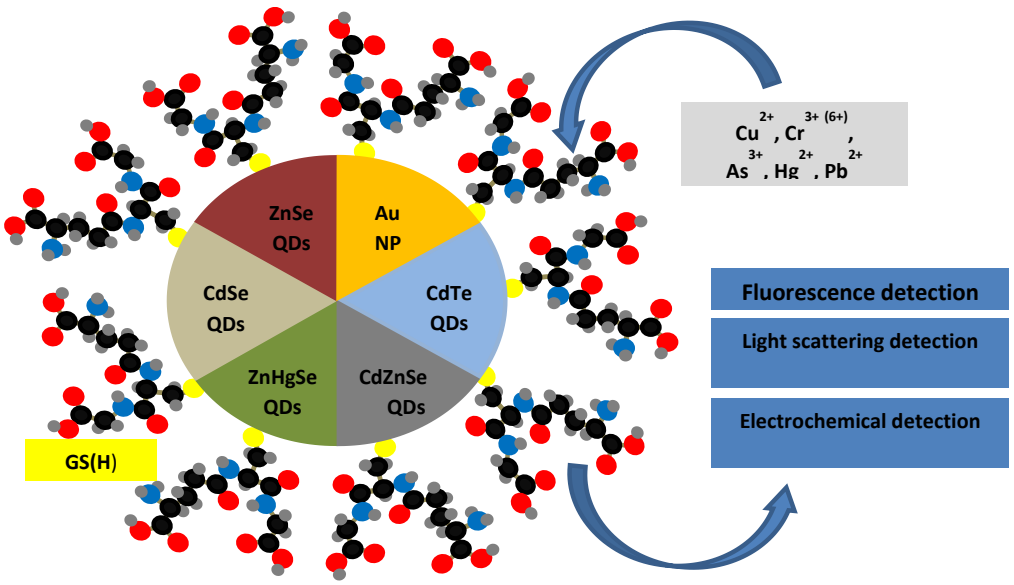

Figure 3. Scheme of various nanoparticles using the GSH modification for the detection of different heavy metals *via* the quantum dots interaction mechanism: Au-NP [262], CdTe QDs [258, 263], CdZnSe QDs [264], ZnHgSe QDs [265, 266], CdSe QDs [259], ZnSe QDs [267, 268].

4.2.2. Detection of Cu Ions

ZnHgSe QDs have been synthesized using GSH as surface-stabilizing agent and have been applied for sensing Cu^{2+} [265]. There are two possible mechanisms of quenching of GSH-QDs. The quenching of QDs can be the result of the strong coordination of Cu^{2+} to the carboxyl group of GSH. The effective electron transfer from GSH to Cu^{2+} occurs onto the surface of QDs and results in the reduction of Cu^{2+} to Cu^{+} [269]. The formation of Cu^{+} induces the effective quenching of the fluorescence of QDs through facilitating non-radiative recombination of excited electron in the conduction band and holes in the valence band [265]. Another cause of quenching is collision of Cu^{2+} with QDs, when Cu^{2+} displaces the Zn and/or Hg in the ZnHgSe QDs and forms extremely low soluble particles of CuSe onto the surface of QDs. These ZnHgSe GSH-QDs bring the advantages of high quantum yields, low toxicity, easy preparation, and high photostability [266]. Another usable sensor for Cu^{2+} detection is GSH-modified Fe_3O_4@ZnS magnetic fluorescent nanosensor (MFNS). MFNS could quantitatively detect Cu^{2+} with high sensitivity and selectivity under a broad pH range (pH 4.6–9) and detection limit of 0.2 μM [270]. GSH capped ZnSe QDs have been synthesized as a fluorescence ions probes for ultrasensitive Cu^{2+} detection with detection limit of 2×10^{-10} M [268].

4.2.3. Detoxification and Detection of Cr Ions

GSH is directly involved in the detoxification of Cr^{6+} compounds in biological systems by the binding of Cr^{6+} by GSH forming metal-GSH complex [271]. The water-soluble and stable CdTe QDs were synthesized in aqueous solution with GSH as the stabilizer and were used as a sensor for Cr^{6+}. The binding of Cr^{6+} on GSH capped-QDs change both the surface and photophysical properties of the QDs. It was found that Cr^{6+} could significantly quench the

fluorescence of these QDs so can be used as fluorescence probes for Cr^{6+} [272]. Fluorescent GSH-Au nanoclusters (GSH-Au NCs) were synthesized using GSH as reducing/protecting reagent. Cr^{3+} and Cr^{6+} show pH-dependent fluorescence quenching capabilities for GSH-Au NCs, and thus selective determination of Cr^{3+} and Cr^{6+} was achieved at different solutions. Under optimized conditions, a limit of detection for Cr^{3+} and Cr^{6+} was 2.5 μg L^{-1} and 0.5 μg L^{-1}, respectively. Reduction of other metal ions interference and good selectivity for chromium ions was ensured by the addition of EDTA [273].

4.2.4. Detection of As ions

GSH modified gold-nanoparticle-based dynamic light scattering assay can be used for label-free selective detection of arsenic. Binding between As^{3+} and the surface-modified gold nanoparticles results in their aggregation as GSH binds to the gold nanoparticle surface through Au-S bonds. Each As^{3+} ion can bind with three GSH gold nanoparticles probably through an As-O linkage [274]. GSH conjugated on the surface of AgNPs for specific binding with As^{3+} ions in aqueous solution through As-O linkage and 4-mercaptopyridine (4-MPY) can be used as a Raman reporter. When As^{3+} ions are added to the system, the binding of As^{3+} with GSH resulted in the aggregation of AgNPs, and excellent Raman signal of 4-MPY reporters is obtained and reflects the concentration of As^{3+} indirectly [275]. GSH as a sulfhydryl-containing tripeptide is an ideal capping agent for the CdTe QDs synthesis. The GSH layer is very important for the stability of the QDs. Therefore, the metal–GSH interaction may be an important parameter for the fluorescence quenching effect, so it is believed that GSH layer is preferentially displaced from the surface of the CdTe QDs upon the binding of As^{3+}. The displacement of GSH creates imperfection on the QDs surface, resulting in fluorescence quenching. Under optimal conditions, the limit of detection for As^{3+} was found to be 2×10^{-8} M [263].

4.2.5. Detection of Hg Ions

Solutions containing Hg^{2+} or Cu^{2+} caused the quenching of the fluorescence of the QDs-multilayer films deposited on quartz slides. When GSH was used to remove Hg (II) or Cu (II) from the QDs-multilayer films due to strong affinity of GSH-metal ions, the photoluminescence of QDs-multilayer films recovers. There are good linear relationships between the metal ions concentration and the photoluminescence intensity of QDs in the quenched and recovered process. Based on different quenching and recovery Stern–Volmer constant between Hg^{2+} and Cu^{2+}, the synchronous detection of Hg^{2+} and Cu^{2+} can be achieved [276].

4.2.6. Detection of Cd Ions

GSH is one of ligands for Cd^{2+} in biological systems since Cd^{2+} preferentially forms complexes with ligands with S-donor atoms [277]. FluoZin-1, reagent forming a fluorescent complex with Cd^{2+} can be used in method for determination of Cd^{2+} content. A mixture of Cd^{2+} and FluoZin-1was titrated with GSH while determining fluorescence intensity of FluoZin-1 to estimate levels of free Cd^{2+} [278].1′-cysteaminecarbonyl-1-glutathionecarbonyl-ferrocene (Fc-GSH) was synthesized from ferrocene dicarboxylic acid and GSH. Fc-GSH was immobilized on the gold electrode surface. As Fc-GSH and Cd^{2+} can form complex, this electrode can be used for Cd^{2+} detection of samples with concentration from 0.1 to 20 nM,

using cyclic voltammetry [279]. Another system for Cd^{2+} measuring was composed form gold electrode covered with 3-mercaptopropionic acid. GSH is then attached to this layer via carbodiimide coupling forming MPA–GSH modified electrode. Voltammetry of the redox couple allows determination of Cd^{2+} concentration. The detection limit was 5 nM after 10 min accumulation and even lower detection limits may be achievable if the accumulation time is increased [280].

4.2.7. Detection of Pb Ions

GSH-capped-ZnSe QDs can be applied for ultrasensitive Pb^{2+} detection. The mechanism of quenching is based on the competitive binding of GSH between QDs cores and Pb^{2+} according to Stern–Volmer equation, when GSH ligands might be displaced from the surface of QDs, resulting in the formation of a complex ligands-quencher and changes in surface of QDs. This change might lead to a quenching. The limit of detection for Pb^{2+} of 0.71 nM was reached [267].GSH functionalized gold nanoparticles (GSH-GNPs) can be used for Pb^{2+} detection using colorimetric detection. Nanoparticles aggregate immediately in the presence of Pb^{2+}, changing their colorimetric response monitored by a UV-vis spectrophotometer or even naked eye. The GSH-GNPs bound by Pb^{2+} shows great selectivity compared to other metal ions. The lowest detectable concentration of Pb^{2+} for this probe was 100 nM [262].

A sensor based on a selective fluorescence quenching of CdTe and CdZnSe QDs capped with GSH shells was developed for Pb^{2+} detection. The detection limit of Pb^{2+} in this assay can be as low as 20 nM. In the presence of other metal ions, this system is still capable of Pb^{2+} detection with a detection limit as low as 40 nM. Only with very high concentration of metal ions mixture (50 µM and higher), system becomes less sensitive. Ag^{+} and Cu^{2+} ions exhibited a similar quenching effect as Pb^{2+} and so they could influence the detection [264].

4.2.8. Detection of Glucose

GSH-capped CdTe QDs can be used as rapid and sensitive assay for glucose detection. This type of QDs exhibited higher sensitivity to H_2O_2, which is produced through an oxidation of glucose catalyzed by glucose oxidase, than other thiols-capped QDs (CdSe/ZnS QDs). The principle of detection is based on the quenching effect of H_2O_2 on GSH-capped QDs. The detection limits for glucose were determined to be 0.1 µM and it was almost a 1000 times lower than other QDs-based optical glucose sensing systems. Small-sized QDs show higher sensitivity [281].

Conclusion

GSH plays a range of significant roles in the physiology of microorganisms, plants, and animals including humans. Over the past decades, its structure, basic behavior and the analytical techniques-assisted determination in various matrices have been studied. Since GSH in the reduced state might be very elusive in the organism it is still a challenge for scientists to develop novel methods for GSH determination. GSH has become an important substance in area of portable or mobile sensors, where the interest in utilization of GSH in the personalized medicine area is rising. On the other hand, the GSH is very valuable for therapy nevertheless the problem, which has to be overcome, is a target delivery of GSH molecule to

the desired tissue. For such tasks, the modern nanotechnology is the right way and will play the important role in further GSH research.

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Chapter 2

Actions of Glutathione in Chronic Inflammatory Diseases, Including Periodontitis: Dietary Agonists

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Abstract

Glutathione (GSH) plays a critical role in cell signalling and antioxidant defences. It plays a significant role in subjects with periodontitis and associated systemic comorbidities. Its depletion leads to oxidative damage. Some of the prevalent redox reactions and interactions with dietary agonists are addressed.

Glutathione may interact directly with ROS / reactive nitrogen species (RNS); or act as an essential cofactor for GSH S-transferases and glutathione peroxidases. Coordinated actions of GSH and its dependent enzymes which constitute the glutathione system, lead to detoxification of reactive oxygen and nitrogen species (ROS/RNS). Therapeutic interventions aimed at enhancing GSH concentrations *in vivo* include N-acetyl cysteine; activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) by folate supplementation and phytochemicals such as curcumin and resveratrol. An antioxidant defence system comprising a range of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) is active in removing ROS accumulating in cells, in addition to vitamin A, vitamin C, α-tocopherol and plant flavonoids which are available as dietary antioxidants.

Oxidative stress plays an important role in chronic periodontitis (CP), the metabolic syndrome (MetS) and associated conditions. There is a significant correlation between SOD activity, triglycerides, high-density lipoprotein and sVCAM-1 levels. The association between SOD activity and MetS components could be the most significant variable parameter in subjects with MetS; it has potential as a predictive tool to determine the degree of oxidative stress in these subjects. The impact of diabetes mellitus (DM) as a

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risk factor for CP in the context of antioxidant enzyme activity, SOD, glutathione reductase (GR), catalase and the marker of free radical damage, malondialdehyde, favours the role of oxidative stress in both DM and CP.

Dietary non-enzymic antioxidants play an important role in interacting with oxidative stress-inducing mechanisms. Their targeted interaction with the glutathione network results in an enhanced antioxidant profile in chronic inflammatory diseases associated with excessive pro-oxidant activities. Dietary agonists are able to overcome proxidant profiles associated with decreased Nrf2 linked to reduced CAT and GPx m RNA expression . Their administration contribute to coordinated cytoprotective responses in tissues.

Introduction

Redox mechanisms play an important role in periodontitis, affecting tooth supporting structures and in systemic inflammatory diseases. The antioxidant glutathione is synthesized in the body and has protective functions in preventing inflammatory oxidative damage to cells. It aids the detoxification process and optimizes host defences. Stressors could contribute to depleted levels of glutathione which require recycling in order to maintain optimal levels for effective cell function. Redox mechanisms, actions of glutathione, associated enzymic networks and their significance in periodontitis and associated systemic diseases are addressed in this chapter. Dietary agonists that enhance glutathione levels constitute an important aspect of maintaining optimal cellular function. Literature searches for original papers and reviews including systematic reviews over the last 15years were done using Medline, Embase and other search engines. Key words representing these topics were used, to include clinical and scientific publications for the purpose of illustrating the concepts covered.

Metabolic activity and environmental factors generate oxidative stress. The pathophysiology of a wide spectrum of inflammatory diseases is driven by it. The antioxidant response element (ARE) genes are induced in response to cellular responses to reactive oxygen species (ROS). They respond to the transcriptional activator Nrf2 and the repressor Bach1. The development of synthetic small molecules have therapeutic applications in activating the antioxidant network in a protective capacity. ARE-regulated gene activation and the repressor Bach1 are potential targets. The endogenous ligand heme of Bach1, inhibits its binding to ARE. Nrf2-mediated gene expression is thus expressed, including that of heme oxygenase (HMOX1), a well-documented target of Bach1. A synthetic small molecule capable of inducing HMOX1 and inhibiting Bach 1 activity has been demonstrated [1]. It acts as a novel agent in activating the antioxidant response by modulating Bach1 binding to the ARE binding site of target genes.

These mechanisms demonstrate the complexity of intracellular redox equilibrium. The enzyme heme oxygenase 1 (HO-1) is induced by oxidative stress and degrades redox-active heme producing agents. Anti-inflammatory and vasodilatory effects are mediated by HO-1 and it is protective of cellular stresses. The expression of the HO-1 gene HMOX1 is highly inducible by a range of pro-inflammatory stimuli via NF-κB in human endothelial cells. HMOX1 is regulated by the ARE genes, with the transcription factor Bach1 acting as repressor and Nrf2 functioning as an enhancer. A TNFα-inducible endothelial microRNA

miR-155, is predicted to bind to the Bach1 mRNA. Oligonucleotides that mimic miR-155 effectively inhibit Bach1 protein translation resulting in increased expression of HMOX1 mRNA and protein in human endothelial cells [2]. These findings indicate that during inflammation, miR-155 is cytoprotective by enhancing HO-1 expression in endothelial cells; and that elevated HMOX1 expression by TNFα is a result of miR-induced repression of Bach1 and not due to direct induction of HMOX1 via NF-κB. It is relevant that Bach1 repression dominates over Nrf2-mediated HMOX1 transcription. Inactivation of Bach1 is a requirement for induction of HMOX1 [3]. In contrast, it is significant that thioredoxin reductase 1 (TXNRD1) is mediated by Nrf2 and not Bach1. Comparison of expression levels of HOMX1 and TXNRD1 indicated that nuclear accumulation of Nrf2 is not a prerequisite for induction of HMOX1. Inactivation of Bach1 permits Nrf2 already present in the nucleus at basal levels to bind to the HMOX1 promoter and induce HNOX1. Bach1 poses another level of regulation of oxidative stress responses via ARE-dependent genes.

ROS are highly reactive molecules containing oxygen and are the most abundant free radicals in cells. They occur during physiological intracellular metabolism and play crucial roles in cell differentiation, proliferation and host defence responses [4]. However, in excess they could have adverse effects. Oxygen-derived free radicals could cause damage to various cell components including the critical processes of lipid peroxidation, DNA damage and protein oxidation. Polyunsaturated fatty acids are the main components of cell membranes which are particularly vulnerable to free radical damage due to their reactive hydrogen atoms associated with an abundant distribution of double bonds. This results in a predisposition to free radical damage, hydroxyl radicals in particular, resulting in compromised cell membrane permeability and cell dysfunction [5]. DNA damage comprising broken strands, cross-linking, base hydroxylation and base excision is also attributed to ROS. When DNA damage is combined with a deficient apoptotic pathway, it could result in their transformation [6].

Proteins are the main targets of free radicals, resulting in their oxidation. Free radical attack of aromatic acids, cysteine and disulphide bonds results in protein denaturation and enzyme inactivation [5]. Denatured protein derivatives thus formed could perpetuate oxidative damage in other cell components by acting as intermediary agonists [7]. Enzymatic and non-enzymatic antioxidant systems prevail in their actions as free radical scavengers [8]. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) comprise the enzymatic antioxidant system. This is the main defence against ROS in vivo. The two main types of SOD consist of CuZnSOD (SOD1) prevalent in the cytoplasm with Cu and Zn at the active site; and MnSOD (SOD2) found in the mitochondrial matrix with manganese at the active site. They catalyse the reaction that converts superoxide anion radicals to H_2O_2, which is subsequently converted to water and oxygen by CAT or GPx.

CAT is mainly found in peroxisome with iron at its active site; and is one of the most effective redox enzymes [9]. In the absence of this enzyme H_2O_2 would be converted to a hydroxyl radical, one of the most damaging radicals to cells. GPx is a selenium containing enzyme which exerts a protective influence from oxidative damage on cells by eliminating H_2O_2 with oxidation of glutathione. GR subsequently converts the oxidized glutathione to the reduced form. The non-enzymatic antioxidant system which serves as a second defence system against free radicals, protects tissue from oxidative damage. These systems also reinforce the actions of endogenous enzymatic antioxidants by scavenging free radicals in a synergistic capacity [10]. Vitamins C and E are the best known antioxidants in this context.

There are several small molecules present in foods such as phenolic compounds, carotenoids and flavonoids which serve as non-enzymatic antioxidants. These food derived phytochemicals are known as nutraceuticals [11], [12].

Mechanisms of Action of Glutathione

Glutathione (GSH) plays a critical role in cell signalling and antioxidant defences. It may interact directly with ROS / reactive nitrogen species (RNS); or act as an essential cofactor for GSH S-transferases and glutathione peroxidases. Coordinated actions of GSH and its dependent enzymes which constitute the glutathione system, lead to detoxification of ROS and RNS; and electrophiles produced by xenobiotics. Adequate GSH levels are essential for T cell activation, differentiation and optimal functions of the immune system. GSH is an ubiquitous regulator of the cell cycle. It also has critical functions in the brain as an antioxidant, neuromodulator and transmitter; and in enabling survival of neurons. GSH depletion leads to damage as a consequence of nitrosative and oxidative stress, hypernitrosylation, raised levels of proinflammatory mediators and inflammatory capacity. Intracellular signalling networks such as p53, nuclear factor-κB and Janus kinases are rendered dysfunctional, with diminished DNA synthesis and cell proliferation, activation of apoptotic mechanisms and compromised epigenetic regulation of gene expression. There are crucial consequences of GSH depletion on homeostatic control of the immune system, mitochondrial survival, regulation of energy production, oxidative and nitrosative stress pathways [13]. GSH depletion is an integral part of diverse neuroimmune doisorders such as depression, myalgic encephalomyelitis / chronic fatigue syndrome and Parkinson's disease concomitant with increased ROS / RNS and mitochondrial dysfunction. Therapeutic interventions aimed at enhancing GSH concentrations *in vivo* include N-acetyl cysteine; activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) via hyperbaric oxygen therapy; dimethyl fumarate; folate supplementation and phytochemicals such as curcumin, resveratrol and cinnamon.

The universally conserved thioredoxin (TRX) and glutathione (GSH) pathways drive a spectrum of cellular functions involving reversible disulfide formation. These pathways are considered in *Saccharomyces cerevisiae* in the context of their cell compartment-specific actions and mechanisms that address differences in redox states between cell compartments [14]. The cytosol has both TRX and GSH pathways of which the former is dominant and the latter functions as a backup. In the mitochondrial matrix where both pathways are represented, GSH has a major role in redox control. There are areas of intense thiol oxidation in the endoplasmic reticulum (ER) and mitochondrial intermembrane space (IMS); thiol-reductase pathways are attributed to GSH. Mitochondria are insulated from other compartments. Cytosol may be involved in entry and exit of reduced and oxidized GSH between compartments and provide reducing power to the ER and IMS. The mechanisms regulating fluxes of GSH and oxidized glutathione between cytosol, ER and IMS with a possible role for peroxisomes require clarification; in order to propose a model for eukaryotic thiol-redox homeostasis which may be extrapolated to mammals.

The small redox proteins glutaredoxins, reduce disulfides and mixed disulfides between GSH and proteins. *Cyanobacterium Synechocystis* sp. PCC 6803 has three genes coding for

glutaredoxines: ssr2061 (grxA) and slr1562 (grxB) code for dithiolic glutaredoxins; while slr1846 (grxC) code for monothiolic glutaredoxin. Analysis of the expression of these glutaredoxins to stresses such as high light, H_2O_2 and heat shock demonstrate that grxA is induced only by heat while grxC is induced by high levels of light and H_2O_2; and repressed by heat shock [15]. In contrast to these findings, expression of grxB was maintained fairly constant throughout all conditions. Experimentation with mutants demonstrated that grxA and grxC participate in independent pathways while grxA and grxB participate in a common pathway for H_2O_2 resistance. These data indicate that glutaredoxins are essential for stress adaptation in cyanobacteria; although their targets and mechanisms of action need further clarification.

Glutathione and Periodontitis

While several agents such as antioxidant vitamins overcome oxidative stress, glutathione is the most important small molecule antioxidant. It is prevalent in its oxidized and reduced forms as GSSG and GSH respectively. In health, higher levels of GSH are indicated, maintaining a reduced state intracellularly. It plays a significant role in antioxidant defences as a scavenger of free radicals and protects tissues via antioxidant networks, cell metabolism, DNA turnover and repair [16]. Periodontal disease severity may be affected by glutathione depletion [17], [18].

Chronic inflammatory periodontitis is characterized by raised levels of reactive oxygen species (ROS) resulting from an oxidative stress-inducing phenotype seen locally and systemically. The role of sulforaphane (SFN) in restoring cellular glutathione levels and reducing the hyperactivity of circulating PMNs in periodontitis was studied [19]. It was demonstrated that the NADPH oxidase complex is elevated by intracellular glutathione depletion; which could result from lipid raft formation as a result of upregulation of acid sphingomyelinase, regulated by thiol. Primary neutrophils from periodontitis subjects were hyper-responsive to stimuli and showed decreased intracellular glutathione. The main regulator of the antioxidant response, nuclear factor erythroid-2-related factor 2 (Nrf2); is reduced in circulating PMNs from subjects with chronic periodontitis. PMNs expressed a low ratio of reduced- / oxidized glutathione (GSH/GSSG) when compared with healthy controls; with reduced expression of of glutamate cysteine ligase- and modifier subunit mRNAs respectively. All the above parameters were improved by pre-treatment of cells with SFN. These findings indicate that deficient Nrf2-dependent pathways could play an important role in the mechanisms that lead to hyper-responsive neutrophils in chronic periodontitis.

The pathogeneses of chronic inflammatory diseases including periodontitis are driven by oxidative stress. Possible links between oxidative stress markers in saliva and periodontal alveolar bone have been studied [20]. Salivary oxidative stress markers 8-hydroxy-deoxyguanosine (8-HOdG) and malondialdehyde (MDA) levels were significantly higher in periodontitis subjects when compared with controls. Bone loss markers such as C-terminal telopeptide of type 1 collagen (CTX1), matrix metalloproteinases-8 (MMP-8), 25-hydroxy vitamin D3 and osteocalcin were also significantly elevated in chronic periodontitis subjects, with significant positive correlations with clinical parameters of periodontitis. There were significant positive correlations between salivary levels of MDA and CTX1. Salivary

activities for uric acid, total antioxidant capacity (TAC) and glutathione proxidase (GPx) were significantly reduced in periodontitis subjects compared with controls. There were significant negative correlations between uric acid and CTX1; and between MMP-8 and uric acid. There are significant associations between oxidative stress markers and alveolar bone loss in saliva of subjects with periodontitis. These findings confirm a pro-oxidant profile in periodontal disease progression.

A correlation between increased lipid peroxidation (LPO) and oxidative stress has been demonstrated in periodontitis associated with raised levels of MDA and SOD. Total oxidative status (TOS), MDA and SOD levels were studied in periodontitis subjects longitudinally throughout periodontal therapy for its impact [21], in serum, saliva and gingival crevicular fluid (GCF). Levels of TOS and SOD were significantly raised in periodontitis subjects over controls, but only MDA in GCF. Following 16 weeks of periodontal therapy, TOS and SOD levels decreased significantly in serum, saliva and GCF and MDA in GCF. Lipid peroxidation is elevated in periodontits with raised levels of TOS and SOD both locally and systemically. Non-surgical periodontal treatment is effective in restoring antioxidant capacity in periodontitis subjects by modifying the levels of MDA, TOS and SOD locally and systemically. The extent of oxidative stress in peridontitis subjects was assessed by measuring levels of thiobarbituric reactive substances (TBARS), enzymic antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (GSHPx) and non-enzymatic antioxidants, vitamins E and C; and reduced glutathione (GSH) [22]. It is relevant that periodontitis subjects showed significantly higher levels of TBARS than periodontally healthy subjects. In plasma and gingival tissues enzymatic antioxidants were significantly elevated, while non-enzymatic antioxidants were markedly lower (apart from reduced glutathione in gingival tissue), relative to those in periodontally healthy subjects. Over-production of lipid peroxidation substances causes a disordered, partly compensatory endogenous antioxidant defence system at inflammatory sites, resulting in elevated oxidative stress in subjects with periodontitis.

Finding a functional and reliable molecular marker of periodontal tissue destruction that is sensitive, with good specificity is a great challenge. The utility of molecular markers of soft and hard tissue destruction of the periodontium has been addressed in a systematic review of human studies presenting with such markers in GCF, saliva and serum [23]. Within limits of the scope of the study, no single or consistent combination of markers could adequately disclose periodontal tissue destruction. The most effective source of molecular biomarkers is closely related to bone and soft tissue destruction, requiring objective confirmation; while clinical measurements remain most reliable. An example is demonstrated here. The effects of initial periodontal treatment on GCF and salivary levels of 8-hydroxy-deoxyguanosine (8-OHdG), as a marker of oxidative DNA damage has been evaluated in subjects with chronic periodontitis [24]. There was a significant positive correlation between GCF 8-OHdG and clinical parameters of periodontal disease with a significant reduction following treatment; with not much change in salivary levels. DNA damage and resulting oxidative stress are seen in periodontal pocket tissue of subjects with periodontitis. Periodontal treatment results in resolution of inflammation and oxidative stress, reflected in GCF levels of 8-OHdG which were a more useful biomarker than saliva. 8-Hydroxydeoxyguanisine is an effective marker of periodontal disease severity and treatment responses.

Redox Mechanisms in Smokers with Periodontitis

Smoking is the most significant preventable risk factor for periodontitis. Smoking has a negative impact on chronic inflammatory diseases such as arthritis and inflammatory bowel disease. Periodontal disease is based on a hyper-inflammatory response leading to destruction of tissues of the periodontium. Changes related to the cellular and molecular aspects of the host response and genetic interactions associated with smoking are addressed in the context of local and systemic host responses in periodontitis subjects [25]. An elevated inflammatory response, decreased leukocyte chemotaxis and immunoglobulin production are some of the effects of smoking. Exposure to cigarette smoke products results in increased tissue destructive substances such as ROS, collagenase, serine proteases and proinflammatory cytokines. Epidemiological studies demonstrate the burden of smoking on periodontal health and potential for improvement in response to smoking cessation. Oxidative stress in periodontitis has potential mechanistic links with commonly prevalent systemic diseases such as type-2 DM and atheromatous heart disease in periodontitis subjects. Examination of serum antioxidant concentrations in periodontitis subjects is relevant in this context. Serum levels of vitamin C, bilirubin and total antioxidant capacity (TAOC) show an inverse correlation with periodontitis, increasing with its severity [26]. Higher serum levels of Vitamin C and TAOC are associated with lower odds ratios for severe periodontitis, more pronounced in never-smokers. Reduced relative risk of periodontitis including never-smokers, is associated with raised serum levels of antioxidants. Smoking is a significant factor in contributing to risk of periodontitis. In addition to increasing oxidative stress levels, it is associated with decreased levels of vitamin C and glutathione, and important antioxidant. This is a mechanism whereby smokers could be subjected to increased risk of periodontitis [27].

The effect of smoking status on local and systemic activities of SOD, glutathione peroxidase (GSH-Px) and catalase; and MDA levels were evaluated in periodontitis subjects [28]. The control groups showed the highest gingival activities of SOD, GSH-Px and CAT when compared with periodontitis groups. Serum MDA levels were elevated in all periodontitis groups, over the periodontally healthy non-smoking group, with a significant difference for former smokers with periodontitis. Amongst the periodontitis groups, smokers showed the highest gingival levels of MDA; and compensatory SOD, GSH-Px and catalase activities. These findings indicate that smoking compounds elevated local and systemic levels of MDA, in addition to periodontitis. Decreased local activities of SOD, GSH-Px and catalase seen in periodontitis subjects may be elevated in response to smoking as an adaptive mechanism which may not be adequate to overcome the effects of smoking on periodontal tissues.

The effect of cigarette smoke extract, nicotine and cotinine on neutrophil superoxide production has been investigated. Superoxide generation was detected by lucigenin chemiluminescence. *Fusobacterium nucleatum*, IgG-opsonized *Staphylococcus aureus* and *Escherichia coli* lipopolysaccharide (LPS) acted as pathologically relevant stimuli.There was significant stimulation of superoxide release from neutrophils in response to smoke extract in a dose-dependent manner [29]. Pre-treatment of neutrophils with smoke extract reduced superoxide generation in response to pathologically relevant stimuli, even in the absence of a continued presence of the smoke extract. Simultaneous exposure to both the extract and stimuli resulted in a similar reduction in superoxide production. Neither nicotine nor cotinine

at <10μg/ml induced superoxide release in stimulated or unstimulated neutrophils. These findings indicate that smoking could initiate and maintain oxidative stress at periodontally healthy sites via potential neutrophil-mediated mechanisms; and contribute to disease progression by attenuating host immune responses. Mechanisms involved in peripheral blood neutrophil hyperactivity in chronic and rapidly progressive forms of periodontitis and ROS generation have been addressed in a comprehensive review [30]. It includes environmental factors associated with compromised plasma antioxidant capacity and its influence on periodontal and systemic diseases. A systematic review has demonstrated that smoking cessation improves periodontal clinical parameters, by reducing periodontal pocket probing depths and improving clinical attachment levels following non-surgical periodontal treatment [31]. It is relevant that GCF total antioxidant status was significantly elevated, following periodontal treatment in smokers with periodontitis, while serum total oxidant status was significantly reduced in smokers and non-smokers following periodontal therapy [32]. These findings indicate that non-surgical periodontal therapy is effective in reducing oxidative stress.

Periodontal disease progression and healing responses are affected by tobacco products. In order to elucidate the role of nicotine a significant component of cigarette smoking, on periodontal destruction; its effects on growth, proliferation and protein synthesis were studied in human periodontal ligament fibroblasts (PDLF). At concentrations greater than 2.5mM, nicotine was cytotoxic to human PDLFs. There was significant dose-dependent inhibition of cell proliferation (by 48% and 86% at 50 and 200microM) and protein synthesis (to 44% at 10mM) when compared with controls [33]. In order to elucidate possible mechanisms involved, the antioxidants SOD, catalase, 2-oxothiazolide-4-carboxylic acid (OTZ): a precursor of cysteine that promotes synthesis of GSH; and buthionine sulfoximine (BSO), a cellular GSH synthesis inhibitor, were added to the cultures. It is relevant that OTZ had a protective effect on nicotine-induced cytotoxicity; on the contrary SOD and catalase did not contribute to decreasing the cytotoxicity induced by nicotine. Equally, BSO an inhibitor of GSH synthesis contributed further to nicotine-induced cytotoxicity. These results indicate that thiol depletion could mediate nicotine toxicity in human PDLFs, manifesting as impaired cell growth, proliferation and protein synthesis with concurrent reduced intracellular thiol levels; suggestive of a significant role for nicotine-induced periodontal destruction during cigarette smoking. Agents and mechanisms that enhance glutathione synthesis in human PDLFs may have applications for preventing or attenuating the damaging consequences of cigarette smoking on periodontal disease progression and response to treatment.

The detrimental effects of nicotine on tissues of the periodontium have been demonstrated by several workers. Cigarette smoking contributes to an increased incidence of periodontitis and a poor response to periodontal treatment. The early stress response c-fos gene was studied in human PDLFs following exposure to nicotine, in order to clarify toxicological implications of cigarette smoking at a molecular level [34]. On exposure of quiescent human PDLFs to 2.5mM and 10mM of nicotine for 2h, there were 2.5- and 4.8-fold increases respectively, in the induction of c-fos mRNA expression; peaking at a concentration of 5 mM nicotine at 2h. There is rapid accumulation of the transcript in nicotine treated cells, with a significant signal at 30 minutes of exposure to nicotine, demonstrated by kinetic studies of c-fos mRNA expression. This increase is transient, returning to baseline values of c-fos mRNA expression, comparable to those of control cells in 8h. In order to establish the role of thiol levels for c-fos induction by nicotine, cells were pretreated with the GSH

precursor OTZ (2-oxothiazolide-4-carboxylic acid), to enhance thiol levels; or BSO (buthionine sulfoximine) to deplete GSH. Results show that OTZ pretreatment decreased c-fos mRNA expression; while BSO pretreatment elevated c-fos mRNA expression following exposure to nicotine. Nicotine also caused significant intracellular GSH depletion in a dose-dependent manner at 5mM and 20mM by 22% and 56% respectively. These findings demonstrate that cigarette smoking could induce early reponse stress genes via the c-fos signal transduction pathway; correlating with intracellular thiol levels in human PDLFs.

The oxidative effects of nicotine in human gingival and human oral periosteal fibroblasts were studied, using androgen biomarkers of wound healing and redox status. The antioxidant glutathione (GSH) was used for confirmation of redox responses [35]. Two radiolabelled androgen substrates were used to evaluate the yields of the antioxidant biomarker 5α-dihydrotestosterone (DHT) in order to validate responses to nicotine, GSH and their combinations in a metabolically active model. Nicotine caused significantly reduced yields of DHT, due to down-regulation of 5α-reductase activity, which was overcome by the antioxidant glutathione indicative of modulation of the pro-oxidant effects of nicotine by GSH. These results could be extrapolated to indicate improved healing responses by overcoming the pro-oxidant effects of nicotine. In a similar study, the oxidative effects of nicotine were validated, in human gingival and oral periosteal fibroblasts by demonstrating the responses to hydrogen peroxide (H_2O_2) an established pro-oxidant, for comparison with nicotine, with GSH as an antioxidant, alone and in combination [36]. Radiolabelled androgen substrates were used and the yields of DHT, an antioxidant marker of redox status and wound healing were assayed in response to the agents tested in a metabolically active model. The yields of DHT were significantly reduced by nicotine and H_2O_2. This was overcome by GSH. It is relevant that when nicotine was added to the neutralized combination of H_2O_2 and GSH, decreased yields induced by nicotine were similar to those induced by H_2O_2. The positive effect of GSH was retained. These results indicate a pro-oxidant role for nicotine, considering that oxidative stress mediated by H_2O_2 was overcome by GSH and recurred when nicotine was added. DHT is a sensitive biomarker of oxidate stress which has implications on wound healing.

Potential oxidative effects of glucose, advanced glycation end products (AGE) and nicotine were studied in human gingival fibroblasts, using the antioxidants glutathione (GSH) and insulin-like growth factor (IGF) [37]. Two radiolabelled androgen substrates were used as substrates and assayed for yields of the the oxidative stress marker 5α-dihydrotestosterone (DHT), in response to the agents tested. Significant reduction in the yields of DHT in response to glucose, AGE and nicotine were overcome by GSH. The stimulatory effects of IGF in combination with AGE were enhanced further by the antioxidant effects of GSH. These findings are suggestive of antioxidant effects of GSH and amelioration of oxidative stress responses to glucose, nicotine and AGE. Results from this experimental model may be cautiously extrapolated to redox responses and healing in uncontrolled diabetic smokers.

The impact of diabetes as a risk factor for periodontitis has been studied in the context of antioxidant enzyme activity, namely superoxide dismutase (SOD) glutathione reductase (GR), catalase and the marker of free radical damage, malondialdehyde; in blood and saliva of periodontitis subjects. MDA levels in both periodontitis groups (CP) with (CPDM) and without DM were elevated in comparison with periodontally healthy subject, although the difference between the two periodontitis groups was not significant. There were significant

differences in all enzyme levels between CP and peridontally healthy subjects except blood levels of SOD. Only salivary SOD and GR activities were significantly different in CP and CPDM groups [38]. These findings favour the role of oxidative stress in both DM and CP. Compensatory host mechanism could fail due to excessive free radical influx during periodontitis compounded by the influx from DM contributing to inflammatory overload.

Antioxidant Effects of Glutathione in Systemic Diseases

A range of functions are carried out by glutathione transferases (GSTs) such as detoxification and beyond, with catalytic reactions affecting metabolic pathways and removal of ROS. Based on previous work, GSTM1 and GSTT1 gene polymorphisms and their association with carotid plaque (CP), biochemical parameters of oxidative stress, lipid profile and inflammation have been investigated, in the context of their modulation of atherosclerosis risk. GSTT1 null genotype patients show significantly lower plama lipoprotein levels than the wild-type genotype carriers [39]. Both GST polymorphisms significantly influenced serum IL-6 levels in subjects with CP. Results indicating significant reductions in GSTT1 deletions in subjects with CP, are indicative of a role for GSTs in carotid astherosclerosis and advanced chronic vascular inflammatory disease.

A protective role for the antioxidant enzyme glutathione peroxidase-1 (GPx-1) has been proposed during atherogenesis. A deficiency of GPx-1 accelerates atherosclerosis and increases cellularity of the lesion in ApoE(-/-) mice. The distribution of GPx-1 within the atherosclerotic lesion and mechanisms involved in the increased macrophage numbers seen, require further clarification. Differential expression of GPx-1 in cells within the atherosclerotic lesion and the relationship between deficient GPx-1, macrophage foam cell formation and cellular proliferation were studied [40]. It was demonstrated by in situ-hybridization and immunohistochemistry that both macrophages and to a lesser extent, smooth muscle cells express GPx-1 within atherosclerotic lesions. GPx-1 deficiency resulted in increased foam cell formation, induced by oxidized low density-lipoprotein (oxLDL); and increased proliferative activity of peritoneal macrophages. Proliferation of peritoneal macrophages induced by macrophage colony stimulating factor (MCSF) and ox-LDL in GPx-1 (-/-) ApoE(-/-) mice was mediated by p44/42 MAPK (p44/42 mitogen activated protein kinase), via ERK 1/2 (extracellular-signal regulated kinase1/2) signalling pathway, demonstrated by inhibitors of this signalling pathway. It is relevant that representative effects of GPx-1 deficiency on both macrophage proliferation and MAPK phosphorylation are overcome by the GPx mimic ebselen. These results demonstrate the significance of GPx-1 deficiency on macrophage foam cell formation and proliferation via the p44/42 MAPK (ERK1/2) pathway; underscoring the potential for new therapeutic strategies in managing atherosclerosis.

Subjects with human immunodeficiency virus (HIV) infection are an effective model for demonstration of pro-oxidant mechanisms. These subjects show a significant reduction in levels of enzymes such as glutathione synthase (GSS), glutamate-cysteine ligase-catalytic subunit (GCLC) and glutathione reductase (GSR); responsible for the synthesis of glutathione. These reduced levels of relevant enzymes correlate with reduced intracellular

levels of GSH [41]. GSH capacity in RBCs is a useful marker for a raised level of oxidative stress and immune dysfunction in response to HIV infection. The results support the hypothesis that reduced levels of GSH-synthetic enzymes contribute to compromised levels of GSH in HIV-infected individuals. Considering the role of GSH in combating oxidative stress and improving immune cell function in HIV subjects, supplementary antioxidants could be beneficial in promoting immune cell function and reducing cell damage.

Glutathione peroxidase 3 (GPx3) plays an important role in eliminating hydro- and lipoperoxides from the body. Several single nucleotide polymorphisms (SNP) at the GPX3 gene and altered concentrations have been linked to vascular diseases, but associations between GPX3 and MetS have not been explored. Serum levels of GPX3 and several GPX3 SNPs in Mexican subjects with MetS have been studied [42]. The MetS group demonstrated increased cardiovascular risk and higher serum levels of GPx3, in comparison with controls. Only three of ten GPX3 SNPs screened were polymorphic, with two observed haplotypes, suggestive of tight linkage disequilibrium at this genetic focus. There were no differences for genotype or allele frequencies amongst the observed groups; however rs8177409 (allele T) is significantly linked to cardiovascular risk (odds ratio: 4.5). These findings indicate that serum levels of GPx3 are raised in MetS subjects; and that rs8177409 SNP is associated with cardiovascular risk in the population under study.

Oxidative stress plays an important role in the metabolic syndrome (MetS) and associated conditions. The number of metabolic syndrome components (ischaemic reactive hyperaemia [IRH], plasma levels of soluble vascular cell adhesion molecule-1[sVcam-1], total nitrite, lipid peroxidation products [LPO], hydrogen peroxide [H_2O_2], superoxide dismutase [SOD] and plasma activities of glutathione peroxidase [GPx]), presenting in the subject was correlated with the degree of oxidative stress [43]. sVCAM-1, H_2O_2 and LPO levels are lower in subjects with 2 or 3 MetS components than in those with 4 or 5 parameters. IRH and total nitrite levels are higher in subjects with 2 or 3 MetS componenets than in those with 4 or 5 parameters. SOD and GPx activities are lower in subjects with 2 MetS components than in those with 4 or 5 MetS parameters. There is a significant correlation between SOD activity; and waist circumference, weight, age, triglycerides, high-density lipoprotein and sVCAM-1 levels. MetS subjects with a greater number of MetS components could have greater oxidative stress levels. The association between SOD activity and MetS components could be the most significant variable parameter in subjects with Mets; it has potential as a predictive tool to determine the degree of oxidative stress in subjects with MetS.

Apocyn (4'-hydroxy-3'-methoxyacetophenone) is a commonly used inhibitor of NADPH oxidase; however due to some of its controversial pro-oxidant effects, its applications raise serious concerns. The effects of apocynin on glutathione metabolism being a key intracellular antioxidant, were studied in a well-established rat model of type 2 DM [44]. The effects of apocynin were also compared with those of melatonin. Compared with untreated lean control rats, untreated obese diabetic rats showed increased Nox activity, accelerated generation of hydroxyl free radicals (HFR) and significantly reduced GSH/GSSG ratio, associated with increased glutathione peroxidase (GPx) and reduced γ-glutamylcysteine synthetase (GCS). In the diabetic animals, apocynin treatment attenuated both Nox activity and HFR formation, restored baseline values for the GSH/GSSG (reduced and oxidized glutathione) ratio (due to increased GSH and decreased GSSG levels), normal GPx and slightly increased GCS activity. There was a similar outcome when melatonin was applied to obese diabetic rats. These findings indicate that in the diabetic rat model used in this study, apocynin has a beneficial

effect on renal glutathione homeostasis. The mechanism involves diminished glutathione peroxidase activity which is stimulated excessively during oxidative stress associated with diabetes mellitus.

Teteracyclines have ameliorating antioxidant properties in tissues, including vascular endothelial dysfunction in diabetes mellitus. Low-dose doxycycline (LDD) was used in diabetic rats for 4 weeks. It is relevant that this treatment normalized elevated lipid peroxidase and cellular GSH levels [45]. The diabetes-induced oxidative stress markers MMP-2 and MMP-9 were also normalized in response to LDD. Its antioxidant actions could be effective in vascular disorders amongst DM subjects. Combined treatment with doxycycline has been shown to restore free and total protein thiol levels in experimental diabetes [46]. Ligature-induced periodontitis rats were treated with LDD in order to assess its effects on oxidative stress induced by periodontitis [47]. Gingival tissues were used to assess lipid peroxidation (MDA), the antioxidant enzymes CAT, GPX and SOD, total oxidant and total antioxidant tstatus (TOS, TAS). There was significant inhibition of MDA, reduced TOS, increased TAS and favourable antioxidant enzyme profiles in response to LDD, reinforcing its effective antioxidant properties.

The anti-inflammatory, proanabolic and anti-catabolic, non-antimicrobial actions of tetracyclines are effective in the adjunctive management of inflammatory periodontitis and associated comorbidities. A dysregulated hyperinflammatory immune response in periodontitis could have an autoimmune element in its progression beyond the presence of an initiating antigenic trigger. Its inflammatory pathogenesis and those of the commonly prevalent comorbidities coronary heart disease, DM and arthritis could benefit from adjunctive management with non-antimicrobial chemically modified tetracyclines (CMTs) to curb an over-exuberant inflammatory response. Tetracyclines and their derivatives interact with matrix metalloproteinases (MMPs), tissue inhibitors of MMPs, cytokines and growth factors. They mediate immunomodulation by affecting the sequence of inflammation, cell proliferation and angiogenesis. CMTs maintain anti-inflammatory, anti-apoptotic and anti-proteolytic actions in organs in a range of chronic inflammatory diseases which reinforces their therapeutic scope. Their specific advantageous effects have been demonstrated in experimental models of ischaemia. Some of these actions and mechanisms involved have been addressed in a recent review [48]. Unique non-antimicrobial actions of tetracyclines in a hyper-inflammatory environment have applications in chronic inflammatory disorders. These advantageous effects are relevant to the adjunctive management of periodontitis subjects presenting with commonly prevalent comorbidities mentioned above.

Redox responses of cultured osteoblasts have been studied, in response to bacterial lipopolysaccharides (LPS), glucose (G), glucose-oxidised low density lipoprotein (GLDL) and minocycline (M), using radioactive substrates and the redox marker of wound healing 5α-dihydrotestosterone (DHT), in a metabolically active model [49]. There were significantly decreased yields of the antioxidant marker DHT in response to LPS, G and GLDL, which were overcome by minocycline. These findings demonstrate its potential antioxidant actions in an environment of oxidative stress and may be extrapolated to periodontitis and co-existing risk markers in cardiometabolic diseases. This has implications on the adjunctive anti-inflammatory and antioxidant therapeutic benefits in the management of periodontitis and prevalent comorbidities. There have been significant advances in adjunctive antioxidant therapeutics in the management of these diseases, in the context of oxidative stress being a common denominator. The same workers have demonstrated that IL-6 and CRP alone and in

combination, caused significant reduction in the yields of the antioxidant marker DHT in monolayer cultures of osteoblasts. These effects were ameliorated by doxycycline, resulting in values of DHT similar to those of control incubations in the absence of agents [50]. The oxidative actions of IL-6 and CRP and antioxidant effects of doxycycline are reinforced by the metabolic yields of DHT in respose to the above agents. DHT directly activates androgen receptor proteins which play an important role as redox regulators via direct actions on glutathione S-transferase [51]. It is significant that mouse stem cells pretreated with DHT which elevates levels of the antioxidant enzyme CAT, are able to overcome the oxidative and apoptotic effects of H_2O_2 leading to reduced levels of DHT, cell cycle regulatory proteins and cell viability [52]. In view of these direct antioxidant effects of DHT, the novel metabolically active model used, demonstrates a closer relationship with *in vivo* conditions; which may be applicable in the context of adjunctive therapeutic applications for periodontitis and prevalent comorbidities such as coronary heart disease and arthritis in periodontitis subjects.

There is a significant prevalence of periodontitis subjects presenting with systemic diseases such as coronary heart disease, insulin resistance and arthritis which present with a pro-oxidant inflammatory profile. Changes in gene expression associated with a pro-inflammatory profile and lipid metabolism in response to periodontal pathogens have been shown in animal models, independent of atherosclerotic lesions. A single nucleotide polymorphism of the TNF-α gene is associated with significant attachment loss of teeth in periodontitis subjects with coronary heart disease. Raised levels of the cytokines IL-1, IL-6 and TNF-α associated with chronic low-grade inflammation and insulin resistance is also relevant to the progression of periodontitis. Uncontrolled periodontitis could contribute to the maintenance of systemic inflammatory loading relevant to rheumatoid arthritis (RA), with increased risk of RA in periodontitis subjects. Concepts linking periodontitis and sytemic diseases associated with a pro-oxidant profile and mechanisms involved have been reviewed recently [53]. Weak but consistent associations are seen for surrogate markers of periodontitis, for example tooth loss with multiple systemic conditions. Effective treatment of periodontitis could be beneficial in reducing systemic inflammatory loading and systemic health. Genetic, epigenetic and other subject variables could account for the lack of a consistent cause and effect relationship between diseases with a predominant pro-oxidant profile.

Physiological metabolic activities of cells result in the formation of ROS as by-products. The damaging effects of ROS are protected by superoxide dismutase (SOD), glutathione peroxidase and CAT. ROS may be produced in response to a range of stimuli such as ultraviolet radiation, cigarette smoking, alcohol, ischaemia-reperfusion injury, chronic infections and inflammatory conditions. Disruption of physiological cellular homeostasis by redox signalling could result in tissue damage and susceptibility to certain diseases. Oxidative stress and ROS release could contribute to gastrointestinal diseases such as peptic ulcers, cancers and inflammatory bowel disease [54]. ROS are produced within the gastrointestinal tract. Despite the protective mucosal barrier, ingested substances and pathogens could contribute to inflammatory, immune-mediated responses involving the epithelium of the gastrointestinal tract. Understanding signalling mechanisms initiated by ROS at a cellular level and the host responses to such stimuli, would advance knowledge of disease pathogenesis for the evolution of appropriate treatment strategies.

Redox control in normal human mammary cells is poorly understood. Purified normal human basal mammary epithelial cells maintain low levels of ROS primarily by a relatively

ineffectual glutathione-dependent antioxidant mechanism that utilizes mitochondrial glutathione peroxidase 2. In contrast, matching luminal progenitor cells consume oxygen at a greater rate and contain oxidative nucleotide damage-controlling proteins. They show increased levels of ROS and demonstrate multiple antioxidants independent of glutathione [55]. These luminal progenitor cells are more resistant to H_2O_2, ionizing radiation and to glutathione depletion than basal cells; including those with proliferation and differentiation activity. Distinct mechanisms for control of ROS in subsets of human mammary cells could have functions related to their state of differentiation, with long-term consequences. Oxidative stress plays a significant role in diseases such as atherosclerosis, heart failure and myocardial infarction. Atopic dermatitis characterised by eczema and pruritis is a chronic relapsing inflammatory disease affecting the skin, in response to irritants, environmental and food allergens. Very little is known about the redox status of subjects with atopic dermatitis. Evaluation of malondialdehyde (MDA) a pro-oxidant, the enzymic antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and the non-enzymatic antioxidants reduced glutathione (GSH), vitamins A, E and C demonstrated that subjects with atopic dermatitis are more susceptible to ROS damage than healthy controls [56]. There were raised levels of malondialdehyde and decreased enzymatic and non-enzymatic antioxidants in the former. Antioxidants could have potential benefits in atopic dermatitis subjects, which requires further study with larger population groups.

Dietary Agonists

It is relevant that periodontitis is associated with reduced serum micronutrient levels [26]. This could be a consequence of dietary, environmental and genetic factors affecting their absorption, biosynthesis, distribution and actions [57]. Potential beneficial clinical effects of powdered fruit and vegetable juice concentrate on periodontitis subjects have been investigated in a randomized double-blind clinical trial. Dietary intake and biochemical nutritional status were assessed [58]. Results showed that there was improved pocket depth reduction in response to non-surgical periodontal treatment with the dietary supplement as an adjunct when compared with treatment combined with a placebo. It would be pertinent to include dietary recommendations for improved consumption of fruits and vegetables, fibre, oily fish and reduced intake of refined sugar. This would be part of a health message for effective prevention and treatment of periodontitis subjects as suggested in the 2011 European workshop on Periodontology [59]. A dsysregulated, hyperinflammatory immune response in periodontitis subjects resulting in a pro-oxidant profile makes it an important area for antioxidant therapeutics as adjuncts. Dietary measures have potential benefits in this context for better outcome measures in the management of periodontitis and prevalent systemic comorbidities.

Growing interest in the role of oxidative stress in the progression of periodontal disease prompted an assessment of potential oxidant / antioxidant interactions of nicotine with coenzyme Q10 (CoQ), Pycnogenol and phytoestrogens in a cell culture model of osteoblasts and periosteal fibroblasts [60]. Metabolic yields of the antioxidant marker 5α-dihydrotestosterone (DHT) from radiolabelled androgen substrates in response to agents tested, were assayed in a metabolically active model. There were elevated yields of DHT in

response to CoQ, Pycnogenol and phytoestrogens; and significantly reduced yields in response to nicotine, which were overcome by combined incubations with CoQ, Pycnogenol or phytoestrogens. These results indicate that the pro-oxidant effects of nicotine could be reversed by the antioxidants CoQ, Pycnogenol and phytoestrogens, which could have potential applications in an environment of oxidative stress. The role of nutritional antioxidants in periodontal and systemic diseases has been reviewed [61], [62]. There are applications for the role of adjunctive nutritional and therapeutic antioxidants in diseases that present with a distinctly pro-oxidant profile such as periodontitis. Accurate therapeutic targeting to complement conventional periodontal treatment for an effective outcome is exacting. Bioactive phytochemicals play a significant role in decreasing oxidative damage in subjects with MetS with accompanying sub-cellular characteristics of atherogenic dyslipidaemia and a proinflammatory state. Optimally targeted, synergistic therapeutic formulations would be potentially effective as adjuncts in the management of periodontitis and associated systemic comorbidities.

Oxidative stress may be reduced by foods rich in antioxidant micronutrients, such as leafy vegetables, berries, kidney beans, red wine and dark chocolate containing more than 70% cocoa. Complex compounds in nuts, olives and oily fish could slow down gastric emptying time, with fewer less significant spikes in serum glucose in response to vitamin C [63], enhanced by the antioxidant efficacy. Oscillating glucose peaks are more damaging to the redox status of endothelium than mean glucose levels. Post-prandial incremental glucose peaks correlate with carotid intima-media thickness in type 2 diabetes mellitus [64]. Diabetic complications are a consequence of long-term hyperglycaemia and elevated levels of reactive oxygen species (ROS). Honey and ginger have antioxidant properties associated with scavenging ROS. The antioxidant and anti-diabetic effects of gelam honey and ginger alone and in combination were studied in diabetic and non-diabetic Sprague-Dawley rats [65]. In order to obtain a metabolic profile, the parameters measured were glucose triglyceride (TG), SOD, CAT, glutathione peroxidase (GPx), reduced glutathione (GSH): oxidized glutathione (GSSG) ratio and malondialdehyde (MDA). Although a combination of gelam honey and ginger did not show hypoglycaemic potential, the combined treatment significantly reduced levels of MDA; while there was significant elevation of GSH and GSH/GSSG ratio in diabetic rats when compared with controls.

Oxidative stress, a cholesterol-enriched diet and raised cholesterol levels result in raised serum total cholesterol levels (TC) and low density lipoprotein-cholesterol (LDL-C), which contribute to atherosclerosis. Antioxidants play an important role in absorbing free radicals which have damaging consequences in tissues. Protective effects of propolis (a resinous hive product collected from plant sources by honey bees) and thymoquinone (TQ) derived from specific plant seeds have been studied, on early atherosclerotic lesions and serum lipid levels in hypercholesterolaemic rabbits [66]. A cholesterol-enriched diet caused significant increases in serum levels of TC, triglycerides, LDL-C, thiobarbituric acid-reactive substances; and significant decreases in HDL-cholesterol and reduced glutathione levels when compared with controls. When the antioxidants propolis and TQ were administered simultaneously with a cholesterol-enriched diet, there were significant reductions in TC, LDL-C, triglycerides and thiobarbituric acid-reactive substances; and increased levels of HDL-C and glutathione, when compared with the high cholesterol control group. Early atherosclerotic changes represented by endothelial damage and thickened foam cells were seen in the high cholesterol group; the antioxidants propolis and TQ provided protection against damage induced by high

cholesterol. Antioxidant mechanisms associated with the latter could provide protection and minimize the risk of atherosclerosis.

The hypoglycaemic and antioxidant effects of shrimp astaxanthin were studied in the kidney of alloxan-induced diabetic rats. *In vitro* anti-diabetic effects of astaxanthin dissolved in olive oil were compared versus controls in plasma and renal tissue of diabetic rats [67]. Antioxidant enzyme activities including CAT, SOD and non-enzymatic levels of reduced glutathione were significantly decreased in plasma and tissue of diabetic rats compared with controls. The above enzyme activities were significantly improved in response to supplementation with astaxanthin with no additional change in response to olive oil alone. These findings demonstrate that shrimp astaxanthin could play an important role in reducing oxidative damage and pathological changes in diabetic rats, indicating potential use for therapeutic applications. The impact of commercially available green and black tea (GT, BT) beverages on oxidative stress and drug-metabolising enzymes was studied in rats [68], in comparison with de-ionised water. In response to both GT and BT, there were significant increases in hepatic microsomal cytochrome P450 (CYP)1A1 and CYP1A2; and a significant decrease in CYP2C, CYP2E1 and CYP3A enzyme activities. There were lower lipid levels in lungs of rats treated with the GT beverage. Feeding both tea drinks to rats results in modulation of drug-metabolising enzyme activity; and reduced oxidative stress in liver and lungs. The GT beverage was more effective in reducing oxidative stress than the BT beverage.

Nutritional assessment of diet in subjects with MetS and a biochemical analysis of redox levels have been carried out in MetS subjects [69] for comparison with those who did not have MetS. Antioxidant capacity showed a normal range in both MetS and control subjects, with no significant differences in SOD levels between the two groups. Mean glutathione reductase levels were significantly greater in controls than in those with MetS. In the context of trends for oxidative stress markers, isoprostane levels were greater in controls than in MetS subjects and oxidized LDL values were higher in MetS subjects; although the differences were not significant. Trends towards a greater degree of oxidative stress, associated with poorer nutritional and biochemical parameters were seen subjects with MetS in comparison with controls.

There is growing interest in the relationship between diet and ageing. Some antioxidants and restriction of dietary calories have been shown to enhance lifespan in experimental models of ageing. As oxygen is the final electron acceptor in mitochondria, it is essential for aerobic organisms. Excess oxygen could be harmful due to generation of reactive oxygen species (ROS) in a continuous cycle; this could contribute to ageing [70]. An antioxidant defence system comprising a range of enzymes such as SOD, CAT, glutathione peroxidase (GPx) and glutathione reductase (GR) are active in removing ROS accumulating in cells. In addition, vitamin A, vitamin C, α-tocopherol and plant flavonoids which are available as dietary antioxidants also have the capacity to scavenge ROS in cells. They could potentially enhance the lifespan of organisms. In this context, several antioxidants including apple polyphenols, blueberry polyphenols, black rice anthocyanins, tea catechins and theaflavins have been shown to enhance lifespans of fruit flies. Some of these trends may be extrapolated to humans.

Resveratrol has strong antioxidant properties and decreases blood glucose levels which could contribute to minimizing complications associated with DM. The effects of resveratrol on catalase (CAT), glutathione peroxidase (GPx) gene, protein expression, their

phosphorylation states and activities were studied in diabetes-induced rats [71]. There is increased total protein phosphorylation in DM, while mRNA expression, protein levels and their activities were similar. Although there is attenuated transcription of GPx in DM, protein levels and actions were not affected. Administration of resveratrol to DM rats resulted in increased pGPx levels. Increased nucler factor kappa B (NFκB) gene expression in DM, decreased Sirtuin 1(SIRT1) and nuclear factor erythroid 2-related factor (Nrf2); are linked to a decrease in CAT and GPx mRNA expression. Nuclear translocation of redox- sensitive Nrf2 and NFκB in DM could be a compensatory mechanism for a reduction in gene expression of antioxidant enzymes. This is seen as increased nuclear protein levels of Nrf2 and NFκB and reduced cytoplasmic levels of the same. These findings indicate that an increased oxidized state in DM, results in altered cellular phosphoryation and regulation of antioxidant enzymes. Administration of resveratrol also results in coordinated cytoprotective responses in tissues.

Regular consumption of wine, a characteristic feature of the Mediterranean diet has been associated with significant health benefits. The non-alcoholic component contains a wide range of phenolic polyphenols with antioxidant properties. Polphenols found in wine could delay the progression of inflammatory intestinal diseases driven by oxidative stress, particularly due to raised levels in the gut, compared with other tissues.They scavenge ROS and modulate specific genes involved in redox signalling in a milieu of inflammation and also act as antimicrobial agents and prebiotics [72].Wine phenolics have potential as alternative adjuncts for the treatment of inflammatory intestinal diseases. Some of the beneficial effects could partly be attributed to the alcoholic component in view of such effects of ethanol and require clarification order to implement a more solid foundation for their applications.

In view of assertions regarding potential toxic effects of synthetic antioxidants, the poultry industry has been seeking natural antioxidant sources alone or in combination with synthetic antioxidants. The status of antioxidant enzymes, fatty acid profile and serum biochemical profile of broilers was determined in response to no addition (T1) and addition of wheat germ oil (natural α-tocopherol T2), synthetic α-tocopherol (T3), α-lipoic acid (T4), and combinations of α-lipoic acid with natural and synthetic α-tocopherol respectively (T5 and T6) [73]. The dietary supplements used, resulted in improved distribution of saturated and unsaturated fatty acids in breast and leg meat. The fatty acid content was significantly greater in broilers receiving T2 and lower in those receiving T6 in their diet. Total cholesterol and triglyceride levels in serum were lowest in those receiving α-tocopherol and α-lipoic acid. Wheat germ oil containing natural α-tocopherol alone or with a-lipoic acid is more effective than its synthetic counterpart in enhancing antioxidant enzyme levels of superoxide dismutase (SOD), CAT and GR (glutathione reductase). This was accompanied by significantly reduced plasma total cholesterol, LDL and triglycerides; and elevated levels of HDL and plasma protein. A combination of wheat germ oil and a-lipoic acid is effective in improving lipid profiles in this context.

ROS and reactive nitrogen species (RNS) contribute to endothelial dysfunction in advancing age and promote the development of coronary heart disease and DM. The discovery of α-lipoic acid as a catalyst for decarboxylation of pyruvate and α-ketoglutarate has generated interest in its efficacy in protecting mitochondrial dysfunction induced by ROS. Both α-lipoic acid and dihydro α-lipoic acid have potent antioxidant actions and account for the benefits of supplementation with α-lipoic acid. Recent clinical work done on its beneficial effects on endothelial dysfunction and possible mechanisms incolved are reviewed [74]. The

redox status of α-lipoic acid depends on the degree of oxidised status of cellular components. The reducing intracellular environment helps to protect from oxidative damage. Younger healthy subjects are able to synthesise α-lipoic acid in adequate amounts for scavenging ROS and increasing the endogenous antioxidants glutathione, vitamins C and E. With advancing age, a significant decline in α-lipoic acid could lead to endothelial dysfunction. Regulation of gene transcription associated with antioxidant and anti-inflammatory pathways are also attributed to α -lipoic acid in several studies.

Chemical redox properties of α-lipoic acid indicate significant antioxidant actions. α-Lipoic acid and its reduced form dihydrolipoic acid are protective against oxidative stress-induced cell damage from reactive oxygen and nitrogen species (ROS, RNS). The role of nonprotein- bound α-lipoic acid as a physiological antioxidant has been questioned due to reduced and transient levels following oral intake. The micronutrient actions of α-lipoic acid could function in influencing cellular oxidative stress response pathways, which would affect antioxidant levels intracellularly and attenuate pro-inflammatory mechanisms [75]. This mode of action would result in more sustained benefits and resistance of cells to oxidative stress-induced pathologies, rather than act as a transient scavenger of ROS.

Yam (Dioscorea batatas Decne) has been used as a health food for its nutritional and anti-inflammatory effects. ROS, implicated in a wide spectrum of diseases are important precursors of carcinogenesis. The modulatory effect of yam on inflammation and antioxidant status in azoxymethane (AOM)-induced colonic cancer has been studied in male rats [76]. The formation of aberrant crypt foci (ACF), haemolysate antioxidant enzyme activities; and gene expression for inflammatory mediators and antioxidant enzymes in colonic mucosa were quantified. Ingestion of yam prior to carcinogenesis caused significant reduction in ACF formation in response to AOM. Erythrocyte levels of glutathione, glutathione peroxidase (GPx) and catalase (CAT) were elevated when rats were fed yam. It also resulted in up-regulation of Cu/Zn-superoxide dismutase (SOD), Mn-SOD and GPx gene expression in colonic mucosa when compared with the AOM group. Gene expression of the inflammatory mediators NF-κB, inducible nitric oxide synthase , COX-2, TNF-α and IL-1β in colonic mucosa was suppressed in response to the diet supplemented with yam. Enhancing antioxidant defences and modulating inflammatory mediators are some of the mechanisms attributed to yam in the prevention of colonic cancer.

The antioxidant effects of bilberries show beneficial preventive actions against inflammatory bowel disease and colonic cancer. The gastrointestinal tract is a potential target for disease prevention. The commercially available anthocyanin-rich bilberry extract (BE) was compared with four diverse BE-loaded microcapsules systems for their antioxidant properties. Intracellular ROS, oxidative DNA damage and total glutathione (GSH) levels were monitored as markers of antioxidant status. Increased cellular glutathione levels and reduced ROS were demonstrated at high concentrations of BE when incubations were made with BE loaded capsule systems [77]. In addition, there was a positive effect on DNA strand breaks. Biological properties of encapsulated BE were comparable to those of non-encapsulated BE. Both forms of BE appear to have beneficial effects on antioxidant activity in preventing oxidative damage of DNA, reducing intracellular ROS and enhancing cellular total glutathione, under the assay conditions used. Direct beneficial effects of dietary components on the antioxidant actions of the glutathione network have potential for their adjunctive applications in chronic inflammatory diseases in addition to conventional therapy.

Conclusion

Dysregulated immune responses with a hyper-responsive pro-oxidant inflammatory profile characterize periodontitis and associated systemic diseases prevalent in these subjects. The relevance of the glutathione system and mechanisms of their actions and interactions are addressed here, demonstrating significant connections between periodontitis and systemic inflammatory conditions. There are distinct mechanisms involving antioxidant response element (ARE) genes in response to ROS, by cells. The complexity of intracellular redox equilibrium is demonstrated by interactions between ARE genes, the repressor Bach1and their modulation by the expression of transcriptional activator Nrf2- and heme oxygenase genes (HOX1). Induction of the latter by a synthetic small molecule enhances antioxidant activity. Synthetic small molecules have therapeutic applications, in activating the antioxidant network to protect tissues and organ systems against oxidative damage. Similarly, dietary agonists could play an important role in attenuating oxidative stress due to their direct chemical antioxidant properties and by scavenging ROS and RNS. The micronutrient α-lipoic acid and its reduced form are potent antioxidants with beneficial effects on endothelial dysfunction. They also regulate gene transcription associated with antioxidant and anti-inflammatory pathways. Collectively, dietary agonists have advantageous interactions with the antioxidant capacity of the glutathione network. These effects have potential adjunctive therapeutic applications requiring focused and targeted delivery.

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Chapter 3

Contribution of Glutathione-S-Transferases to the Pharmacogenetics of Azathioprine

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Abstract

Azathioprine is a purine antimetabolite drug commonly used as immunomodulator in the treatment of various chronic inflammatory diseases, such as inflammatory bowel disease (IBD). Azathioprine is activated *in vivo* after reaction with reduced glutathione (GSH) and conversion to mercaptopurine. Although this reaction may occur spontaneously, the presence of the enzyme glutathione-S-transferase (GST), in particular of isoforms GST-A1/GST-A2 and GST-M1, can increase its speed, leading to a faster activation of azathioprine to active thioguanine nucleotides. Moreover, GSTs may contribute to azathioprine effects by modulating GSH consumption, oxidative stress and apoptosis. Indeed, in young patients with IBD, deletion of GST-M1, which determines reduced enzymatic activity, was recently associated with reduced sensitivity to azathioprine and reduced production of its active metabolites. Therefore, genetic polymorphisms in genes for GSTs may be useful to predict response to azathioprine even if more *in vitro* and clinical validation studies are needed.

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Introduction: Azathioprine in Inflammatory Bowel Disease

Azathioprine, the 1-methyl-4-nitroimidazol-5-yl derivative of mercaptopurine, is a purine antimetabolite drug commonly used to treat inflammatory bowel disease (IBD). Despite the introduction of effective biological treatments, such as antibodies against TNF-alpha, azathioprine is still a mainstay for maintenance therapy of severe IBD. Azathioprine is a prodrug and requires complex conversion to active metabolites (Figure 1). The first step in this conversion is the reaction with reduced glutathione (GSH), to yield the prodrug mercaptopurine and a nitroimidazole derivative/conjugate of GSH. Even though this reaction can occur spontaneously [1] the presence of the enzyme glutathione-S-transferase (GST) may increase its speed, as discussed later in this chapter [2]. After oral administration, intact azathioprine is undetectable in blood because of extensive first pass metabolism [3]. Even mercaptopurine needs metabolic conversion to thioguanine nucleotides (TGNs), catalyzed by the enzymes of the purine salvage pathway. Moreover, mercaptopurine is inactivated in the liver mainly by xanthine oxidase (XO), while in the extra hepatic tissues mercaptopurine catabolism involves predominantly genes that display genetically determined polymorphic activity, such as thiopurine-S-methyltransferase (TPMT) and inosine triphosphate pyrophosphatase (ITPA).

Mercaptopurine's cytotoxic effects are mainly the consequence of the incorporation of the active TGNs in the nucleic acids and of the consequent interference with the function of DNA processing enzymes and, to some extent, of *de novo* purine synthesis inhibition, mainly operated by methylated precursors of TGNs.

While mercaptopurine pharmacokinetics and pharmacogenetics have been characterized extensively, the mechanism of conversion of azathioprine to mercaptopurine and its clinical implications for therapy personalization have not been completely elucidated.

Involvement of the Enzyme GST in the Conversion of Azathioprine to Mercaptopurine

GSTs are enzymes responsible for the inactivation of endogenous and exogenous electrophilic substances, by catalyzing their reaction with GSH. Human cytosolic GSTs are encoded by 17 genes and the proteins derived can be classified into 7 distinct classes based on their amino acid sequences. The most abundant GSTs in human cells are those of class P, M and A. GST-P1 is the principal isoform in most tissue, such as the small intestine and erythrocytes, but is not detectable in normal liver cells; on the other hand, GST-M1, GST-A1 and GST-A2 are highly expressed in hepatocytes, while they are not expressed in erythrocytes. GST-T1 is expressed in the liver, intestine and erythrocytes even if its level of expression is lower compared to GSTs of the other classes [4].

All the genes for these GST classes display common genetic polymorphisms that influence the activity of the enzyme in some individuals. For GST-M1 and T1, common deletions are present in humans, so that about respectively 50% and 20% of Caucasians lack activity of these enzymes because of these genetic variants. In patients of African and Asian ancestry, frequency of GST-T1 deletion is higher than Caucasian, reaching around 50%, while frequency of GST-M1 deletion is similar [5]. GST-P1 displays a common coding non-synonymous variant, the rs1695, an A-G transition at base 1578, resulting in the amino acid change Ile105Val in the substrate binding site of the enzyme: the allele frequency for this polymorphism in Caucasian and African populations is similar (41-39%), while in Asians the percentage is lower (13%) [6, 7].

GST-A1 contains three linked base substitutions in the proximal promoter in the 5'-noncoding region, at positions -567, -69 and -52, commonly characterized through SNP rs3957357 (also known as -69 C>T), that determines variable enzymatic expression [8]. Indeed, GST-A1 protein expression in liver cytosol decreases significantly according to genotype with rs3957357 CC (GST-A1*A/*A) > rs3957357 CT (GST-A1 *A/*B) > rs3957357 TT (GST-A1*B/*B) and about 25% reduction in expression for each copy of the variant allele [9-11]. The GST-A1 polymorphism shows different frequencies for the variant allele between Caucasian and African (respectively 17 and 11%) and Asian (1%) populations. For GST-A2, three coding non-synonymous SNPs have been described: rs2234951 G>A, rs2180314 C>G, rs6577 T>G that lead to changes respectively in the residues on GST-A2 protein at position 110, 112 and 210. In Caucasians, the most common residues at these position are P110, T112 and E210 (GST-A2*C allele); the variant residues defined by the three most common GST-A2 SNPs are S110 (GST-A2*E), S112 (GST-A2*A) and A210 (GST-A2*B). These polymorphisms modulate GST-A2 protein concentration in the liver: the homozygous genotype A2*C, the most common in Caucasians, has been reported to present three fold lower expression than combination of the genotypes A2*A/B/E [11]. Frequencies of these GST-A2 SNPs differ widely among the three major ethnic groups as reported in Table 1 [12-14].

Table 1. Glutathione-S-transferases (GST) polymorphisms and their functional consequences and frequencies in different ethnic groups

Gene	Polymorphisms	Allele defined	Functional consequences	Variant allele frequency		
				Caucasians	Africans	Asians
GST-M1	deletion	GST-M1 null	Loss of enzymatic activity	50%	50%	50%
GST-T1	deletion	GST-T1 null	Loss of enzymatic activity	20%	50%	50%
GST-P1	rs1695 (A>G)	GST-P1 Ile105Val	Loss of enzymatic activity	41%	39%	13%
GST-A1	rs3957357 (C>T)	GST-A1*B	Each copy of the variant allele reduced GST-A1 expression of 50%	42%	31%	12%
GST-A2	rs6577 (T>G)	GST-A2*B	Altered enzymatic expression	7%	77%	23%
	rs2180314 (C>G)	GST-A2*C	Altered enzymatic expression	57%	23%	66%
	rs2234951(C>T)	GST-A2*E	Increased enzymatic activity toward azathioprine	5%	0%	12%

Genetic polymorphisms in GSTs, determining the interindividual variability in the activity of these important metabolic enzymes, have been related to the incidence of several pathologies, in particular oncological, and to altered sensitivity to medications, including azathioprine [15-18].

Oxidative Damage

Considering the involvement of GST enzymes in the metabolism of thiopurines, especially azathioprine, it is reasonable to suggest that these agents are able to induce oxidative stress, mediated primarily by GSH consumption during the metabolic conversion of azathioprine to mercaptopurine [19, 20]. Cellular redox balance is largely determined by GSH; depletion of such cellular antioxidant defenses allows the accumulation of significant amounts of reactive oxygen species (ROS), as demonstrated in several systems [21, 22], which, in turn, have been suggested to act as a signal for apoptosis induction [23]. Among the many secondary effects attributed to ROS, a role in mediating an anti-proliferative effect has been demonstrated. Indeed, the cellular redox balance fluctuates during cell cycle, so that the redox state modulates cell cycle progression from one phase to the next [24]. In this scenario, a significantly higher GSH content in the G2 and M phases compared with G1 was found in chinese hamster ovary fibroblasts [25]. Hence, it is reasonable to hypothesize a role for ROS in affecting the anti-proliferative effect of thiopurines, which are cell cycle S-phase specific agents, and especially of azathioprine, which consumes GSH during its conversion to mercaptopurine.

In vitro azathioprine exposure induced a rapid depletion of GSH in hepatocytes before any loss of viability and addition of exogenous GSH or N-acetylcysteine protected against cell death. Lee and co-workers [19] suggested that oxidative stress induced by GSH depletion is able to induce mitochondrial damage, opening of mitochondrial permeability transition pore (MPTP) and rapid consumption of ATP. In accordance, in the human hepatic HepaRG cell line, azathioprine and mercaptopurine treatment induced a rapid ATP depletion, observable already after 24 h exposure, earlier then the cytotoxic effects that appeared only after 96 h exposure [26]. As demonstrated in rat hepatocytes, changes in cellular ATP levels might suggest that mitochondrial injury is involved in the mechanism of thiopurine hepatotoxicity [27]. On the other hand, ATP depletion could be the result of a deficient mitochondrion as a consequence of GSH depletion and therefore of oxidative stress condition. Indeed, some speculations have been made on the effective role of oxidative stress in the effects of azathioprine, mainly relating to GSH depletion. For instance, the fact that, under the same experimental conditions, mercaptopurine was not able to reduce hepatocyte viability as azathioprine did, allows suggesting that the activating steps triggered by GST and the associated GSH depletion could be crucial in azathioprine cytotoxicity *in vitro*. Moreover, even metabolism of thiopurines by XO may generate ROS, as assessed on primary cultures of rat hepatocytes [20]. XO, which metabolizes mercaptopurine, converting it into thiouric acid, is a well know producer of ROS, such as superoxide anion [28], whose accumulation could worsen the oxidative stress induced by GSH depletion. However, allopurinol, a XO inhibitor, has been shown to restore response to thiopurines in patients with IBD unresponsive to thiopurines and with unfavorable metabolic ratio, increasing the concentration of active TGNs

and decreasing those of the methylated nucleotides, likely because of inhibition of TPMT [29].

To confirm a direct role of azathioprine in ROS production, a recent *in vitro* study [30] has shown that in rat hepatocytes treated with a high concentration of azathioprine (400 μM, corresponding to the EC_{50} after a 2 hour exposure in this model system), a significant increase in azathioprine-induced cytotoxicity and ROS formation was present when GSH depleted cells were used. The addition of N-acetylcysteine decreased cytotoxicity and ROS formation. XO inhibition by allopurinol decreased azathioprine-induced cytotoxicity, ROS, hydrogen peroxide (H_2O_2) formation and increased mitochondrial membrane potential. Addition of N-acetylcysteine and allopurinol together caused nearly complete cytoprotection against azathioprine-induced hepatic cell death. Antioxidants such as DPPD (N,N'-diphenyl-p-phenylenediamine), Trolox (a water soluble vitamin E analogue) and mesna (2-mercaptoethansulfonate) or TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl), a known ROS scavenger and superoxide dismutase mimetic, also reduced hepatocyte death and ROS formation. Taken together, results from this study indicate that azathioprine-induced cytotoxicity in isolated rat hepatocytes may be at least in part due to ROS formation and GSH depletion, determining oxidative stress and mithocondrial injury [30].

Azathioprine half-life is very short [3] and its therapeutic effects on lymphocytes are likely due to the metabolites produced after first pass metabolism in the intestinal and liver cells. However, azathioprine cytotoxic effects due to GSH consumption are likely to play an important role on the development of cytotoxicity in intestinal and liver cells, supporting a role in the induction of adverse events in these organ systems. To elucidate the mechanism underlying azathioprine induced hepatic injury an *in vivo* study was carried out on female BALB/c mice.

After azathioprine oral administration, plasma levels of alanine aminotransferase and aspartate aminotransferase, markers of a hepatic damage, were increased, and liver damage was confirmed through a histological evaluation. In addition, the hepatic GSH levels and superoxide dismutase activity were significantly decreased. ROS plasma levels were significantly increased during the early phase of azathioprine-induced liver injury, and the hepatic mRNA levels of immune- and inflammation-related factors were also significantly changed. In conclusion, this *in vivo* study revealed that oxidative stress and the subsequently activated immune- and inflammation-related factors are involved in azathioprine-induced liver injury [31]. An association between ROS plasma concentration and azathioprine induced hepatic adverse events could be verified even in patients with IBD, in order to establish ROS as a biomarker of azathioprine induced liver injury. However, the fact that on hepatocytes azathioprine is able to modulate a necrotic cell death through a cyclosporine-A sensitive MPTP opening, rises some doubts on the actual role of oxidative stress [19] and more studies are required to clarify if ROS could represent an alternative mechanism of cytotoxicity induced by azathioprine and its clinical relevance.

Modulation of Apoptosis

Azathioprine and mercaptopurine act as immunosuppressants by inducing apoptosis in activated lymphocytes. TGNs effects in terms of blockade of DNA replication do not fully explain this effect, and an additional role for azathioprine and its metabolites in inducing

apoptotic cell death by inhibition of intracellular signaling pathways has been shown. Tiede and co-workers found that successful azathioprine treatment in patients with IBD leads to an increased number of apoptotic T cells in *in vitro* stimulated peripheral blood mononuclear cells as compared with untreated patients. Furthermore, they observed that in patients affected by ulcerative colitis, mercaptopurine was capable of inducing apoptosis in activated lamina propria lymphocytes isolated from gut specimens, suggesting that the beneficial effect of this drug in IBD could be due to the induction of local T cell apoptosis in areas of intestinal inflammation [32]. The authors clarified the basis of the mechanism leading to the mitochondrial apoptotic pathway, demonstrating the specific binding of 6-thioguanosine triphosphate (6-ThioGTP) to the small GTP binding protein Rac1, with consequent suppression of survival pathways mediated by Rac1 target genes (e.g.: MEK, NF-κB, and the antiapoptotic bcl-xL) [32, 33].

In the last decades, evidence arose about the function of some GSTs as regulators of signal transduction pathways and thus as putative modulators of cell death processes [34-37]. GST-P1 was the first isoenzyme found to play such a role [38] and so far is the most important one. This function is achieved independently from the well-known GSH conjugating activity and occurs through a physical protein-protein interaction with c-Jun N-terminal Kinases (JNKs), a family of mitogen-activated protein kinases (MAPKs) that are activated by stressful and inflammatory stimuli and regulate cellular responses such as proliferation, differentiation, and apoptosis. Stress signals (UV, H_2O_2, ROS, heat or osmotic shock, inflammatory cytokines) are able to induce the GST-P1/JNK complex dissociation, and thus the accumulation of GST-P1 as oligomers in the cytosol and the activation of released JNK with phosphorylation of the transcription factors c-Jun. Therefore, by sequestering JNK, GST-P1 maintains the basal activity of JNK at a low level and acts as an endogenous negative regulator of downstream c-Jun mediated events directly contributing to the stress response through changes in the cell cycle, DNA repair, or apoptosis. Similarly, GST-A1 was shown to physically interact with JNK. The suppressive role of GST-A1 in the activation of JNK signaling by a proinflammatory cytokine and oxidative stress was demonstrated by Romero and coworkers in human epithelial colorectal adenocarcinoma cell line (Caco-2) with variable GST-A1 expression at different stages of confluence and in mouse 3T3 embryonic fibroblast-derived cell line with a tetracycline regulated transactivator to induce GST-A1 expression. As a further confirmation, authors investigated also butyrate-induced cell death, taken as an indicator of JNK activation, and found that the apoptotic response was reduced in Caco-2 cells with high GST-A1 expression [39]. In mouse liver tissue extracts, endogenous JNK was co-immunoprecipitated with GST-A4 isoform, suggesting that also GST-A4 might be an endogenous regulator of JNK activity by direct binding [40]. The structural homology and the overlapping specificity between GST-P and GST-A family members may explain the functional analogy in sequestering JNK.

Besides JNK, GST-P1 was reported to regulate also other cell signalling pathways, including the ERK1/ERK2 pathway [41] and TNF-alpha-triggered signalling [42]. Experiments performed by Wu and co-workers showed that GST-P1 physically associated with the tumor necrosis factor receptor-associated factor 2 (TRAF2) *in vivo* and *in vitro* with an interaction independent by its GSH-conjugating activity: overexpression of GST-P1 inhibited JNK and p38 (another MAPK) TRAF2-mediated activation and attenuated the TRAF2-enhanced autophosphorylation of apoptosis signal-regulating kinase 1 (ASK1), a MAPK kinase kinase (MAPKKK) that plays an important role in oxidative stress-induced

apoptosis and activates both JNKs and stress responsive p-38 kinase. By interfering in TRAF2 and ASK1 interaction, GST-P1 inhibited TRAF2-ASK1-induced cell apoptosis. Conversely, silencing of GST-P1 expression resulted in increase of TNF-alpha-dependent TRAF2-ASK1 association, followed by hyper-activation of ASK1 and JNK [42].

While JNK basal activity is directly controlled by GST-A1 and GST-P1, ASK-1 is physically sequestered by endogenous GST-M1 [43]. Stress triggers, such as heat shock or the pro-inflammatory cytokines TNF-alpha and interleukin-1β, promote the dissociation of GST-M1 from ASK1, resulting in oligomerization of GST-M1 and in the activation of released ASK-1 leading to the phosphorylation-dependent activation of downstream MAPK (JNK and p38) [44]. The final cell fate (proliferation or apoptosis) depends on the strength and duration of the oxidative stress. In rat hepatocytes, the expression of GST-M1, GST-M2, GST-A1 and GST-A2 has been reported to drop under apoptotic conditions and their overexpression was able to block apoptosis in cells overexpressing ASK-1 [45].

So far, modulation of apoptosis by GSTs has not been taken into account to explain azathioprine effects in IBD patients. However, studies on hepatocytes revealed that the drug's effects on cell viability were partially abrogated by JNK and p38 inhibitors, thus suggesting a correlation between azathioprine mechanism of action and the activation of stress-activated protein kinase pathways regulated by GSTs [27]. Even consumption of GSH catalyzed by GSTs could make cells of patients with IBD particularly sensitive to the cytotoxic effects of thiopurines, potentially leading to an increased incidence of adverse events. Matsumaru et al. reported that depletion of cytosolic GSH could sensitize murine hepatocytes to apoptosis induced by TNF-alpha [46, 47]. Interestingly, clinical studies have shown that TNF-alpha protein and mRNA levels are elevated in serum, intestinal tissue and stools of active IBD, in correlation with disease activity [48].

Enzymatic Conversion of Azathioprine to Active Thioguanine Nucleotides

Azathioprine conversion to mercaptopurine can occur spontaneously [1], however in the presence of specific GST classes and at physiological pH values, the reaction catalyzed by the enzyme may be prevalent.

Kaplowitz described an initial report on the enzymatic contribution on the conversion of azathioprine to mercaptopurine in rat liver homogenates. While at relatively high pH levels (i.e., 8.0) the non-enzymatic reaction and the enzymatic one occur in similar proportions, at lower pH levels (i.e., 6.5 to 7.4), closer to physiological values, the enzymatic reaction prevails [49]. The same reaction has been demonstrated in homogenates of human livers: in these samples, mainly from kidney transplant donors, conversion of azathioprine to mercaptopurine was inhibited by treatment with furosemide, a GST inhibitor [50]. Additional evidence obtained in animal models supports a significant contribution of the enzymatic conversion of azathioprine to mercaptopurine *in vivo*. Indeed, pretreatment of rats with probenecid, a GST inhibitor, determines a greater proportion of unmetabolized azathioprine in the liver and less hepatic GSH depletion. Bilirubin is also a GST inhibitor and, in a model of hyperbilirubinemic rat (Gunn rat), less hepatic GSH depletion was found during exposure to azathioprine [51]. These observations indicate that the conversion of azathioprine to mercaptopurine *in vivo* is mediated enzymatically by the GSTs. After oral administration of azathioprine this reaction likely occurs mostly in the liver: indeed after oral administration

azathioprine is undetectable in plasma, while mercaptopurine appears after either oral or i.v. azathioprine administration [3]. In addition it has been shown that after i.p. injection of azathioprine in rats, GSH was depleted rapidly in hepatocytes but not in other tissues (i.e., erythrocytes, kidneys and intestine) indicating that after administration of azathioprine, the hepatic contribution to total GSH consumption may be predominant [52].

Eklund et al. have shown that among 14 GSTs tested, GST-A1, GST-A2 and GST-M1 display the highest activity on the catalysis of azathioprine to mercaptopurine; these enzymes are all highly expressed in human hepatocytes and therefore in these cells the uncatalyzed reaction of azathioprine with GSH was estimated to be less than 1% of the GST-catalyzed biotransformation. Among the different human GSTs, GST-A2 has the highest catalytic activity toward azathioprine, with a specific activity of 0.53 μmol mg^{-1} min^{-1}, while GST-A1 and GST-M1 display a specific activity respectively of 0.24 and 0.17 μmol mg^{-1} min^{-1}. All other GSTs display much lower catalytic activity toward azathioprine.

Interestingly, GST-M1 is polymorphic, with a deletion of the gene present in about 50% of Caucasian, and GST-A1/GST-A2 also display genetically determined variable expression levels as already described in this chapter (Table 1). Genotyping of patients for GST-A2 aside from providing hints about its expression levels, allows estimation of specific activity of GST-A2 toward azathioprine. Indeed, steady-state kinetic parameters were determined and were similar for all except A2*E, which had three- to fourfold higher catalytic activity: therefore, variant A2*E catalyzes this reaction three to fourfold faster than the other variants of GST-A2 and about six-fold faster that GSTs A1 and M1 [53].

To account for the final level of total GST activity available to convert azathioprine to mercaptopurine, even differences in tissual expression between the different GSTs have to be considered, in particular at the level of the small intestine and liver, organs involved in azathioprine absorbance and first-pass metabolism after oral administration of the drug. Indeed, GST-A1 is expressed almost 5 times more in the small intestine and almost 2 times more in the liver than GST-A2 [14].

These differences in GST activity may result in interindividual differences in the conversion of azathioprine to mercaptopurine. Individuals with high levels of GST could be particularly sensitive to azathioprine and potentially more prone to adverse effects during treatment with azathioprine, because of both increased concentrations of free mercaptopurine and of a more pronounced GSH depletion [4].

Effects of GST Polymorphisms on Azathioprine Efficacy and Metabolism in Patients with IBD

The hypothesis that patients with reduced levels of specific GST isoforms, due to genetic polymorphisms, may present decreased sensitivity to azathioprine because of a reduced enzymatic conversion of azathioprine to mercaptopurine, was tested recently by our team in young patients with IBD. The deletion of GST-M1, GST-T1 and the coding non-synonymous SNP rs1695 in GST-P1 were evaluated [54, 55]. An initial study [55] considered a cohort of 70 young patients (median age 16.2 years, 36 females) with IBD (41 Crohn's disease, 29 ulcerative colitis). Among these, 15 patients developed adverse events during treatment with azathioprine: in particular, there were three cases of bone marrow suppression, three cases of

liver toxicity, seven cases of pancreatic toxicity, one case of neuropathy and one case of arthralgia; all these side effects resolved completely after the reduction or interruption of azathioprine administration: azathioprine was therefore considered the main determinant of the adverse effects. Interestingly, the candidate genetic association analysis in these patients revealed that frequency of GST-M1 deletion was significantly lower in patients that developed an adverse event in comparison to patients that tolerated azathioprine treatment with no adverse event (frequency of deletion respectively 26.7% vs 67.3%, p-value = 0.0072). Moreover, the incidence of mild lymphopenia (lymphocytes count under 1000/mm^3), that was considered a marker of efficacy during azathioprine treatment, resulted associated with GST-M1 genotype: indeed, among patients tolerating azathioprine treatment and with lymphopenia, frequency of the deletion was 28.6% in comparison to 72.9% among patients tolerant to azathioprine but that did not present this drug effect (p-value = 0.032) [55]. Taken together, these results are in agreement with a model in which patients with GST-M1 deletion are less sensitive to the effects of azathioprine, putatively because of the contribution of this enzyme on the conversion of azathioprine to mercaptopurine. In a recent study [54], we evaluated the effects of GST polymorphisms on azathioprine metabolism in a cohort of 75 young patients (median age 15.2 years, 36 females) with IBD (46 Crohn's disease, 29 ulcerative colitis) tolerant to azathioprine therapy (taking azathioprine for more than 3 months). Azathioprine metabolites were measured on samples collected from these patients using a high performance liquid chromatography assay [56]: 150 measurements of azathioprine metabolites were done; on average, 2 samples per patient were collected. Patients with the deletion of GST-M1 tolerated a dose of azathioprine significantly higher in comparison to patients with normal GST-M1 (mean dose of azathioprine 2.1 mg/kg/day vs 1.8 mg/kg/day, p-value = 0.022). Moreover, the amount of active TGNs generated in patients with the deletion of GST-M1 was significantly decreased in comparison to patients with a normal genotype (mean amount of TGNs metabolites concentration for mg/kg of azathioprine: 252 pmol/$8x10^8$ erythrocytes vs 164 pmol/$8x10^8$ erythrocytes, p-value = 0.0030). Multivariate analysis confirmed that this effect was independent from that of other genes with a significant effect, such as TPMT, the main gene known to influence mercaptopurine metabolism [54]. This study therefore supports a role of GST-M1 on azathioprine efficacy, mediated by an increased conversion of azathioprine to mercaptopurine. The reaction catalyzed by GST-M1 likely occurs after oral administration mainly in the intestine and the liver, modulating the amount of mercaptopurine and TGNs that are released in the main circulation.

These studies considered even the effect of GST-P1 and GST-T1 polymorphisms on azathioprine effects and metabolism but did not detect any significant association. The lack of association may be due to the tissue distribution of GST-P1 and GST-T1, which are not highly expressed in the liver, but even to the lack of specific activity of these enzymes toward the catalysis of the reaction of azathioprine with GSH [4]. Presently, there are only two additional clinical studies that considered GST-M1 genetic polymorphisms as a candidate involved in azathioprine activation [57, 58]. One report considered 51 Asian patients with systemic lupus erythematosus (SLE) and the effect of polymorphisms in the ITPA, TPMT, GST-M1 and GST-T1 genes on the response to a low dose of azathioprine (0.97 mg/kg/day). Response to therapy, evaluated as a change in disease activity index, was associated with ITPA genetic polymorphism but not with the other ones. A clear interpretation of this paper's results may be difficult because of the lack of data on azathioprine metabolites concentrations

in these patients. However, the lack of an effect of GST-M1 on azathioprine efficacy in these patients may be due to the very low dose of drug used. This study indicated that in Asian patients with SLE the effect of ITPA might be predominant on those of TPMT and GST-M1 when azathioprine is used at very low doses; indeed, even TPMT genetic polymorphism was not associated with azathioprine efficacy. It is known that in patients with Asian ancestry, the frequency of variant TPMT is very low, while that of variant ITPA is increased [59, 60].

Another recent study examined whether gene polymorphisms in GST-M1, GST-T1 and TPMT, combined with various clinical parameters, can predict thiopurine induced serious adverse events. A retrospective cohort of 176 Crohn's disease patients treated with thiopurines (131 with mercaptopurine and 45 with azathioprine) was genotyped for common polymorphisms in GST-M1, GST-T1 and TPMT. Clinical data including serious adverse events, age, ethnicity, gender and smoking status were extracted from patient charts. Adverse drug reactions evaluated were myelosuppression, hepatotoxicity and pancreatitis. Associations between demographic, clinical, and genetic variables and thiopurine induced serious adverse drug reactions were assessed. Twenty-four patients (14%) developed thiopurine-related adverse drug reactions, revealing a significant association between GST-M1-null genotype (P=0.05), older age (P=0.016) and active smoking status (P=0.043) and serious adverse events. In this study of thiopurine-treated adult Crohn’s disease patients, active smoking and GST-M1-null genotype appear to be risk factors for thiopurine induced serious adverse events [58]. While this study considered the association of GST polymorphisms on the incidence of adverse events during thiopurine therapy in Crohn’s disease, only 25% of patients were treated with azathioprine and the majority with mercaptopurine. On the basis of this study it is possible to speculate that in adult patients treated with thiopurines, particularly with mercaptopurine, GST-M1 polymorphism may modulate thiopurine sensitivity mostly through its role as an inhibitor of apoptosis, since patients with GST-M1 deletion presented more adverse events than patients with wild-type GST-M1. The interaction between smoking status, thiopurine induced adverse events and GST genotype should be further characterized by additional studies.

Conclusion

It would be important that other studies validate the observation of the increased conversion of azathioprine to mercaptopurine in patients with normal GST-M1, resulting in increased sensitivity to the medication in patients with IBD, both clinically and using *in vitro* experiments; in particular, the contribution of azathioprine induced oxidative stress should be evaluated *in vivo*, to test whether, in patients, the level of ROS production correlates with azathioprine effects, in particular liver toxicity.

Presence of polymorphisms of GST-A1 and GST-A2 has been shown to be of pharmacogenetic relevance for medications such as cisplatin and busulfan [61, 62], however no study has been reported on the effects of GST-A on azathioprine in IBD. Therefore, since GSTs of the A class are highly expressed in the liver, have catalytic activity on the conversion of azathioprine to mercaptopurine and display genetically determined polymorphic activity, it would be important to evaluate the role even of variation in genes for the A1 and A2 class of GST on azathioprine pharmacokinetics and efficacy.

Further insights on the role of genetic polymorphisms of GST and other enzymes on azathioprine pharmacogenetics could come from the use of innovative and more sensitive methods for the measurement of azathioprine metabolites. Indeed, most of the research published so far, including the papers mentioned in this report, have characterized thiopurine metabolites in erythrocytes from patients with IBD, using HPLC methods that group all thionucleotides as TGNs and methylated nucleotides [56, 63], without distinguishing the degree of phosphorylation, which may be of relevance for thiopurines' cellular effects [64]. Given the complexity of thiopurines' metabolism, methods with increased sensitivity, such as those based on mass spectrometry, allowing to assess the nucleotides' degree of phosphorylation and potentially the identification of additional relevant species, are of great interest [65]. Moreover, these methods with increased sensitivity should allow the use of very small volumes of patients' samples and this is particularly relevant for pediatric patients [66, 67].

The increasing complexity [68] of thiopurines' pharmacogenetics has been consolidating: while TPMT is the strongest determinant of variability in the pharmacokinetics of these medications [69] and is currently used in several clinical protocols to adjust treatment with thiopurines, even other genes, such as ITPA, have been shown to be of relevance [70]. Based on the clinical and *in vitro* evidence described in this chapter, it seems that for azathioprine GST-M1 genetic polymorphism could also enter in a useful multi-locus genotype to predict patients' response to this medication. However, the association of GST-M1 with azathioprine efficacy in patients with IBD still needs to be supported mechanistically by *in vitro* studies and validated by adequately sized prospective clinical trials.

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Chapter 4

Glucocorticoids Inhibit Programmed Cell Death Induced by Glutathione Depletion in Neuronal-Like PC 12 Cells

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Abstract

We described previously in PC 12 cells that inhibition of glutathione biosynthesis induced by high concentrations of glutamate or addition of buthionine sulfoximine led to a rapid oxidative shock followed by a slowly progressing apoptosis. On the other hand, the effects of glucocorticoids on brain cell survival or death remain a matter of controversy since both neuroprotective and neurotoxic actions have yet been described by different groups in various experimental models.

In the present paper we have thus studied the effect of dexamethasone, a potent synthetic glucocorticoid, on the toxicity induced by glutathione depletion in PC 12, a neuronal-like cell line.

We first observed that Dex was able to markedly decrease the deleterious effect of buthionine sulfoximine addition on cell survival but failed to protect PC 12 cells from an oxidative shock induced by either cumene hydroperoxide or 6-hydroxy-dopamine.

The characteristics of Dexamethasone action, i.e. time-course, dose-response curve and antagonism by mifepristone, strongly suggested that it represents a typical receptor-mediated event. We also demonstrated that PC 12 cells contained immunoreactive glucocorticoid receptors.

Dexamethasone failed however to alter intracellular glutathione content and did not significantly influence two of the major cellular defences against oxidative shock: glutathione peroxidase and superoxide dismutase.

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Although the precise mechanism of the protection provided by dexamethasone remains to be defined, we consider that our model may represent a useful tool to investigate the effects of glucocorticoids on brain cells survival.

1. Introduction

Glutathione (GSH) is the most important non-protein thiol found in mammalian cells where it is usually present at concentrations within the millimolar range. Due to the presence of its sulfhydryl group, GSH is extremely reactive and fullfill several important functions (Cooper and Kristal 1997, Forman et al. 2009, Luschak 2012).

In biological fluids, cysteine is fairly unstable and could easily be oxidized into cystine. GSH thus represents an useful storage form of cysteine inside the cells and continuously provides cysteine for the synthesis of cellular proteins.

It is also known for decades that GSH is a key player in the cellular defence against reactive oxygen species produced under both physiological and pathophysiological situations. As a substrate of glutathione peroxidases and periredoxins, GSH contributes to the reduction of hydrogen peroxide and organic peroxides produced during oxidative shocks (Brigelius-Flohe 1999).

Under the action of glutathione-S-transferase, GSH forms conjugates with a great variety of electrophiles thus enabling the excretion of toxic compounds via transporters such as the multidrug resistance proteins.

GSH may also participate to the regulation of nitric oxide homeostasis, to the modulation of the activity of proteins via post translational modifications, to the control of the activity of neurotransmitter receptors, particularly NMDA receptors and metabotropic glutamate receptors, and is also able to bind free metal ions such as copper and iron but also zinc (Gow et al. 2000, Maher and Schubert 2000, Poulsen et al. 2000, Thannikal and Fanburg 2000, Deng et al. 2004)

During oxidative stress, GSH is consumed and oxidized to give GSSG which could then react with a protein thiol to form a protein-mixed disulfide. Formation of such protein-mixed disulfides with enzymes or transcription factors but also redox modification of protein sulfhydryls by reactive oxygen and nitrogen species (S-thiolation, S-nitrosation) have been shown to play an important role in signal transduction and participate to the control of cell cycle and proliferation, in the modulation of the mitochondrial transition pore, cytochrome c release and apoptosis as well as in the control of redox sensitive gene expression (Sen 1998, Arrigo 1999, Biswas et al. 2006, Matés et al. 2008, Nakamura and Lipton 2011, Finkel 2012).

It is generally accepted for more than 30 years that oxidative stress participates to the onset and/or progression of situations leading to brain tissue dysfunctions and neuronal death. Oxidative stress likely contributes to age-related neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease but also represents a key event in neuronal degeneration associated with brain hypoxia and ischaemia reperfusion (Bains and Shaw 1997, Bossy-Wetzel et al. 2004, Lee et al. 2008, Niizuma et al. 2010). This assumption relies in part on numerous experimental results showing that oxidative stress is generally associated with alterations of glutathione metabolism and concentration (Jenner and Olanow 1998, Chi et al 2007, Martin and Teismann 2009)

Riederer and coworkers (1989) have for exemple described at autopsy, in patients with moderate to severe Parkinson's disease, a significant decrease in the GSH content of various brain areas. A similar glutathione depletion has also been observed along with oxidative stress and brain damage in various animal models of hypoxia and focal ischaemia such as bilateral carotid occlusion or four vessels occlusion. (Mizui 1992, Shivakumar et al. 1995, Namba et al 2001) Several groups have also described in cellular models that neuronal death induced by various oxidative agents is often preceded by or associated with marked glutathione depletion, whereas glutathione depletion triggered in neuronal cell lines by buthionine sulfoximine (BSO), a potent and selective inhibitor of GSH biosynthesis, leads to cell apoptosis or potentiates the deleterious action of oxidative agents (Andersen et al. 1996, Froissard et al. 1997, Sagara et al. 1998, Merad-Boudia et al. 1998). Conversely, compounds which improve glutathione biosynthesis or spare GSH consumption, such as N-acetylcysteine, N-acetylcysteine-amide, β-mercaptoethanol or dithiothreitol, often protect neuronal cells from oxidative agents, both in vivo or in vitro (Yan et al. 1995, Wang et al. 2007, Welin et al. 2009, Vimard et al. 2011. On the other hand, the effects of glucocorticoids on brain cell survival or death remain a matter of controversy since both neuroprotective and neurotoxic actions were described. Almost thirty years ago, Sapolsky (1985) demonstrated that a prolonged exposure to glucocorticoids led to hippocampal neuronal loss in male rats. Since then, several groups have determined that glucocorticoids may significantly increase the deleterious effects of various stimuli both in vivo and in vitro. These include hypoxia-ischaemia-induced brain damage, excitotoxicity of glutamate agonists, neuronal death triggered by reactive oxygen species, as well as neurotoxicity induced by β-amyloid or gp 120 (Flavin 1996, Goodman et al. 1996, Semba et al. 1996, Adachi et al. 1998, Brooke et al. 1998, Xiao et al 2010). More recent experiments have also shown that acute neonatal exposure to glucocorticoids induced a rapid and selective apoptosis of cerebellar neuronal progenitor cells in rat and led to long term motor and cognitive impairment (Noguchi et al. 2008).

Conversely, Sloviter et al. (1989) demonstrated long ago that ablation of adrenal glands induced a selective degeneration of granule hippocampal neurons in adult rats. This cellular loss could be prevented by administration of corticosterone, thus suggesting that physiological levels of glucocorticoids are required to sustain neuronal survival. Glucocorticoids have also been shown to exert protective actions against glutamate toxicity and ischaemia-induced neuronal degeneration (Unlap and Jope 1994, Macaya et al. 1998, Bertorelli et al. 1998).

We have previously described in a neuronal-like cellular model (PC 12) that treatment by high concentrations of glutamate (5-10 mM) or by buthionine sulfoximine induced a depletion of intracellular glutathione associated with an oxidative shock and led to a slowly progressing apoptosis process (Froissard and Duval 1994, Froissard et al. 1997). We also describe that cell death could be prevented by addition of thiol donors (N-acetylcysteine, β-mercaptoethanol, dithiotreitol...) able to restore intracellular GSH content (Le Foll and Duval 2001). We have thus decided to investigate in this cell model the effect of dexamethasone (Dex, a potent synthetic glucocorticoid) on BSO-induced cellular apoptosis.

Materials and Methods

Materials

Dulbecco's Eagle's minimal essential medium (DMEM), antibiotics solution, steroids and L-glutamine were obtained from Sigma Chemical Co (L'isle d'Abeau, France). Buthionine sulfoximine (BSO) was from Sigma Aldrich (St Quentin Fallavier, France). Alexa fluor 488 coupled to streptavidin was purchased from molecular Probes (Leiden, Nederland). Horse serum and fetal bovine serum were from Life Technologies (Cergy Pontoise, France). The primary antibody raised against glucocorticoid receptors (rabbit antiGR) and the secondary biotinylated antibody (goat anti-rabbit) were purchased from Santa Cruz Biotechnology (Ca, USA).

Cell Culture

We used a subclone of PC 12, able to grow on plastic without polylysine or collagen coating. The cells were grown routinely in DMEM supplemented with 10% heat-inactivated calf serum, 5% heat-inactivated horse serum, 1% of a solution containing 10µg/mL penicillin G, 10µg/mL streptomycin and 25µg/mL amphotericin B in 0.9% Nacl. Sera were pretreated by charcoal-dextran to eliminate steroids. Cells were subcultured twice a week by gentle scraping and dilution at a concentration of 8-10 x 10^3 cells/cm^2 in six well plates (Falcon, 1.5 mL/well). The effects of the drugs tested were generally assessed 48 h after seeding.

Determination of Cell Viability

The viability of the cells was monitored using the trypan blue exclusion procedure. In each sample the number of cells taking up the dye was counted under a light microscope, using a Malassez chamber, and expressed as a percentage of the whole cell population present in the culture well (i.e. total number of cells present in the culture supernatant plus cells adherent to the bottom of the well).

Immunocytochemistry

Cells were first fixed by incubation with a 4% paraformaldehyde solution and then extensively washed with phosphate buffered saline (PBS, pH 7.4). The primary antibody was used at a dilution of 1/100 and the corresponding secondary, biotinylated, antibody at a dilution of 1/1000. Revelation was made using streptavidin conjugated to alexa fluor 488. Negative controls were obtained in parallel incubations carried out in the absence of the primary antibody.

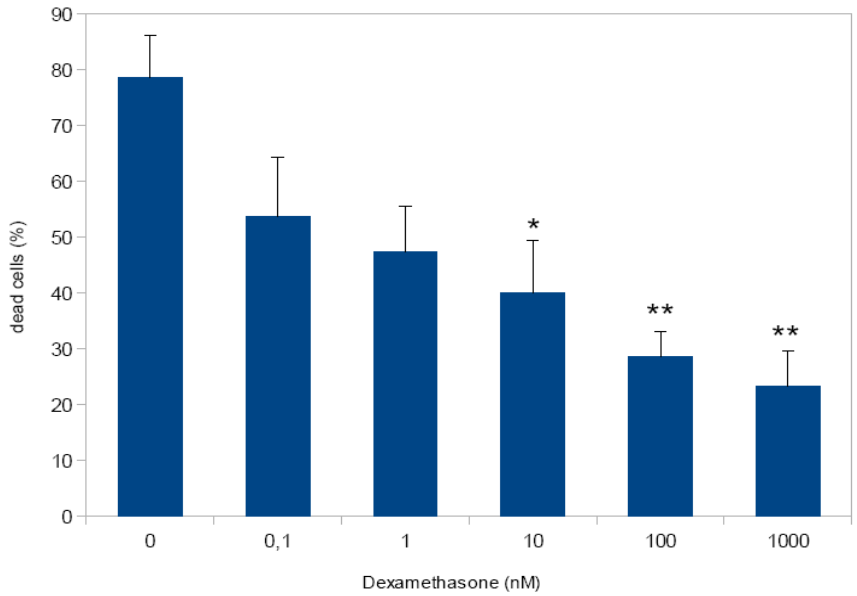

Figure 1. Dexamethasone significantly protects PC 12 cells from an oxidative shock induced by buthionine sulfoximine. Cells were preincubated for 24h in the presence of increasing dexamethasone concentrations (10^{-10} to 10^{-6} M) and then received buthionine sulfoximine (BSO, 300 µM). At the end of an additional 24h incubation, the proportion of dead cells in control (BSO, no dexamethasone) and dexamethasone treatment was determined by trypan blue dye exclusion. Each value, expressed as a percentage of dead cells in the whole population represents the mean (± s.d.) of 3-5 distinct experiments. * $p < 0.05$ vs control, ** $p < 0.01$ vs control.

Determination of Cellular Glutathione Content

The amount of intracellular glutathione [reduced glutathione (GSH) + oxidized glutathione (GSSG)] was measured using the method of Tietze (1969) as modified by Froissard et al. (1997). Cellular protein concentration was determined using the BCA protein assay kit (Pierce, Interchim, Montluçon, France) and bovine serum albumin as a standard.

Determination of Enzyme Activities

Superoxide dismutase (SOD) specific activity was assayed using the method described by Marklung and Marklung (1974), whereas glutathione peroxidase (GPx) activity was determined using the method described by Paglia and Valentine (1967), as modified by Chaudiere and Gerard (1988)

Statistical Analyses

Each of the experimental results presented in the figures represents the mean value (± s.d.) of at least 3 different determinations obtained in distinct experiments. Statistical significance was determined using the Student's t test.

Results

The cells were first preincubated for 24h in the presence of increasing concentrations of dexamethasone (10^{-10} to 10^{-6} M) and then treated by 300 µM BSO for an additional 24h period. At the end of the experiments, the percentage of dead cells in control (BSO alone) or Dex-treated samples was determined using the trypan blue dye exclusion procedure. As shown in Figure 1, BSO treatment led, as previously described, to a marked decrease in the viable cell population, since almost 80% of the whole cell population were stained by the dye at the end of the BSO treatment. In contrast, preincubation with Dex significantly reduced the proportion of BSO-induced cell death. This protective effect increased with increasing concentrations of Dex to reach a maximum at 10^{-6} M (Figure 1). Dex alone failed, at any concentration, to alter cell viability.

We have also tested in parallel the effect of Dex treatment on the deleterious actions of two different agents known to trigger oxidative shocks, 6-OH-dopamine and cumène hydroperoxide. As shown in Figure 2, 6-OH-dopamine (100 µM) and cumene hydroperoxide (100 µM) both induced a significant reduction in the viability of PC 12 cells over 24h and 2h incubation periods respectively, but preincubation in the presence of Dex (10^{-6} M) failed, in both cases, to reduce drug toxicity.

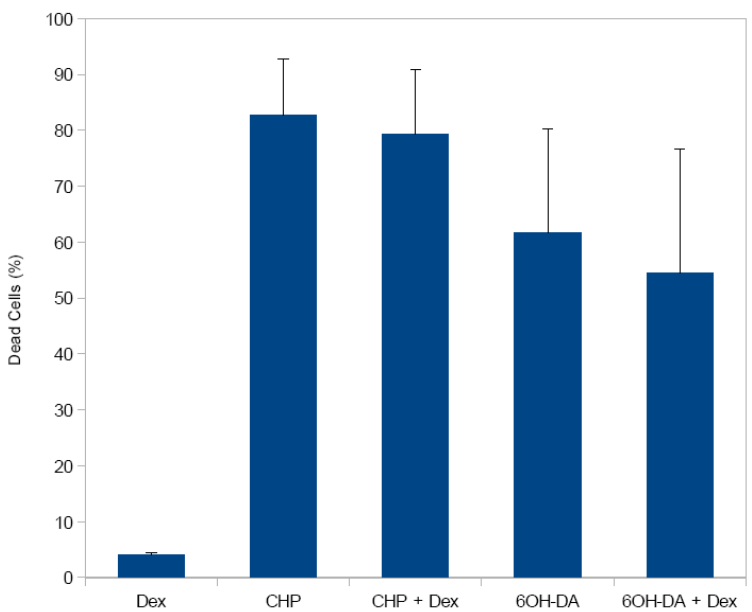

Figure 2. Dexamethasone (Dex) fails to protect PC 12 cells from oxidative shocks induced by cumene hydroperoxide (HPC, 2h, 100 µM) or 6-hydroxydopamine (6OH-DA, 24h, 100 µM). After a 24h incubation in the presence of 10^{-7} M dexamethasone, cells were treated by HPC or 6OH-DA. At the end of each experiment, the percentage of dead cells in Dex-treated samples was monitored by trypan blue dye exclusion and compared to that in samples incubated in the absence of Dex. Each value, expressed as a percentage of dead cells, represents the mean (± s.d.) of 3 distinct experiments. NS: not significant.

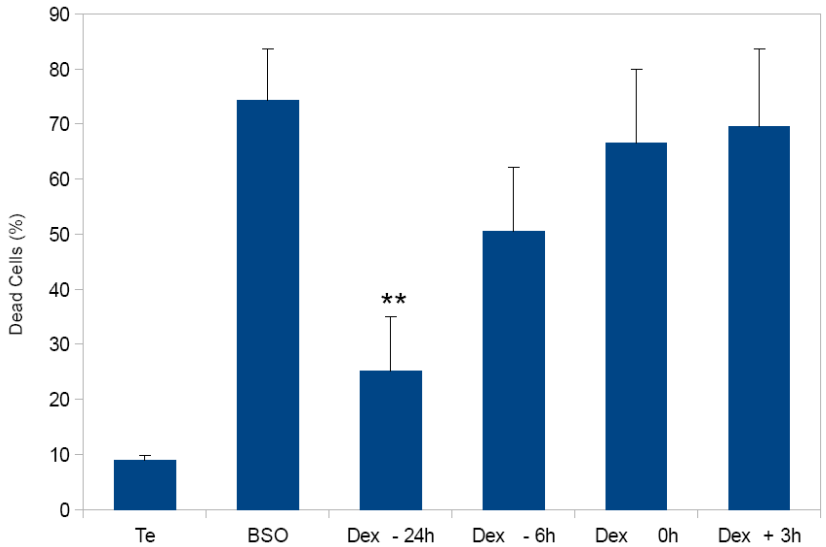

Figure 3. The protective action of Dex against BSO is only observed after a 24h incubation period. Cells were either preincubated for 6 and 24h in the presence of 10^{-7} M Dex or were treated by the steroid at the time or 3h after BSO addition. Twenty four hours after BSO addition (300 µM), the percentage of dead cells was determined in each sample by trypan blue dye exclusion. Te: untreated cells, BSO: treatment by BSO alone. Each value, expressed as a percentage of dead cells, represent the mean (± s.d.) of 3 distinct experiment. ** p <0.01 vs BSO-treated cells.

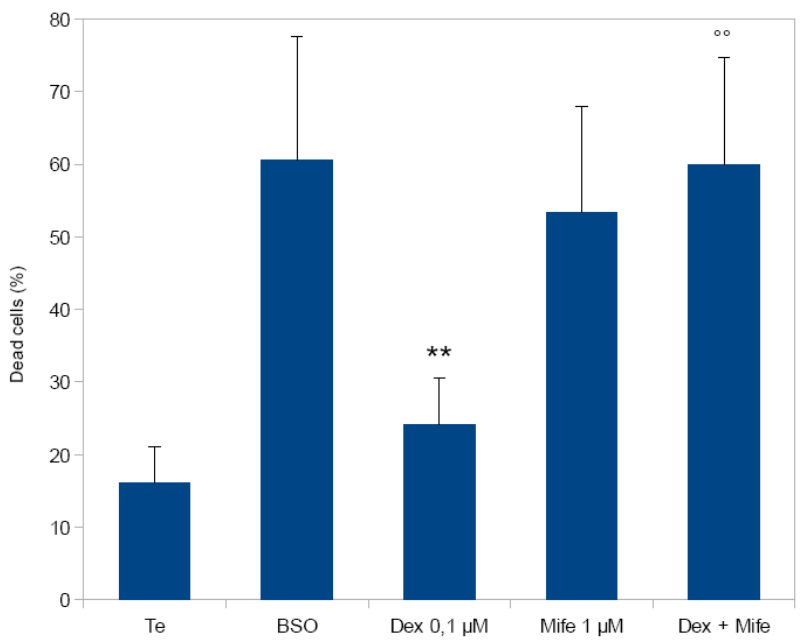

Figure 4. The neuroprotective action of Dex is abolished in the presence of mifepristone. The cells were preincubated either with Dex alone (10^{-7} M), mifepristone alone (1 µM), or Dex plus mifepristone , and then received BSO (300 µM) for an additional 24h incubation. At the end of the experiment, the proportion of dead cells was determined by trypan blue exclusion. Each value represents the mean (± s.d.) of 3 distinct experiments. ** p < 0.01 vs BSO treated cells, °° p < 0.01 vs Dex + BSO.

We have then determined the time-course of the action of Dex. The cells were pretreated with 10^{-7} M Dex for 6 and 24h or received the steroid at the time of BSO addition or 3h later. As shown in Figure 3, a significant steroid-induced protection was only observed when Dex was added 24h before BSO addition but not when addition was carried out 6h before or at the time of BSO addition (Figure 3).

To gain insight in the mechanism of action of dexamethasone, we determined whether its protective effect could be abolished in the presence of mifepristone, a potent competitive inhibitor of glucocorticoid receptors. As shown in Figure 4, mifepristone (10^{-6} M), which by itself did not protect cells from the toxic action of BSO nor alter the viability of control cells, completely abolished the protective action of dexamethasone.

We also tested by immunocytology the presence of glucocorticoid receptors (GR) in PC 12 cells. Figure 5 shows that fixed cells were indeed labeled by an antibody raised against GR. Fluorescence can be observed in most of the cells and appeared diffuse in the cells, mainly in the cytoplasm.

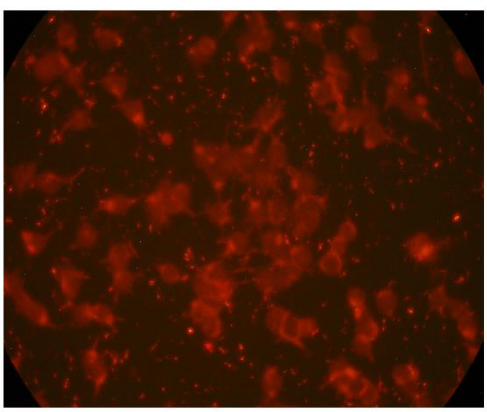

Figure 5. PC 12 cells express immunoreactive glucocorticoid receptors (GR).
Fixed cells were incubated overnight in the presence of a rabbit polyclonal antibody raised against rat GR (1:100)n and revelation carried out using a secondary antibody coupled to Alexa 488.
Magnification x 200.

Table 1. Dex treatment does not modulate SOD and GPx activities

	Control	**Dexamethasone**
SOD Units/mg protein	0.394 ± 0.076	0.411 ± 0,087 N.S.
GPx nmol/min/mg protein	0.477 ± 0.304	0.391 ± 0.266 N.S.

We have already described in PC 12 cells that BSO induces within 4-6 h a stricking depletion in the intracellular content of glutathion, a phenomenon which appears responsible and mandatory for its toxic action (Le Foll and Duval 2001). Inhibition of BSO-induced glutathione depletion in PC 12 cells would therefore be a potential explanation for the protective action of Dex. When measuring the effect of BSO on intracellular GSH content in

cells pretreated by Dex, we observed however that BSO-induced decrease in cellular GSH content remained unaltered in the presence of dexamethasone (results not shown).

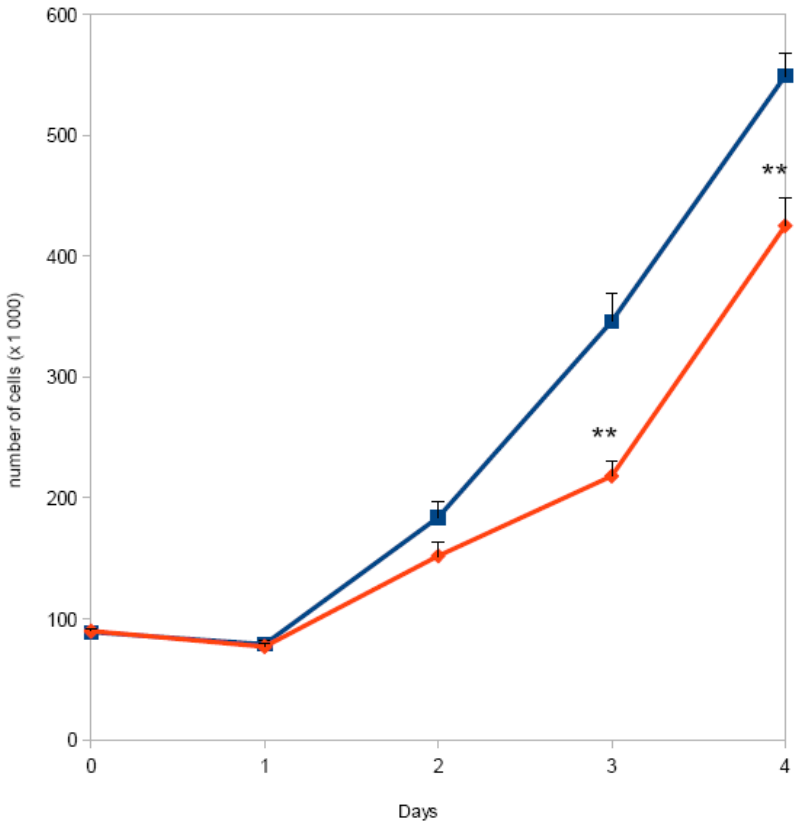

Figure 6. Dexamethasone slowed down PC 12 cells proliferation. Cells were seeded at a concentration of about 8x103 cells/ml in 35 mm Petri dishes and received Dex (final concentration 10^{-7} M) on the day of seeding. At daily intervals, the number of cells in control and Dex-treated samples was determined by scrapping off the adherent cells (viable) and counting them under a light microscope. Each value represents the mean number of cells/dish (± s.d.) in 3 distinct experiments. ** $p < 0.01$ vs control.

Several authors have previously suggested in other cellular models that glucocorticoids may enhance the activity of some enzymes involved in the cellular defence against oxidative damages such as superoxide dismutase (SOD) or gluthatione peroxidases (GPx). We have thus measured the effect of glucocorticoid treatment on the activities of these enzymes. As given in Table 1, Dex treatment only induced a slight and not significant decrease in GPx activity and no change in SOD activity.

We have also measured in parallel the growth rate of cultures incubated for 96h in the absence or presence of 10^{-7} M Dex. As shown in Figure 6, dexamethasone induced within 24h a progressive reduction of cell proliferation. This effect was significant over 48h, but Dex-treated cells then appeared to resume their proliferation at a rate similar to that of untreated controls.

Discussion

Our results demonstrate that glucocorticoids are indeed able to significantly reduce the toxicity induced by BSO treatment in PC 12 cells. This action appears rather selective for this mode of insult, since dexamethasone pre-treatment fails to protect the cells from necrosis triggered by either 6-OH-dopamine or cumene hydroperoxyde (Vimard et al. 1996).

Several of the characteristics of dexamethasone action strongly suggest that it is indeed mediated through a classical, receptor-mediated, regulation of gene transcription and protein synthesis. The dose-response curve first indicates that steroid action occurs at relatively low concentrations (1-10 nM), and increases with increasing drug concentration to reach a maximum at 0.1-1 µM, a value close to the concentration of corticosterone currently observed in rodent plasma during the circadian rythm and sufficient to achieve receptor saturation (Duval et al. 1979). We then showed that Dex only exerts its neuroprotective activity when incubated with the cells for more than 6 hours and not when the drug is added at the moment of BSO addition or 3 h later. We finally observed that PC 12 cells contain immunoreactive glucocorticoid receptors and that mifepristone, a well known competitive inhibitor of glucocorticoid receptors, completely abolishes the protective effect of dexamethasone. It was also shown earlier in the same model that glutamate-induced cell death can be abolished in the presence of known inhibitors of macromolecule synthesis, cycloheximide and actinomycin D (Serghini et al. 1994).

Depletion of intracellular glutathione plays a pivotal role in the apoptotic process induced by high concentrations of glutamate or BSO treatment and we have thus determined whether or not dexamethasone might interfere with GSH metabolism. Our results indicate that Dex pretreatment does not significantly enhance cell GSH content and also fails to block the decrease in GSH content induced by BSO, thus underlining the fact that glucosteroids do not interfere with the regulation of cellular glutathione metabolism or the inhibitory effect of BSO on γ-glutamylcysteine synthetase activity.

Given the reports suggesting that steroids may modulate several of the enzymes involved in the detoxification of reactive oxygen species (Mc Intosh et al. 1998, Zafir and Banu, 2009, Sato et al. 2010, Assaf et al. 2012) we have determined if Dex may alter either glutathione peroxidase or superoxide dismutase. We only observed a very slight but not significant decrease in GPx activity and no change in SOD activity. Taken together, these results suggest that glucocorticoids do not interfere primarily with the cellular defence against reactive oxygen species but more likely interfere downstream in the pathway(s) linking GSH depletion to apoptosis.

We have also tested in parallel the effect of Dex on other mediators possibly related to cell death and failed to observe any effect of the steroid on either NO synthesis or eicosanoid release (Results not shown).

On the other hand, we have recently described in the same experimental model that deferoxamine, a potent and selective iron chelator, is able to completely block cellular death, even when added 9-12h after BSO addition (Chouraqui et al. 2013), thus suggesting that alteration of iron metabolism could be involved in a late step of the apoptotic process. One possible explanation of the neuroprotective action of Dex would be the modulation of one/several factors governing metabollicaly available iron release. Such effects of glucocorticoids on iron regulatory proteins and iron accumulation have been demonstrated in

rat livers by He and coworkers (2011), who showed that glucocorticoids may promote iron accumulation by up regulating iron regulatory protein 1 and transferrin receptor 1 and down regulating ferritin expressions. Using human macrophages Vallelian et al. (2010) showed that glucocorticoids shift iron homeostasis toward an increased cellular export of heme-derived iron, in particular through an enhanced expression of ferroportin. Glezer and coworkers (2007) have studied the expression of numerous genes regulated by LPS treatment in the presence or absence of glucocorticoid receptor signaling (pretreatment by RU 486/mifepristone) in mouse brain. They clearly pointed out that a subset of the genes modulated by LPS, including ceruloplasmin, is clearly implicated in iron homeostasis and that the ability of glucocorticoids to control these genes is important for the mechanisms leading to protection or damage during inflammatory reaction in the central nervous system. So far, we have no precise informations concerning the effect of Dex on ceruloplasmin expression or on the expression of other iron regulatory proteins but Maines and coworkers (1996) have previously described that glucocorticoids can enhance the expression of heme-oxygenase 2 in rat brain, an enzyme which is also present in PC 12 cells (Leon et al. 2003). Clearly additional experiments are now required to determine more precisely the genes regulated by Dex in PC 12 cells and their role in BSO-induced apoptosis.

In conclusion, glucocorticoids exert a striking protective action on PC 12 cells challenged by an oxidative shock leading to glutathione depletion. This protective action appears mediated by the interaction of the drug with glucocorticoid receptors and likely involves regulation of gene transcription. Although the nature of the biochemical pathways triggered by glucocorticoids and leading to neuroprotection remains yet poorly defined, this model may provide an useful tool to investigate the role of glutathione in neuronal cells as well as its modulation by pharmacological agents.

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Chapter 5

Thiol Metabolic Changes Induced by Oxidative Stress and Possible Role of B-Vitamins Supplements in Esophageal Cancer Patients

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Abstract

Rationale: Reduction-oxidation reactions determine cell homeostasis and free-radicals productions are invariable components of the aerobic metabolism processes. The cells have an elaborate defense against free-radicals and the imbalance resulting in excessive accumulation of free-radicals, defined as oxidative stress which plays a key role in promotion of pathological processes including cancer. Hence physiological levels

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of free-radicals mediate crucial intracellular signaling pathways and are essential for cell survival whereas excess generates cell damage and death.

Thereby, "hormetic" responses to free-radicals are resulting from the constant ongoing battle between the production of oxidants and the antioxidants defenses. Among the oxidative stress-dependent compounds are the thiol-antioxidants having glutathione (GSH) as its major representative intracellularly. Alterations in GSH levels are associated with human diseases including cancer where it has "double-edge sword" actions by protecting non-tumor cells against oxidative stress and by removal and detoxification of carcinogens. However, at the other end of the scale GSH protects tumor cells from apoptosis by increasing the resistance to cancer chemotherapeutic agents. By its physiological importance GSH levels can be controlled endogenous and exogenously by changing its biosynthesis with nutrients such as amino acids and vitamins. Almost none has been found in the available literature about B vitamin related-GSH metabolism in esophageal cancer . Nutritional deficits in fresh fruits, vegetables and dietary fiber are commonly referred as associated with the presence of esophagus cancer (EC). Moreover heavy consumption of alcoholic beverages and tobacco might interfere with vitamins and dietary components with potential anti-carcinogenic effects. To our understanding the adequacy of B-vitamins would allow the full effects of the sulfur-containing antioxidative defenses.

Methods: Twenty-six patients with EC (58.4 ± 11.8 years) and a control group of 20 healthy subjects (27 ± 8.4 years) were assessed for nutritional and biochemical markers at baseline (Mo). The EC patients were distributed in two groups G1/G2 to be either supplemented with placebo or vitamins B_2, B_6, B_{12} and folate during 15 days (M1) followed by cross-over for the same period (M2). The results were statistically analyzed.

Results: The EC patients were predominantly males addict to smoke and alcoholism, diagnosed with squamous-cell carcinoma, stage IV. Their food intake was inappropriate, particularly energy resulting in 46% with Body Mass Index (BMI) <18 kg/m^2 (15.9 ± 1.7 kg/m^2) presenting a body-weight loss of 21.3 ± 13.4% during the last 6 months. However plasma albumin and glucose were similar to controls. No significant difference was also found for cholesterol, folate, and levels of Methionine (Met), Homocysteine (Hcy), Glutamic acid (Glu), and in the glutathione disulfide/glutathione ratio (GSSG/GSH). After the intervention it was observed an increasing of B_{12} vitamin and decreased levels of Hcy.

Conclusion: EC in its advanced stage has a different pattern of thiol pathways with the most preserved amino acids being methionine, homocysteine and glutamate. Met/Hcy (transmethylation/remethylation) cycle was maintained whereas Hcy/Cys (transsulfuration) was reduced therefore accumulating Hcy. However even in the presence of lower Cys it seems that there is an effort of the host to generate GSH by uptaking more GSH- precursors (Cys and Glu) from the GSH-gamma GT cycle for keeping controlled the GSSG/GSH ratio. Short-term B-vitamin supplementation led to increased plasma vitamin B12 which together with normal folate contributed effectively for reducing Hcy. By keeping controlled Hcy and GSH/GSSG the cell would tend to reduce the oxidative stress and probably the tumor progression, what could be attributed presently to the supplemented vitamins.

Keywords: Cell oxidation-reduction homeostasis, oxidative stress in cancer, sulfur antioxidant pathway in esophageal cancer, dietary risk factors for esophageal cancer, B-vitamin supplementation in esophageal cancer

Introduction

1. The Cell-Hormetic Responses to Oxidative Stress

Reduction-oxidation reactions determine cellular homeostasis. Free radicals are defined as molecules having an unpaired electron in the outer orbit of electron shell. Aerobic life is connected with continuous production of free-radicals which are invariable components of the aerobic metabolism in the processes of respiratory chain, autacoids biosynthesis, P450 cytochrome activity and respiratory burst of phagocytes. On the other hand, cells have an elaborate defense against (ROS/RNS) free-radicals composed by enzymatic and non-enzymatic mechanisms. Thus, in every cell, there is an ongoing battle between the production of various oxidants and the anti-oxidant defenses. Oxidant-antioxidant imbalance resulting in excessive accumulation of ROS is defined as oxidative stress which is consequent to either an excessive production of ROS or a deficient antioxidant system.

Physiological levels of ROS mediate crucial intracellular signaling pathways and are essential for cell survival. However, an excess of ROS generates cell damage and death [1]. To prevent the irreversible cell damage, the increase of ROS induces an adaptive response, consisting in a compensatory up regulation of antioxidant systems aimed to restore the redox homeostasis [2].

Many types of cancer cell have increased levels of free radicals compared with their normal counter parts [3] and oxidative stress is considered to play a key role in promotion of various pathological processes including cancer [4].

DNA accumulates oxidative damage induced by ROS generated by endogenous and exogenous sources. This damage is a major contributor to diseases such as cancer, cataracts, heart diseases and brain dysfunction. Numerous factors contribute to the initiation and development of cancer, including genetic, environmental and dietary influences. Studies investigating the contribution of genetic polymorphisms to the development of cancer have looked at many genes including oncogenes, tumor suppressor genes, DNA repair genes and genes encoding phase I and phase II enzymes [5]. It is widely accepted that reactive species (RS) produce a broad range of DNA damage including base and sugar modifications, base-free sites, DNA-protein crosslinks, and strand breaks [6].

The most prevalent damage to purines is 7,8-dehydro-8-oxoguanosine (8-oxoG), while the most common damage to pyrimidines is the formation of thymine glycol (Tg). Modified bases generated by RS are highly mutagenic and can induce base-mispairing during DNA replication, generating mutations that affect cellular physiology [7]. It is estimated that the number of oxidative hits to DNA per cell per day is around 10,000 in the humans. It is possible that oxidative lesions in mammalian DNA exceeds 100 different types, of which 8-hydroxyguanine (8-oxoG) is one of the most abundant [8].

In normal functional cells, DNA repair enzymes efficiently remove most of the lesions formed by ROS. However increased ROS generation in cancer cells leads to the accumulation of oxidative products of DNA, proteins, and lipids in tissues, and their release into the blood and urine. DNA oxidative products (8-oxoG), and lipid peroxidation have been detected in many cancer tissues [9].

Cellular damage by oxidation of macromolecules such as DNA, proteins or membrane lipids is an important process during early carcinogenesis [4, 10, 11]. ROS and RNS are

important agents of DNA damage. They are involved in both initiation and promotion of multi step carcinogenesis [4].

Besides ROS also the generation of halogens (RHS) contribute to tumor development, this time by inducing synthesis of pro- inflammatory molecules by macrophages. Hypochlorous acid (HOCl) is a strong oxidant and cytotoxic agent. HOCl can halogenate/oxidize pyrimidine and purine bases of DNA. HOCl is found in phagocytes cells as the final product of the reaction between H_2O_2 and chloride ion that is catalized by myeloperoxidase (MPO). High levels of HOCl production are associated with chronic inflammation. Hence inflammatory-mediated chlorination of pyrimidine (cytosine) residues in DNA may account for several DNA alterations observed in human tumors [12]. A significant imbalance between ROS/RNS production and antioxidant defense can explain all findings associating with tumor growth and a state of high oxidative stress [13]. The magnitude of the damage is therefore dependent on the body's defense mechanisms against free radicals. The metabolism of ROS in cancer cells is drastically altered favoring at least two mechanisms; cancer cells produce large amounts of ROS compared to non-neoplasic cells and suppression of antioxidant system in cancer cells.

Cancer develops from a clonal proliferation of altered cells at the sight of local tissue injury, inflammation, and genomic instability. Modulation of gene expression by oxidative damage affects carcinogenesis by altering the epigenetic effects and chromosomal rearrangements. Epigenetic effects on gene expression stimulate growth signals and proliferation, while chromosomal rearrangements contribute to neoplasic progression [14].

The main cause of cancer related deaths is metastasis of the primary tumor. The cascade involves detachment of cells from the original tumor, invasion through the basement membrane, intravasation into the blood stream and extravasation from the blood at distant site. Therefore successful metastasis requires remodeling of the extracellular matrix changes in expression and localization of key tumor-related and cytoskeletal proteins, proteases and growth factors. There are findings highlighting the roles of intracellular and extracellular redox state in the induction and maintenance of oxidative stress associated with cancer and metastasis via activation of survival pathways, disruption of cells death signaling and increase in cell proliferation [15].

Studies with primary cancer tissues revealed increased levesl of ROS-scavenging enzymes and antioxidant compounds [16] which could be a result of an adaptive response to intrinsic ROS stress [8].

Thus there are two facets of free-radicals in biology, they serve as signaling and regulatory molecules at physiologic levels but as highly deleterious and cytotoxic oxidants at pathologic levels [17-19]. This behavior fits pro oxidative/antioxidant mechanism as an hormesis phenomenon.

In toxicology hormesis is used to refer to a biphasic dose response to an environmental agent characterized by a low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect. In the fields of biology and medicine, however, hormesis is defined as an adaptive response of cells and organisms to moderate (usually intermittent) stress. The cellular signaling pathways and molecular mechanisms that mediate hormetic responses typically involve enzymes such as kinases and deacetylases, and transcription factors such as Nrf-2 and NF-kB. As a result cells increase their production of cytoprotective and restorative proteins including growth factors, phase 2 and antioxidant enzymes and protein chaperones [20].

A better understanding of hormesis mechanisms at the cellular and molecular levels is leading to and to novel approaches for prevention and treatment of many different diseases [20].

Oxidative stress has long been implicated in cancer development and progression suggesting that antioxidant treatment may provide protection from cancer [21, 22]. On the other hand, pro oxidant therapies, including ionizing radiation and chemotherapeutic agents, are based on the rationale that a further oxidative stimulus added to the constitutive oxidative stress in tumor cells should, in fact, cause the collapse of the antioxidant systems, leading to cell death [23].

2. The Role of Antioxidant Defenses

Upon exposure of cells to oxidative stress, signaling pathways such as protein kinase C, phosphatidylinositol-3 kinase, and MAP kinase, phosphorylate the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2). After phosphorylation Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE) within the promoters of genes encoding antioxidant enzymes and detoxifying enzymes. Key Nrf2 target genes include glutathione peroxidases (GPx), glutathione S-transferase (GST), superoxide dismutase (SOD), cytochrome P450, NAD(P)H quinone oxidoreductase and heme oxygenase(HO).

Superoxide dismutase (SOD) alters toxic superoxide radicals to H_2O_2. Catalase (CAT) converts H_2O_2 to molecular oxygen and water. Glutathione peroxidase (GPx) also catalyses the convertion of H_2O_2 to water. There are also non-enzymatic radical scavengers like vitamins, different thiols, ferritins and others located intra and extracellularly [24]. Depending on the order of their corresponding redox potentials, it is common for one antioxidant to regenerate another one from its oxidized species. Studies showed that SOD enzyme activity increases when the effectiveness of other enzymes decrease. The induction of SOD in turn leads to the protection of GPx activity and glutathione (GSH) has a mild sparing effect on vitamins C and E through its role as a reducing agent [4].

2.1. Sulfur-Containing Antioxidants

Among the enzymatic systems involved in the maintenance of the intracellular redox balance, a main role is played by glutathione [7]. Glutathione (L-gamma-glutamyl-L-Cysteinylglycine) is the principal tripeptide thiol involved in the antioxidant cellular defense [5] and a major hydro-soluble component of the cellular antioxidant system. GSH plays important roles in nutrient metabolism and regulation of cellular processes, including cell differentiation, proliferation and apoptosis [8]. Due to different roles of ROS in cell signaling and many human pathological processes, imbalance of GSH is observed in a wide range of pathologies including cancer, neurodegenerative disorders, cystic fibrosis, HIV, and aging [5].

The term thiol refers to compounds containing sulfur. Sulfur-containing compounds are found in all body cells and are indispensable for life. Some of sulfur-containing antioxidant compounds are cysteine (Cys), methionine (Met), taurine (Tau), glutathione (GSH), lipoic acid and mercaptopropionyl glycine. Sulfur, as a part of sulfydryl groups, forms thioester linkages that are necessary for the activation of molecules such as acetate. Sulfur atoms are also important in the iron-containing flavoenzymes, such as, succinate dehydrogenase and

NADH dehydrogenase. In addition, sulfur atoms in Cys are responsible for the major covalent cross-links in protein structures, by the formation of disulfide bridges between two Cys molecules, which are important in stabilizing protein conformation.

The thiol-antioxidative activities follow a general trend, the more highly reduced forms are stronger antioxidants. Dihydrolipoic acid is the most effective antioxidant. It contains two sulfhydryl groups and can undergo further oxidation reaction to form lipoic acid.

Among plasma thiols, total Cys is the most abundant, followed by the Hcy and GSH. These thiols are in a dynamic relationship through thiol-dissulfide exchanges and redox-reactions. The albumin cysteine-34 SH group is believed to be important for protection against oxidative stress. The antioxidant role of albumin in plasma is fortified by its Cys-34 residue, which can directly participate in radical scavenging.

Tau, a non-protein sulfur amino acid, is the most abundant free amino acid in the body. Tau is present in high concentrations in most tissues, particularly in pro-inflammatory cells, such as polymorphonuclear phagocytes and in retina. Tau scavenges hypochlorous acid and therefore partially scavenges ROS and prevents changes in membrane permeability following oxidant injury.

2.2. Glutathione Functions

GSH is an abundant natural tripeptide found within almost all cells. It is highly reactive and instills several vital roles within a cell including antioxidation, maintenance of the redox state, modulation of the immune response and detoxification of xenobiotics [5].

In mammalian cells, GSH reacts widely to form a number of different metabolites. These reactions can be divided into those involved with the sulfhydryl moiety or with the gamma-glutamyl portion of the tripeptide. In the former are included the oxidation-reduction reactions and the nucleophilic reactions in which the reduced sulfhydryl reacts with electrophiles to form a thioester [25].

In its antioxidant performance, oxidation of the reduced form of glutathione (GSH) to form GSSG is carried out either by direct interaction with free radicals or, more often, when GSH acts as a cofactor for antioxidant enzymes such as GSH-peroxidases. Hence cytosolic GSH peroxidase reacts in peroxisomes with the hydrogen peroxide produced during the aerobic metabolism. In this reaction GSH is oxidized to GSSG. In order to prevent oxidative damage, the GSSG is reduced to GSH by (riboflavin-dependent) GSSG-reductase at the expense of NADPH (generated by pentose-shunt pathway), forming a redox cycle.

In extreme conditions of oxidative stress, the ability of the cell to reduce GSSG to GSH may be less, inducing the accumulation of GSSG within the cytosol. In order to avoid a shift in the redox equilibrium, the GSSG can be actively transported out of the cell or react with protein sulfydryl groups and form mixed disulfides [1].

GSH is found free or bound to proteins. Free form is present mainly in its reduced form (GSH) which can be converted to the oxidized form (GSSG) during oxidative stress.

In normal conditions the GSH predominates over the GSSG form with the ratio GSH/GSSG exceeding 100 in a normal resting cell. The ratio decreases to values between 10 and one in oxidative stress [8]. Intracellularly, the majority of GSH is found in the cytosol (90%), while mitochondria contains nearly 10% and the endoplasmic reticulum contains a very small percentage [26].

An extension function of GSH is the maintenance of the intracellular redox balance and the essential thiol status of proteins. In the reaction the oxidized protein (protein-SSG) is

reduced (protein-SH) and the reduced glutathione (GSH) is oxidized (GSSG). The equilibrium of this reaction depends on the concentrations of GSH and GSSG [27].

The reversible thiolation of proteins is known to regulate several metabolic processes including enzyme activity, signal transduction and gene expression through redox-sensitive nuclear transcription factors such as AP-1, NF-kB and p53 protein [28]. In fact DNA-binding activity of transcription factors often involves critical Cys residues, and the maintenance of these residues in a reduced for, at least in the nuclear compartment, is necessary. AP-1 is a transcription factor whose DNA-binding activity can be diminished if Cys-252 is oxidized similarly p53 which contains 12Cys residues in its amino acid sequence, and oxidation of some of these inhibits p53 function [1].

The molecular mechanism of how GSH modulates cell proliferation remains largely speculative. A key mechanism for GSH's role in DNA synthesis relates to the maintenance of reduced glutaredoxin or thioredoxin, which is required for the activity of ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis [1].

Storage of Cys is another important function of GSH because Cys is extremely unstable extracellularly and rapidly auto oxidizes to Cystine in a process that produces potentially toxic oxygen-free radicals [1].

The gamma glutamyl cycle allows GSH to be the main source of Cys. In this cycle, GSH is released from the cell and the ectoenzyme gamma-glutamyl transferase(yGT) transfers the y-glutamyl moiety of GSH to an amino acid (the best acceptor being Cys) , forming y-glutamyl-amino acid and cysteinyl-glycine [25]. Cysteinyl-glycine is broken down by dipeptidase to generate Cys and Gly. Once inside the cell, the majority of Cys is incorporated into GSH, some being incorporated into protein, and some degraded into sulfate and Tau. The y-glutamyl-amino acid (Gln) can be transported back into the cell and once inside can be converted to Glu and used for GSH synthesis [1, 29].

Plasma GSH arises largely from the liver. The content of GSH in mammalian cells is dynamically maintained by the gamma-glutamyl cycle, using GSH as a substrate for transpeptidases. Tissues that present low transpeptidase activity (e.g. liver, pancreas and muscle) export GSG through the blood to cells that have high transpeptidase activity such as kidney [30].

However the major function of GSH probably is the detoxification of xenobiotics and some endogenous compounds. These substances are electrophiles and form conjugates with GSH in reactions catalyzed by GSG-S-transferases(GST). The conjugates formed are usually excreted in the bile, but can also undergo modification to mercapturic acid [1].

GSH deficiency contributes to oxidative stress, which plays a key role in pathogenesis of many diseases, one of which is cancer [8].

GSH is able to detoxify liver, intestinal tract, lungs and RBCs. It also removes a wide range of toxins, including those produced by heavy metals, cigarette smoke, alcohol, radiation and cancer chemotherapics. Hence just as low intracellular GSH levels decrease cellular antioxidant capacity, elevated GSH levels generally increase antioxidant capacity and resistance to oxidative stress in a phenomenon known as multidrug and radiation resistant cancer [8]. The increase in GSH is a major contributing factor to drug resistance by binding to or reacting with drugs, interacting with ROS, preventing damage to proteins or DNA, or by participating in DNA repair processes [1].

GSH is involved in a variety of cell functions such as DNA repair, cell cycle, regulation of cell signaling and transcription factors. Hence GSH can modulate the genes of cell

proliferation, differentiation and apoptosis. A higher level of GSH is important for normal cellular functions, signal transduction and protection against certain carcinogens. When intracellular GSH levels are low the cells are more vulnerable to ROS attacks. Increased ROS might activate different intracellular oncogenic pathways or mutate a tumor suppressor gene pathway, which will activate a tumorigenesis process [31].

In many normal and malignant cells, increased GSH level is associated with a proliferative response and is essential for cell cycle progression. From the redox-sensitive nuclear transcription factors AP-1 is related to tumor progression and the tumor suppressor p53 is known as "guardian of the genome" [1].

Because the increase of ROS in cancer cells may be part of the initiation and progression of cancer, such intrinsic oxidative stress is often viewed as an adverse event. However, as excessive levels of ROS stress can also be toxic to the cancer cells, further ROS induced by exogenous drugs will be effective as cancer treatments. Otherwise high cancer-cell GSH levels can slow down any effective cancer treatment that works by increasing intracellular ROS. Cancer cell lines containing low GSH levels have been demonstrated to be much more sensitive than control cells to the effect of irradiation. Therefore changing ROS levels by GSH modulation is a way to selectively kill cancer cells without causing significant toxicity to normal cells [32].

Therefore GSH metabolism is able to play both protective and pathogenic holes. It is crucial in the removal and detoxification of carcinogens, and alterations in this pathway, can have a profound effect on cell survival. It has be seen that a breakdown in detoxification mechanisms often correlates with a higher risk of cancer. At the other end of the scale, however, increased levels of GSH are often associated with an increased resistance to cancer chemotherapeutic drugs via GSH conjugation and detoxification [5].

In liver cancer and metastatic melanoma cells, GSH status is correlated with growth and has a direct correlation with metastatic activity [33]. Therefore maintenance of high intracellular levels of GSH could be critical for the extravascular survival and growth of metastatic cells. Mitochondrial dysfunction is a common event in the mechanism leading to cell death [34]. Thus the impairment of GSH uptake by the mitochondria may be important to sensitize invasive cancer cells to prooxidant compounds capable of activating the cell death mechanism [1].

GSH is effluxed by cells through γGT-mediated metabolism, allowing a "GSH cycle" to take place, which is implicated in tumor development [35]. Increased levels of γGT have been observed in several cancers. Elevated γGT activity has been found accompanying increased invasive growth of melanoma cells [36] and γGT expression was related with unfavorable prognostic signs in human breast cancer. Thus the modulatory effects of γGT-mediated prooxidant reactions could contribute to the resistance phenotype of γGT-expressing cancer cells, by regulating both signal transduction pathways involved in proliferation/apoptosis balance, as well as by inducing protective adaptations in the pool of intracellular antioxidants [1].

In melanoma cells, GSH depletion and γGT inhibition significantly increased cytotoxicity via oxidative stress [37]. In addition γGT-overexpressing cells were more resistant to hydrogen peroxide and chemotherapics, such as doxorubicin, cisplatin, and 5-fluorouracil [1].

2.3. Glutathione Modulation

Alterations in GSH levels are associated with numerous human disease, including cancer. Considering the homeostatic redox buffer function of GSH and its role in inactivating some carcinogens and protecting cells against DNA-damaging free radicals and lipid peroxidation, it is plausible that tumor cells may need more GSH for their survival than other cell types [12]. Tumors that are multidrug and radiation resistant are found to have high GSH content. Under metastatic conditions, high levels of GSH can support a rapid cell cycle, an elevated rate of DNA synthesis and a block in cell apoptosis [38]. Thus, GSH can be characterized as a "double edge sword", protecting non-tumor cells against oxidative stress induced by metabolism (e.g. Hcy) or exogenous compounds and at the same time, protecting tumor cells from apoptosis and chemotherapeutic treatments, though furthering tumor development and metastasis [12].

Cell GSH levels can be controlled either by its synthesis or its removal. GSH biosynthesis can be controlled either by their enzymes or by its constituent amino acids.

GS(SG)H is effluxed by cells through yGT-mediated metabolism, allowing a "GSH-cycle" to take place, which is implicated in tumor development, suggesting the hypothesis of yGT as an early marker of neoplastic transformation [39]. The implications of yGT activity in the resistance phenotype of cancer cells suggest a potential use of yGT inhibitors associated with chemotherapeutics in order to deplete intracellular GSH and/or inhibit extracellular drug detoxification [40].

GSH is synthesized in the cell by the sequential actions of y-glutamylcysteine synthase (GCS) and GSH synthase (GS) in a series of six-enzyme-catalysed reactions [5]. Elevated GSH levels are observed in various types of tumors, and this makes the neoplastic tissues more resistant to chemotherapy. The content of GSH in some tumor cells is typically associated with higher levels of GSH-related enzymes, such as GCL, and yGT activities, as well as a higher expression of GSH-transporting export pumps [38]. Therefore it has been some attraction for depleting GSH by a specific inhibition of GCL, as a medical intervention against cancer progression and chemoresistance [1]. Recently, GSH analogues have been employed in order to sensitize tumors to cytotoxic effects of anticancer agents, by depleting GSH-related cytoprotective effects and some of them aims to induce GSH depletion acting at the transcriptional level (Nrf2-ARE) of genes encoding GCL and GST [41, 42].

However, under normal physiological conditions, the rate of GSH synthesis is largely determined by GCL activity and Cys availability. Cys is normally derived from diet, protein breakdown and in liver, from Met via trans sulfuration (conversion of Hcy to Cys) [26].

Sulfur containing amino acids play a role in determining the flux of Cys between Cys catabolism and GSH synthesis. In stressed and inflammatory states, sulfur amino acids metabolism adapts to meet the increased requirements for Cys as a rate-limiting substrate for GSH. Therefore Cys is now widely recognized as a conditionally essential (or indispensible) sulfur-amino acid. It plays a key role in the metabolic pathways involving Met, Tau and GSH (figure 1), and may help fight chronic inflammation by boosting antioxidant status [43].

In vivo studies have shown that when healthy adults are fed with diets, either deficient in sulfur amino acids or containing reduced amounts of total protein, GSH turnover is suppressed. Moreover the flux of non-essential amino acids, such as glutamate, cysteine and glycine) consists of its release from protein breakdown and from de novo synthesis. Thus seems that GSH deficiency is in large part to decreased synthesis secondary to a decreased supply of the precursor amino acids [44, 45].

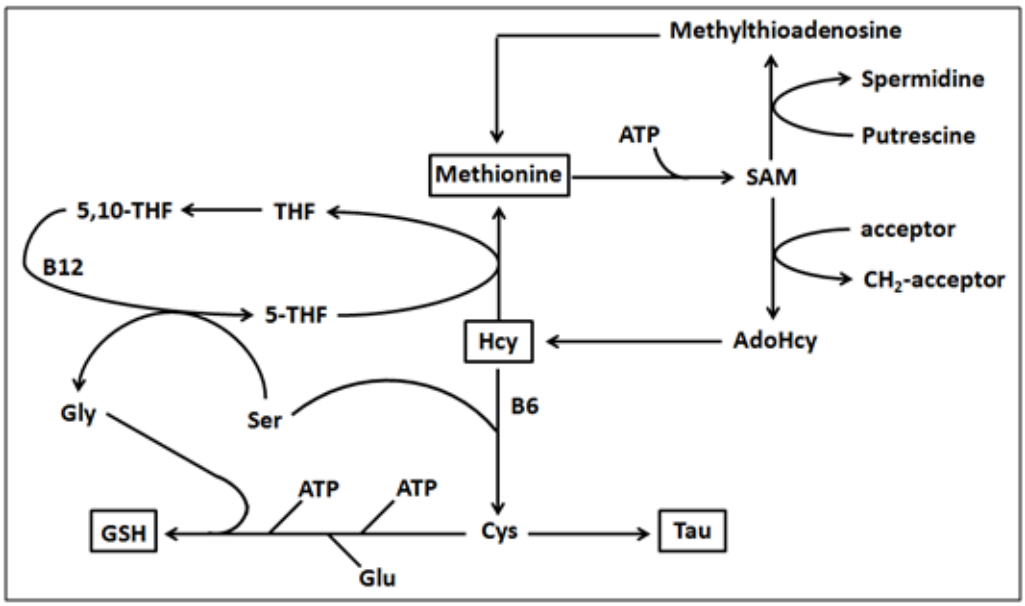

Figure 1. The methionine cycle and derived synthesis of homocysteine, glutathione and taurine.

Unlike humans, plants can use inorganic sulfur to synthesize sulfur-containing amino acids. Therefore, plants are an important source of sulfur for humans. Fruits and vegetables contribute over 50% of dietary GSH, while meats contribute less than 25%. Improving cysteine availability is the most extensively studied approach for enhancing the cell GSH pool. Among the agents tested are N-acetylcysteine, lipoic acid, cysteamine and 2-oxothiazolidine 4-carboxylate. Oral L-glutamine also increases plasma GSH in healthy subjects and patients [30, 46-48].

Cys can be endogenously synthetized from the Met-transmethylation/trans sulfuration - pathway. In fact the GSH synthesis pathway is one of the five major biochemical pathways of the methionine metabolism (figure 1). GSH synthesis is preceded by transmethylation/remethylation pathway (through folate/B12 dependent reactions) and trans-sulfuration pathway (through vitamin B6 dependent reactions). Hcy is formed from transmethylation of Met to S-adenosyl-methionine (SAM) and Hcy is mostly remethylated to Met and minorly trans-sulfurated to Cys which can be further incorporated to protein or GSH or metabolized to Tau (figure 1).

The Met cycle is responsible for synthesis of SAM, the major biological methyl donor for molecules such as DNA (trans-methylation pathway). The Met cycle is also responsible for producing Hcy, for converting 5MTHF into THF which is used in the remethylation reaction of Hcy to Met [49]. The trans-sulfuration pathway connects the Met metabolic pathways to the generation of Cys, GSH and Tau. The first step of trans-sulfuration involves the formation of cystathionine from Hcy and Ser. Once formed cystathionine is cleaved releasing free Cys [12] (figure 1).

When Hcy levels are low, Cys flux through the trans-sulfuration pathway becomes down regulated in order to conserve Hcy for the Met cycle. In the presence of high Cys levels cystathionine is directed into the GSH and Tau synthesis pathways. Thus, Cys levels are considered the limited step in liver GSH synthesis (major source of plasma GSH).

Approximately half of the Cys used for GSH anabolism is derived from Met that was synthesized from the trans-sulfuration pathway. On the other hand, half of the circulating Cys is derived from GSH breakdown.

Hcy can enter the trans-sulfuration pathway (10%) and be metabolized into cystathionine or be re-methylated (90%) into Met (figure 1). The existence of multiple routes for Hcy metabolism is consistent with the requirement for efficient product removal to prevent its accumulation in the cell [12].

The trans-sulfuration pathway is sensitive to pro- and antioxidants, which enhance or diminish Hcy flux [50]. On the other hand, trans-sulfuration pathway provide the amount of Cys required to synthesize the cellular redox-controlling molecules like GSH and Tau, that protect the molecular constituents of cells against RS-induced damage.

Alterations in intracellular redox homeostasis increase the rate of base mispairing due to base oxidation, alterations in gene expression and chronic inflammatory processes thus inducing tumor progression [12]. The increase in oxidized protein level resulted by the depletion of functional proteins by RS leads to higher recycling of oxidized proteins by the ubiquitin system, increasing the amino acid requirement for protein synthesis, specially Met and Cys [51].

The Met cycle plays an important role in cell physiology. It is place where SAM biosynthesis occurs in a reaction catalyzed by methionine adenosyl transferase. SAM is the major biological methyl donor for other molecular such as DNA [52] resulting Hcy from this transmethylation reaction (figure 1). Furthermore Met is produced by remethylation of Hcy in a Met synthase reaction using tetrahydrofolate (THF) as cofactor (figure 1) [29].

The Met salvage pathway is important for Met conservation in cells that synthesize large amounts of polyamines (putrescine and spermidine) (figure 1) for the cells cycle [53]. Deficiencies in the salvage pathway are responsible for many kinds of tumor. The Met salvage pathway is important for Met conservation in cells that synthesize large amounts of polyamines (putrescine and spermidine) (figure 1) for the cells cycle [53]. Deficiencies in the salvage pathway are responsible for many kinds of tumor.

It is demonstrated that approximately 50% of all tumors are incapable of proliferation when Met is replaced by Hcy, resulting in cell arrest, and eventual death [54]. It is likely that the elevated production of Hcy by cancer cells is an adaptation that has allowed tumor cells to survive and colonize [12]. The absolute requirement for Met by tumor cells is known as "Met-dependency" or "Met-stress" [55]. The molecular mechanism is not completely known but may be triggered by high transmethylation rates and hyper production of Hcy [53]. The accumulation and export of Hcy probably impairs Cys biosynthesis possibly affecting the major enzymes in the trans-sulfuration pathway [12, 29].

Hcy has auto-oxidative potential, since free Hcy contains a free thiol group, this molecule can react with itself to form homocystine or albumin and Cys to increase ROS/RNS production to enhance oxidative stress [56].

The first step of trans-sulfuration pathway (cystathionine β-synthase; CS) is activated by SAM in order to remove Hcy [29]. Redox regulation of CS activity to pro- and antioxidants, enhances or diminishes Hcy flux through the trans-sulfuration [50].

The trans-sulfuration pathway is necessary to maintain intracellular redox homeostasis controlling the tumor progression. This pathway is necessary for cellular physiology because it connects the Met metabolic pathways to the generation of Cys, GSH and Tau as well as

conserving or accelerating Hcy utilization. Moreover Cys is the amino acid limited for GSH formation and half of Cys levels are maintained by the breakdown of GSH [57].

The infiltration of macrophages into the cellular mass is a common characteristic of tumors and it has been shown that these cells play an important role in initiating and promoting tumor [58, 59]. The induction of a chronic inflammatory process by tumor itself or its leukocyte-infiltrate may accelerate cancer growth and metastasis [60]. In both cases, the combined effects of ROS, cytokines as well as angiogenic factors produced by tumor-associated macrophages and other inflammatory cells explains the abnormal growth of healthy cells [58, 61].

The precise molecular mechanisms of macrophage inducing tumor remain poorly understood, but it is well established that once a cellular mass becomes infiltrated by macrophages, the ability of tumor tissue to survive the immune response increases exponentially. Hence, considering that Met-dependent tumors produce large amounts of Hcy from Met, it is plausible that Hcy is one of the signals required to recruit macrophages to the site of tumor cells [12].

Since reduced free Hcy contains a free thiol group, this molecule can react with itself to form homocystine and increase RS production. Plasma levels of reduced free Hcy are found to enhance oxidative stress [62].

It is possible that tumor cells induce a local hyper homocyst(e)ine that recruits a sub population of macrophages. Once established, the macrophages trigger a series of biochemical events that result in the increased synthesis of pro-inflammatory molecules, RS, halogenated pyrimidines, angiogenic and growth factors, and create an optimal cellular microenvironment. This may lead to the selection and stimulation of cells with the ability to evade the immune system and colonize other tissues. Thus metabolic deficiencies in the trans-sulfuration pathway, especially those resulting cellular homocyst(e)ine accumulation, are directly correlated with chronic inflammation and it is likely that the elevated production of Hcy by Met-dependent cancer is an adaptation that allows tumor cells to survive and colonize a constantly changing, biological environment [12].

On the other hand, under normal conditions Tau controls cells and tissue levels of chloride and HOCl by reacting with these molecules to generate taurine chloramines (Tau-Cl). Hence Tau can protect cells from oxidant-induced injury by forming Tau-Cl. Tau-Cl is formed by the direct reaction of Tau with hypochlorous acid, which is generated by the myeloperoxydase-catalyzed oxidation of H_2O_2 during the respiratory burst. Chlorotaurine decreases both NO and TNF secretion by the activated macrophages in a manner that involves changes at the transcriptional and translational levels of inducible nitric oxide synthase (iNOS) and TNF expression respectively, as well as by inhibiting iNOS itself [62].

Tau-Cl down regulates the immunologic response by reducing the production of pro-inflammatory mediators like nitric oxide, tumor necrosis factor alpha, prostaglandin E2 and monocyte chemotactic protein 1, which blocks the cascade effect of chronic inflammation that leads to tumor progression [12].

Thus it seems that tumor cells control their proliferation by increasing Met availability and transmethylation while the higher Hcy formation is usefull to attract residual macrophages and blood phagocytes that allow growth factors and cytokines necessary for inflammation and tumor progression. The trans-sulfuration pathway (through Hcy removement and formation of Cys and Tau) along with folate-stimulated remethylation of Hcy would impair these tumor-favored redox homeostasis probably favoring the tumor [29].

3. Esophageal Cancer

Cancer remains one of the top killers despite the many breakthroughs in our understanding of the biology and molecular pathogenesis of this disease. Increased oxidative stress associated with disturbances in antioxidant defense system has been implicated in the pathogenesis of several diseases, most notably oral cancer [4].

The primary targets of peroxidation by ROS are the polyunsaturated fatty acids in the membrane lipids. The decomposition of these lipids yields a variety of end products such as lipid hydroperoxides and malondialdehyde (MDA). The levels of these end products indicate the extend of lipid peroxidation and serve as a marker of cellular damage caused by free radicals. Increased levels of MDA and NO indicated an increase in the oxidative stress in oral-squamous cell carcinoma (OSCC) patients associated with a deficient antioxidant defense mechanism.

Among the different types of cancer that affect men and women, esophageal cancer (EC) ranks 8th in incidence and 6th in mortality in the world [63]. It is known for its marked variation by geographic regions, race ethnicity and gender [64, 65]. In the US it accounts for only 1% of all diagnosed cancers, however, it is the seventh leading cause from cancer among men. In Brazil it is 8th in incidence, with an estimated risk of 8/100.000 new cases for males and 3/100.000 new cases in females [66]. In western countries the most frequent histological type of EC squamous-cell carcinoma (SCC) seems to be decreasing, contrary to adenocarcinoma (ACA) [67]. Hence the incidence of esophageal ACA has increased a 500% in the last decades. Differently from SCC, ACA incidence is higher in caucasian males than in afro-americans [67].

The etiology of EC involves an association of various intrinsic risk factors such as family history, genetics, age, and extrinsic factors such as alcohol intake, tobacco use, intake of very hot beverages and/or food, exposure to nitrosamines, local fungal infections, and vitamin deficiencies. Other factors, like inflammation caused by gastroesophageal reflux and Barrett's esophagus can also initiate the carcinogenesis process [67-69].

Unfortunately these patients seek health services only when with an advanced stage of the disease (stage III and IV according to TNM/UICC) and thus oftenly with a severe body-weight loss (>10%), which contribute to post-surgery complications and premature death [70].

3.1. Nutritional Risk-Factors

The highest incidence of esophageal SCC is found in northwestern China were the main risk factors are described of drinking very hot and salted tea, boiled with milk, a diet rich in meat, specially salted, dry and/or smoked meta, and dairy products [71]. In general the use of tobacco, moderate to heavy alcohol ingestion, low income and infrequent consumption of raw fruits and vegetables accounted for more than 98% of the SCC rates among both white and African American men [72].

Dietary differences may account for at least some of the disparity in esophageal cancer incidence among racial and ethnic groups. Moreover it is difficult to disentangle the influence of dietary and nutritional factors from the potent effects of alcohol and tobacco. In particular, heavy consumption of alcoholic beverages can interfere with the consumption and use of a variety of nutrients, including fat-soluble and hydro-soluble vitamins, zinc and protein. Smokers appear to have lower intake of several nutrients including vitamin C, than non-

smokers. Nutritional deficits in fresh fruits, vegetables and dietary fiber are commonly referred as associated with the presence of esophagus cancer in Americans [71], Chinese [63] and Germans [73].

The protective effects of fruits and vegetables were seen specially those eaten raw. Fruits and vegetables contain various micronutrients and dietary components with potential anti-carcinogenic effects. One of these micronutrients, vitamin C, blocks the endogenous formation of N-nitroso compounds, which are linked to risk of esophageal cancer [71].

The consumption of fresh food, mostly raw fruits and vegetables, is an important vehicle of antioxidant compounds capable to prevent and/or diminish the impairment of the antioxidant defense relatively to the production of reactive species of Oxygen and Nitrogen (ROS/RNS) as well as other free radicals. These radicals, when in excess generally, react with DNA, RNA, proteins and/or lipids contributing to important damage to cellular metabolism leading to malign transformation [4, 11].

Low levels of ROS/RNS are well tolerated by cells; however the creation of an excess of these species although, including hydrogen peroxide (H_2O_2) and the superoxide anion (O_2^-) induce oxidative stress. Glutathione, together with superoxide dismutase (SOD), catalase, thioredoxin reductase and other intracellular compounds with a redox function act as ROS/RNS detoxifiers and protect cells from oxidative damage. GSH can directly remove free radicals and peroxides which accumulate in cells during oxidative stress by giving rise to glutathione disulfide, i.e. oxidized glutathione (GSSG). This redox role of GSH is its most important function, its reactions being catalyzed by glutathione peroxidase (GPx) and glutathione reductase (GR) [10]. GSH synthesis takes place at the end of Met metabolism, whose pathway is dependent on some B-complex vitamins (B_2, B_6, B_{12} and folate).

It was found that EC patients show high plasma concentrations of Tau and Hcy [74]. Hyperhomocysteinemia may point out some vitamin deficiency(mainly folate) given that these patients frequently present with food deprivation due to dysphagia, and hypovitaminosis (B_2, B_6, B_{12} and folate) is common among alcohol consumers.

Thus the EC, a disease that can be ranked as chronic inflammation, it is expected a predominance of the pro-oxidative status. Its sufferers generally present with important nutritional deficiencies, which contribute to the impairment of the antioxidant capacity and of the immunological status, resulting in decreased life quality and low survival. In the literature there is evidence of an association between dietary pattern change and EC. Given that nutritional deficiency can affect the sulfur-containing amino acids pathway with antioxidant and immunological consequences.

Therefore a supplementation of vitamins involved in the cellular protection system, such as the GSH system, could improve the organic capacity against cancer worsening. Moreover we did not find any study reporting a role of B-complex vitamins (B_2, B_6, B_{12} and folate) in EC and/or in the GSH/GSSG system.

Objectives

a) To investigate the pattern of sulfur-amino acids pathways in esophageal cancer patients, comparatively to healthy controls

b) To study the possible effects of short-term B-vitamins supplementation on oxidative stress-induced changes in plasma thiol pathways in patients in the late stage of esophageal cancer.

Methods

The sample was casual and sized according to demand of patients admitted to the General Surgery ward of the Botucatu Medical School-General Hospital from May 2009 until May 2011. The sample size was 26 patients (24 males and 2 females), with an average age of 58.4±11.8 years. The healthy control group consisted in 20 adults (10 males and 10 females) with a mean age of 27±8.4 years. The Project was submitted and approved by the Ethics Committee of the UNESP Botucatu School of Medicine, in accordance with Resolution 196/96 of the National Health Council (CNS).

The patients were assessed at baseline (Mo) by means of endoscopy, biopsy, bronchoscopy, and thoracic abdominal computerized tomography to check the EC diagnosis and stage, according to TNM [66]. After defining the diagnosis, patients as well the controls underwent to a nutritional assessment and blood analysis for general chemists and plasma sulfur-containing compounds.

Thereafter the patients were randomized distributed in two groups, one receiving vitamin supplementation (G1) and the other placebo (G2) both for 15 days followed immediately by anthropometric and plasma assessments (M1). Then the two groups crossed over the supplementations and continued for the next 15 days finishing up by another set of assessments (M2).

Patients had their follow-up (M1 and M2) on an outpatient ward. Vitamin supplementation was administered in accordance with Dietary Reference Intakes (DRIs) recommendations for male and female adults. Each participant received 15 vials with either vitamins or placebo.

The vitamin vials contained 10 mL of syrup with riboflavin (1.3 mg), pyridoxine (1.7 mg), cobalamin (2.4 mg) and folic acid (400 ug). The placebo vials contained 85% sucrose solution having the same color, taste and texture as the vitamin syrup. Every patient was advised to ingest the contents of one vial per day, together with its oral or enteral diet, and to keep the empty vials and hand them back to the researcher at next visit.

The nutritional evaluation included anthropometry for weight, height, and BMI calculation (kg/m^2) classified as the World Health Organization (WHO) [75] standards. Weight loss percentage (WL%) calculation was estimated by the equation: usual body weight – current body weight x 100 ÷ current body weight. Body composition was obtained by electrical bio -impedance analysis (BIA) using a Biodinamics® apparatus (model 450, USA).The muscle-mass index(MMI) was calculated by dividing the muscle mass per height(kg/m^2).

Subjective Nutritional Assessment (SNA) was collected, as well as diet history by means of a 24-hour diet recall, in which domestic measurements were converted to grams or milliliters, with an aim to allow the chemical analysis of intake food, using the program Nutrilife version 8.

The reference values for B-complex vitamins were established according to DRIs. For Total Energy Intake (TEV) we used the recommendation of 20 to 45 kcal/kg/day and, for proteins, we followed the recommendation of 1.0 to 2.0 g/kg/day according to the National Consensus in Oncological Nutrition [66].

Biochemical analyses were performed on blood samples collected by venipuncture after 12-hour fasting. The markers of glomerular filtration (creatinine and urea), liver function (γ-GT), albumin, calcium, glucose, lipid (triacylglycerols and cholesterol), and uric acid were measured within 4 hours of collection, by means of dry chemistry (Vitros System, Johnson & Johnson, USA). High-sensitivity C-reactive protein (CRP), insulin, B_{12} vitamin and folate were analysed by chemiluminescence (Immulite 2000, Siemens Healthcare Diagnostics, Germany). Analyses of the amino acids Met, Cys, Ser, Gly, Gln, Glu, Hcy, Tau, as well as MDA, GSH, and GSSG analyses, were performed by High Performance Liquid Chromatography (HPLC) using a Shimadzu system.

Results are presented as mean ± standard deviation. For each subject, the relative variation of variables was calculated, defined as $G1 = X_{15days} - X_{0days} / X_{0days}$ and $G2 = X_{30days} - X_{15days} / X_{15days.}$ The Kolmogorov-Smirnov test was used to check the sample homogeneity. To compare supplementations (vitamin and placebo) between G1 and G2, a variance analysis (ANOVA – one way) was performed, followed by a *post hoc* Tukey's HSD test. To compare both stages of supplementation a paired Student's t-test was done.

A significance level of $p<0.05$ (5%) was chosen. The statistical data processing software used was STATISTICA 5.0.

Results

EC patients were predominantly males (92.3%), white (84.6%), smokers (88.5%), and alcoholics (84.6%). The predominant clinical-pathological characteristics of the tumor were the middle esophagus location, the SCC histological type, and stage IV. The most frequent symptoms were dysphagia and odynophagia, lasting on average 3.6 ± 2.7 months.

Nutritional diagnosis showed an average BMI of 20.5 ± 5.9 kg/m^2 (Table 1). A protein-energy malnutrition was observed in 46% of patients, presenting with BMI under 18 kg/m^2 (15.9 ± 1.7 kg/m^2). Regarding weight loss (WL), the average WL% was 21.3 ± 13.4% during the last six months to diagnosis. The Subjective Nutritional Assessment showed that 100% of patients changed from a hypocaloric diet to a liquid one, with 58% (n=15) of them being seriously malnourished and 40% (n=11) moderately malnourished according to said diagnostic model.

The 24-hour diet recall at M0, M1 and M2, showed energy intake changing from 650 kcal/day to 1,851 kcal/day, the average being of 1,200 ± 372 kcal/dia. The average protein intake was 1.1 ± 0.6 g/day, but the distribution of carbohydrates, lipids, and vitamins B_2, B_6, B_{12} was within DRIs recommendations for the patients' age, excepted the folate intake (182 ± 81µg/d), which was under the recommended level (DRI 320 µg/d). In comparison with the control group, patients were older, had a lower BMI, and similar levels of albumin, glucose, cholesterol, triglycerides, folate, and plasma concentrations of nitrogenous metabolites (Table 1). The patient group showed differences relative to the control group in vitamins B_{12} e γ-GT (Table 1). Further to this, both groups were similar for Met, Hcy, Glu; and the ratios 0f

GSH/GSSG, Met/Hcy and Gln/Glu. The remainder amino acids and GSH and GSSG, were lower in patients than the control group (Table 2). Both vitamin and placebo supplementations resulted in weight loss and increased plasma levels of urea (Table 3). Furthermore vitamin supplementation increased vitamin B12 and reduced Hcy (Table 4).

Table 1. Comparison of markers between the control and patients groups at basal visit (M0)

	Control	Patients	*p*
Age (years)	27.3 ± 8.4	58.4 ± 11.8	< 0.001
BMI (Kg/m^2)	25.01 ± 3.2	20.5 ± 5.9	0.004
Glucose (mg/dL)	87.3 ± 5.0	91.7 ± 34.8	0.06
Albumin(g/dL)	4.1 ± 0.3	3.8 ± 0.8	0.07
Triacylglycerols(mg/dL)	88 (67 - 118)	107 (92 - 160)	0.05
Total chol. (mg/dL)	166 ± 27	187 ± 50	0.09
HDL-c (mg/dL)	52.5 ± 20	48.2 ± 23.4	0.52
LDL-c (mg/dL)	90.5 ± 32.9	114 ± 34.7	0.03
Urea(mg/dL)	29 ± 7.5	33.6 ± 16.9	0.26
Creatinine(mg/dL)	0.94 ± 0.19	0.85 ± 0.22	0.14
Uricacid(mg/dL)	4.7 ± 1.2	5.0 ± 1.7	0.49
γ-GT (UI/L)	20.0 (16.5 - 29)	50 (28 - 96.7)	< 0.001
Folate(µg/mL)	8.02 ± 3.12	8.76 ± 4.28	0.64
B_{12} (pg/mL)	288 ± 130	533 ± 230	< 0.001

Tests: Independent t-Student test (normal distribution variables) and Mann-Whitney test (non normal distribution variables).$p<0.05$.

BMI: Body Mass Index; Total chol: total cholesterol; HDL-c: High Density Lipoprotein; LDL-c: Low Density Lipoprotein; γ-GT: Gamma-glutamyltransferase.

Table 2. Comparison of amino acid and oxidative stress marker levels between patients and control groups at basal visit (M0)

	Control(µmol/L)	**Patients**(µmol/L)	*P*
Methionine	30.8 ± 8.9	26.7 ± 10.2	0.13
Homocysteine	13.9 ± 5.5	15.2 ± 3.2	0.26
Serine	108.2 ± 7.8	45.3 ± 38.2	<0.001
Cysteine	533.6 ± 53.9	198.4 ± 84.7	<0.0001
Taurine	63.3 ± 3.9	27.0 ± 14.5	<0.001
Glutamine	305.1 ± 50.2	106.4 ± 65.8	<0.001
Glutamate	56.8 ± 9.6	93.6 ± 98.5	0.051
Glycine	485.1 ± 68.510	164.5 ± 115.6	<0.001
GSH	9.21 ± 2.1	4.85.± 1.9	<0.001
GSSG	0.81 ± 0.92	0.26 ± 0.1	0.006
GSSG/GSH	0.06 ± 0.04	0.08 ± 0.07	0.37
Met/Hcy	2.56 ± 1.22	1.94 ± 0.82	0.22
Hcy/Cys	0.03 ± 0.01	0.08 ± 0.03	<0.001
Gln/Glu	5.54 ± 1.34	2.17 ± 1.70	<0.001

Test: Independent t-Student test. $p<0.05$.

Table 3. Comparison of results in patients between basal visit (M0) and after supplementation (M1)

	M0	Placebo	Vitamin suppl.	*P*
MMI	63.6 ± 20.3	54.4 ± 12.9	55.4 ± 12.6	0.05
BMI (kg/m^2)	21.8 ± 7.4	18.6 ± 4.8	19.0 ± 4.8	0.11
Creatinine (mg/dL)	0.76 ± 0.11	0.82 ± 0.35	0.80 ± 0.25	0.76
Urea (mg/dL)	27.5 ± 5.6 a	41.4 ± 16.6 b	36.2 ± 19.0 b	0.04
Uricacid (mg/dL)	4.5 ± 1.5	4.2 ± 1.4	4.7 ± 1.5	0.48
Calcium (mg/dL)	9.5 ± 0.5	9.0 ± 1.0	9.5 ± 0.3	0.16
Albumin (g/dL)	4.4 ± 0.7	3.9 ± 0.5	4.1 ± 0.4	0.11
CRP (mg/dL)	1.5 (1.1 - 1.7)	1.4 (0.6 - 1.9)	1.4 (0.6 - 2.1)	0.97
γ-GT (U/L)	52.0 (27.3-73.5)	52.5 (33.0-85.5)	58 (42.5-76.0)	0.96
Glucose (mg/dL)	95.9 ± 23.3	92.9 ± 50.6	90.0 ± 29.7	0.94
Insulin (mIU/L)	6.06 ± 3.26	7.00 ± 3.36	6.53 ± 3.25	0.76
HOMA-IR	1.42 ± 0.69	1.93 ± 0.95	1.46 ± 1.15	0.15
TG (mg/dL)	118.2 ± 47.3	108.9 ± 41.4	105.8 ± 60.3	0.68
Total chol (mg/dL)	196.3 ± 52.5	196.0 ± 50.9	213.5 ± 50.1	0.42

Tests: one-way ANOVA (normal distribution variables) and ANOVA on-ranks (non normal distribution variables). $p<0.05$.

MMI: Muscle Mass Index; BMI: Body Mass Index; hs-CRP: high-sensitivity C-Reactive Protein; γ-GT: gamma-glutamyltransferase; TG: triacylglycerols; Total Chol: total cholesterol.

Table 4. Comparison between amino acid and oxidative stress marker levels after crossover (M2)

	Placebo (G1)	**Vitamin (G2)**	***p***
Methionine(µmol/L)	-0.0036 ± 0.25	0.120 ± 0.26	0.21
Homocysteine(µmol/L)	0.236 ± 0.56	-0.311 ± 0.16	0.01
Serine(µmol/L)	0.260 ± 0.95	0.477 ± 0.95	0.89
Cysteine(µmol/L)	0.194 ± 0.43	0.180 ± 0.57	0.97
Taurine(µmol/L)	-0.050 ± 0.27	0.004 ± 0.03	0.72
Glycine(µmol/L)	0.379 ± 0.65	0.034 ± 0.76	0.68
Glutamine(µmol/L)	0.719 ± 2.80	0.668 ± 1.37	0.97
Glutamate(µmol/L)	0.017 ± 0.76	0.779 ± 1.17	0.09
GSH (µmol/L)	0.131 ± 0.33	0.106 ± 0.37	0.75
GSSG (µmol/L)	0.055 ± 0.13	-0.005 ± 0.15	0.36
MDA (µmol/L)	-0.136 ± 0.19	0.424 ± 0.83	0.10
Folate(µg/mL)	0.059 ± 0.26	0.241 ± 0.26	0.08
B_{12} (pg/mL)	-0.250 ± 0.40	2.07 ± 4.55	0.02

Test: Dependent t-Student test. $p<0.05$

GSH: glutathione; GSSG: glutathione disulfide; MDA: malondialdehyde.

Discussion

This study followed a crossover design with the aim to assess the effects in of a supplementation of vitamins participating in sulfur-containing amino acids metabolism in EC

patients. The supplemented vitamins used were those participating in remethylation (folate, B_2, and B_{12}) and trans sulfuration (B6) pathways of Hcy.

The demographic characteristics of patients in this study were similar to those in previous studies [70, 74] and are in agreement with other national and international studies involving a majority of white male patients, over 50 years of age, with an excessive consumption of alcohol and tobacco, and with stage III or IV tumors (TNM-UICC classification) [63, 64, 66].

Dysphagia is the most frequent symptom reported in the literature. In this study dysphagia and odynophagia were reported by all patients at diagnosis. These symptoms are common in people suffering EC given that the esophagus does not have a serous layer, which leads to circumferential tumor growth, thus impeding food passage, with solid and gradually liquid food becoming difficult to swallow.

The silent development of EC, whose symptoms only appear when tumor growth causes obstruction of food passage through the esophagus, which further affect the diets of the patients. They first change from solid to pasty meals, and later change food composition to filtered broth and diluted fruit juices, with a significant reduction in total protein and energy intake. The energy value of the diet in these patients amounted to, on average, 60% of caloric requirements in this population but the protein intake was within the recommended levels. The significant difference in nutritional status such as weight loss and BMI reflects the effects of both the dietary change and the progression of tumors per se [60,52]. In addition, most patients are discharged with an enteral diet prescription. The enteral diet prescription is a standard in these patients, since most of them, due to disease seriousness or prognostic, undergo palliative therapies such as ostomies, which are effective in the reinstatement of an alternative feeding way [64, 76]. Therefore, the present study also showed a significant difference in BMI between patients and the control group, indicating the differences among patients in adapting to the enteral feeding, and enteral feeding helped weight preservation in some patients [50], since most of them, due to the advanced stage of disease received palliative therapies such as ostomies, which are effective in the reinstatement of an alternative feeding way [50,51].

Albumin and glucose plasma levels (markers of protein energy chronic malnutrition) in these patients were within the acceptable limits of body composition. Similar data was obtained in a previous work [74] which included 18 EC patients and showed an average albumin plasma level of 3.5 g/dL. Similar levels were found previously by Marin *et al.* [70] from reviewing 101 clinical records of EC patients and founding average plasma albumin as 3.47 g/dL.

Concentrations of Met, Hcy and Glu were similar in both patients and the control group. On the other hand, the rest of amino acids showed lower levels in patients. Concerning vitamin B_{12}, in spite of different group levels, both were considered to be within the normal range (117 –1158 pg/mL) [78]. Also regarding the aging difference between groups does not seem to influence plasma concentration of amino acids and other chemists once both groups are considered in a mature age.

The contrasted behavior of the cancer profile of Met, Hcy and GSH in relation to other amino acids might be related to the Met-dependency of the tumor [55] whose metabolism is accompanied by higher formation of Hcy, keeping unchanged the ratio Met/Hcy. Moreover Hcy by its auto-oxidative potential enhances the oxidative stress [56] and signals the macrophage recruitment to the site of tumor cells [12]. Once established, the sub population

of macrophages triggers biochemical events with higher synthesis of pro-inflammatory molecules that allows tumor cells to colonize other tissues [12].

The accumulation and export of Hcy probably impairs not only Cys biosynthesis [12] and plasma levels (as seen in our case) but also GSH affecting the antioxidant capacity. For the tumor cell, under metastatic conditions, high levels of GSH can support a rapid cell cycle, an elevated rate of DNA synthesis and a block in cell apoptosis [38] furthering tumor development. The present data showed patients with similar GSH level and thiol antioxidant/oxidant capacity (GSH/GSSG ratio) to the healthy controls.

Similarly to Hcy also folate concentrations were equivalent between groups, allowing Hcy remethylation to Met. This process controlled by folate with a role of Ser, constitutes the main pathway of endogenous Met formation, while it reduces simultaneously intracellular Hcy (and pro-oxidative status). In this study the patients presented a remethylation (Met/Hcy ratio) similar and a trans sulfuration (Hcy/Cys ratio) lower than the controls. Even under low Cys the higher yGT activity presented by the patients would suggest an effort to provide Cys and Glu to the cell for keeping the GSH/GSSG under control.

Metabolic deficiencies in the trans-sulfuration pathway, especially those resulting cellular homocyst(e)ine accumulation, are directly correlated with chronic inflammation in cancer [12]. Here, the plasma systemic marker for inflammation (CRP) did not varied between groups.

In the present work the only effective change seen after B-vitamins supplementation was the increase of vitamin B12 and decrease in Hcy what would promote economy of Met by converting Hcy in the remethylation pathway with participation of vitamins folate and B12. Besides keeping Met controlled the consumption of Hcy would decrease the oxidative stress and lower chemo attraction of macrophages, chronic inflammatory activity and tumor progression [12].

Protein-energy malnutrition in EC patients is associated with increased weight loss and decreased food intake. These patients are not only subject to dietary effects of their food obstruction, they suffer as well alterations that can be caused by the anorexia mediated by cytokines, hypermetabolism, and protein metabolism changes [64]. Lower GSH values were observed together with proportionally lower values of its oxidized form (GSSG/GSH ratio), as well as of other GSH precursor amino acids, such as Cys and Gly. In the present case, these precursor deficiencies can be attributed to synthesis decrease in the cases of Cys (since Met and Hcy levels are similar to the control group) and of Tau (reduced) and to an increased metabolization of Gly, owing to an increased synthesis of Ser required for Hcy remethylation and transsulfuration (to create Cys). The lower levels of Gln can be attributed to its increased metabolization to synthesize (and normalize) Glu, trying to restore a normal GSSG level.

The majority of patients (73%) in the present study were subjected to chemo/radiotherapy. Given that such treatments involve drugs that are metabolized by the liver, the significant rising of γ-GT relative to the control group could be linked to the liver damage these patients are caused. Prospectively studying Japanese subjects over 9 years, Tsuboya *et al*. [79] found a positive correlation between higher γ-GT levels and the incidence of tumors linked to daily alcohol consumption, as happens in the majority of our patients. On the other hand, evidence is reported in the literature of γ-GT participation in oxidative stress. The main function of γ-GT is its use as a precursor of amino acids (cysteine) for intracellular GSH synthesis. The γ-GT increase could be interpreted as a mechanism of antioxidant

defense, since it could decrease oxidative stress, promoting intracellular GSH regeneration [80].

The literature provides evidence of a higher incidence of renal insufficiency among cancer patients, in connection with approximately 50% of anticancer drugs being mostly excreted in urine [80]. The evolution of EC favors loss of weight and, consequently, of lean body mass. The increase of urea levels in patients could be reflecting weight loss as a consequence of patients' catabolic status. On the other hand, it is common to attribute the lower glomerular filtration to aging, which consequently favors an increase of plasma solutes. Considering the age range of the population in this study, one could infer that the observed urea increase in patients would be expected more in view of age than in view of lower glomerular filtration. To this reasoning contributes the fact that creatinine, another marker of glomerular filtration, had a similar level to that of the control group after vitamin supplementation.

The majority of tumors are catabolic, consuming lean mass and leading to weight loss. This could be observed in the present study (by MMI decrease) during disease evolution, irrespective of supplementation type.

The present study has limitations regarding the sample size and homogeneity and the length of vitamin supplementation. The reduced number of patients and their severity were regarding the fact they show up spontaneously looking for treatment only when the disease is on its advanced stage. At that time their survival is minimal even for accomplishing this 30-day intervention. Moreover some of them feel clinically better after the baseline (M0) or M1 interventions and did not come back for the next evaluation. Hence we began the experiment (M0) with 26 patients and finished up the interventions (M1, M2) only with 8.

Conclusion

Evidence was obtained that EC in its advanced stage has a different pattern of sulfur-containing compound pathway with the most preserved amino acids being methionine, homocysteine and glutamate. Met/Hcy (transmethylation/remethylation) cycle was maintained whereas Hcy/Cys (transsulfuration) was reduced therefore accumulating Hcy. Even in the presence of lower Cys it seems that it is an effort of cancer- cells to generate GSH by uptaking more precursors (Cys and Glu) from the GSH-gamma GT cycle at least to keep GSSG/GSH ratio controlled. Short-term B-vitamin supplementation led to increased folate and vitamin B12 levels contributing effectively for reduction of Hcy. Hence the other variables resulted unchanged. By keeping controlled Hcy and GSH/GSSG the cell would try to reduce the oxidative stress and therefore the tumor progression, what could be attributed presently to the supplemented vitamins.

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Chapter 6

Glutathione As Preventive and Therapeutic Target of Adult T-Cell Leukemia/Lymphoma and Its Regulation by Carnosol, a Functional Food Ingredient

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Abstract

Adult T-cell leukemia/lymphoma (ATL) is a fatal malignancy caused by infection with human T-cell leukemia virus type Ⅰ (HTLV-1). Worldwide, 10 to 20 million people are infected with HTLV-1 and a part of viral carriers (6-7% for men and 2-3% for

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women) develops ATL after a long latent period (at least 20-30 years). There is much room for improvement in accepted curative therapy for ATL and the development of new therapeutic and preventive strategies is necessary. Functional foods and their ingredients are focused as natural resources for the prevention and treatment of life style-related diseases. Considering that only a part of viral carriers develops ATL after the long latent period, it is speculated that ATL onset is influenced by a diet taken daily. Recently, we have reported that carnosol, which is an ingredient contained in rosemary (*Rosmarinus officinalis*), induces apoptosis in ATL cells via glutathione depletion. This suggests that glutathione depletion caused by functional food ingredients may be a possible new target for the prevention and therapy of ATL. In this review, we present an overview of the developmental mechanism of ATL, glutathione as new preventive and therapeutic target of ATL, and glutathione regulation by carnosol.

Introduction

Adult T-cell leukemia/lymphoma (ATL) is a fatal malignancy caused by infection with human T-cell leukemia virus type Ⅰ (HTLV-1) [1]. Worldwide, 10 to 20 million people are infected with HTLV-1 and endemic areas for the virus are unevenly distributed, which include south west Japan, the Caribbean islands, South America, and a part of Central Africa [2]. The HTLV-1 infection does not necessarily lead to the development of ATL and the majority of viral carriers remain asymptomatic throughout their lives. The estimated lifetime risk of developing ATL in viral carriers is 6-7% for men and 2-3% for women in Japan and it occurs mostly in adults, at least 20-30 years after the HTLV-1 infection [3]. Once developed, ATL has a poor prognosis despite the operation of various therapies such as combination chemotherapy [4]. Recently, mogamulizumab, humanized monoclonal antibody against CC chemokine receptor 4 (CCR4) that is expressed in ATL cells, has been reported to show potential efficacy for ATL [5]. Although mogamulizumab is expected to provide new, promising treatment options in ATL patients, there is much room for improvement in its efficacy of therapy. Additionally, the preventive strategy is inadequate and many HTLV-1 carriers live with apprehension about developing ATL. Also, the virus is associated with multiorgan inflammatory disorders, including HTLV-1−associated myelopathy/tropical spastic paraparesis (HAM/TSP) and uveitis [6−8]. The South Kyushu area of Japan, including Miyazaki prefecture, has a high prevalence of HTLV-1, and it is now recognized as a social problem. The development of new therapeutic and preventive strategies against ATL and other HTLV-1−associated diseases is necessary.

Because most HTLV-1 carriers are asymptomatic and medical intervention to them is restricted despite the overwhelming difficulty in ATL therapy, the prevention in asymptomatic phase is very important. Several indicators for the progression to ATL have been reported from epidemiological studies and one of them is the HTLV-1 proviral load level [3, 9]. It is speculated that the onset of ATL is influenced by the decrease of viral load level. Considering that only a part of viral carriers develops ATL after the long latent period, a diet taken dairy may play a considerable role in the prevention of ATL in a similar manner to that of life-style-related diseases such as diabetes and cancers.

Functional foods and their ingredients are focused as natural resources for the prevention and treatment of cancer [10, 11]. Dietary patterns, foods, nutrients and other dietary constituents are closely associated with the risk for several types of cancer and it has been

estimated that 35% of cancer deaths may be related to dietary factors [12]. Recently, dietary polyphenols derived from various fruits and vegetables are suggested to be effective for cancer prevention. Thus, we screened local agricultural products (1700 samples from 283 species) grown in Miyazaki prefecture for their growth-inhibitory activity against ATL cells and HTLV-1−infected cells. We found that carnosol, which is an ingredient of rosemary (*Rosmarinus officinalis*), induces apoptosis in ATL cells via glutathione depletion [13]. This suggests that glutathione depletion caused by functional food ingredients may be a possible new target for the prevention and therapy of ATL.

In this review, we present an overview of the developmental mechanism of ATL, glutathione as a new preventive and therapeutic target of ATL, and glutathione regulation by carnosol.

1. Discovery of HTLV-1 As the Pathogen of ATL

ATL was first reported as a distinct clinical entity in Japan in 1977 [14]. ATL patients clustered in southwest part of Japan and its epidemiologic clustering instigated Japanese investigators to discovery the pathogen of ATL. After that, the Caribbean islands, South America, and a part of Central Africa were also reported as the other endemic areas of ATL [2]. In parallel with these epidemiologic studies, identifying a pathogen of ATL was challenged and then HTLV-1 was discovered as the causative virus for ATL in early 1980s [15, 16]. HTLV-1 is the first retrovirus proven to be associated with human disease.

The etiological association of HTLV-1 with ATL was established on the basis of the following findings [3]: (1) geographical areas of high incidence of ATL patients correspond closely with those of HTLV-1 carriers [17], (2) all ATL patients have antibodies against HTLV-1 [1, 18], (3) HTLV-1 immortalizes human $CD4^+$ T cells *in vitro* [19], and (4) ATL cells having genomic DNA in which HTLV-1 proviral DNA is integrated are monoclonal [20]. Investigations on the natural history of HTLV-1 and the underlying molecular mechanism are indispensable for understanding the pathogenic mechanism of ATL.

2. Natural History of HTLV-1 and Developmental Mechanism of ATL

HTLV-1 belongs to the family of Retroviridae and is an enveloped virus with single-stranded RNA as viral genome [15, 16]. HTLV-1 is transmitted primarily in three ways: mother-infant (mainly through breast feeding), sexual contact, and parenteral transmission [21]. Although HTLV-1 has the potential to infect various cell types such as T cells, B cells, macrophages, and dendritic cells, the virus can induce transformation almost exclusively in $CD4^+$ T-cells [22]. Once a retrovirus enters into host cells, viral genome RNA is reverse-transcribed into double-stranded DNA and then integrated into the host chromosomal DNA by the viral integrase, being termed provirus [15, 16]. HTLV-1 provirus plays a central role in the viral replication. As shown in Figure 1, the provirus is transcribed into RNA, which serves as both viral genome for progeny virions and mRNA for the synthesis of viral specific proteins. HTLV-1 provirus has four structural genes (*gag*, *pro*, *pol*, and *env*) necessary for the production of infectious virions [23]. *Gag*, *pro*, *pol*, and *env* code for internal proteins of virion, the viral protease, the reverse transcriptase, and envelop proteins, respectively.

Additionally, HTLV-1 uses alternative splicing and internal initiation codons to produce several regulatory (Tax and Rex) and accessory (p12, p13, and p30) proteins located in the *pX* region.

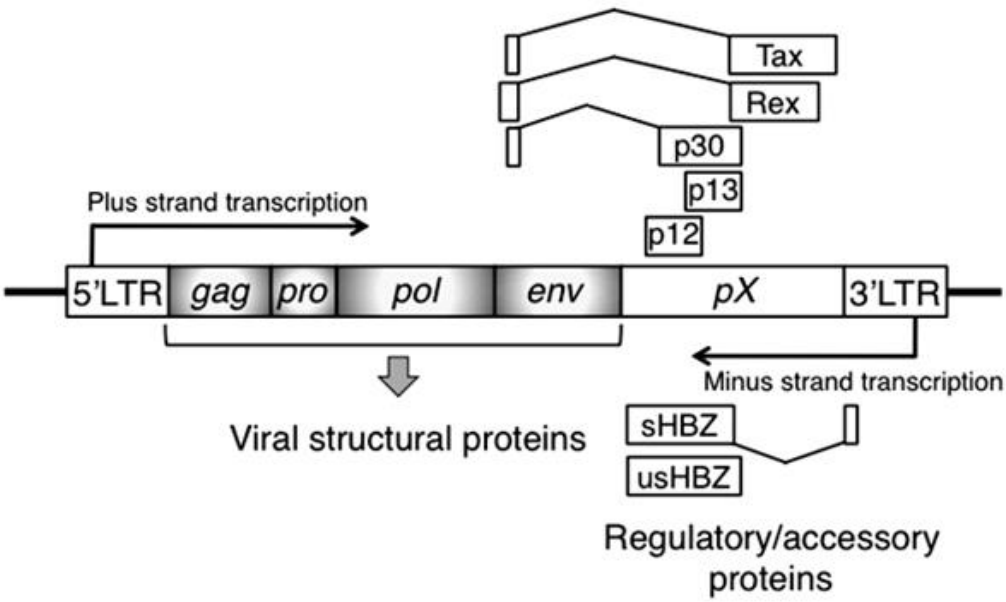

Figure 1. Structure of HTLV-1 provirus. Position and relative sizes of open reading frames for viral specific proteins are shown as boxes. While viral structural proteins for progeny virions are coded by four genes (*gag*, *pro*, *pol*, and *env*; shaded gray boxes), most amino acid sequences of viral regulatory proteins (Tax and Rex) and accessory proteins (p12, p13, and p30) are coded by *pX* region. These proteins are synthesized from the plus strand transcript. In contrast, another regulatory protein, HBZ, which has spliced form (sHBZ) and unspliced form (usHBZ), is synthesized from the minus strand transcript. The 5' terminal and 3' terminal of HTLV-1 provirus have 5' LTR and 3' LTR, respectively.

While all these proteins are synthesized from the plus strand transcript, another regulatory protein, HTLV-1 bZIP factor (HBZ) is synthesized from the minus strand transcript. HBZ has two isoforms: spliced form (sHBZ) and unspliced form (usHBZ).

Figure 2 shows the natural history of HTLV-1 infection. After an individual is infected with HTLV-1 via mother-infant, sexual contact, and parenteral transmission (Figure 2A), the infected cell expansion occurs in the living body by two patterns: *de novo* infection from infected cells to uninfected cells and clonal expansion of infected host cells [21]. Unlike many other viruses, HTLV-1 has been reported to spread not via free viral particles but via virological synapse formed on cell-to-cell contact (Figure 2B) [24]. Tax enhances the transcription of viral structural genes (*gag*, *pro*, *pol*, and *env*) by being recruited to 5' long terminal repeat (LTR) and is also necessary for the formation of cell-cell contact sites. Viral spread via cell-to-cell contact is thought to contribute to initial establishment of a population of HTVL-1−infected cells (Figure 2C). While Tax immortalizes human primary T cells, the cells expressing Tax could be eliminated by the host cytotoxic T lymphocyte (CTL) response after establishment of host immunity against HTLV-1 (Figure 2D). Thus, HTLV-1 replicates predominantly using the second form of replication, clonal expansion of infected cells [25, 26]. In order to escape from the host immunity, HTLV-1 replicates as a provirus by

increasing the number of infected host cells. HBZ is a regulatory protein needed for the proliferation and maintenance of infected cells and has low immunogenicity [27].

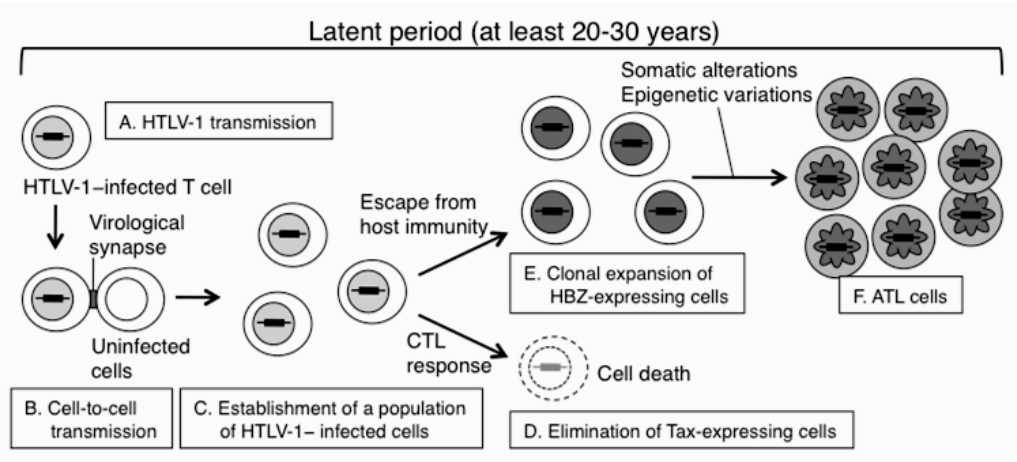

Figure 2. Natural history of HTLV-1 infection. After an individual is infected with HTLV-1 (A), the virus spreads via virological synapse formed on cell-to-cell contact (B), contributing to initial establishment of a population of HTLV-1−infected cells (C). While Tax immortalizes human primary T cells, Tax-expressing cells are eliminated by CTL response (D). HBZ-expressing cells escape from the host immunity due to the low immunogenicity of HBZ and proliferate by clonal expansion (E). HBZ works synergistically with other viral proteins to modulate host cell factors and the cells survive long enough to go through somatic alterations and epigenetic variations. After a prolonged asymptomatic period (at least 20-30 years), aneuploid ATL cells (flower cells) emerge in approximately 2.5% to 5% of infected individuals (F). Most HTLV-1−infected individuals remain life-long asymptomatic carriers.

Further, HBZ inhibits Tax-dependent viral transcription and modulates Tax activity in the infected cells via various signal transduction pathways [28]. Several accessory proteins (p12, p13, and p30) also modulate Tax expression [28]. Collectively, the survival of HBZ-expressing cells is enhanced on behalf of Tax-expressing cells (Figure 2E). In ATL cell lines and ATL cells from the patients, HBZ expression is constitutively detectable whereas Tax expression is frequently suppressed or diminished [29]. It is considered that this expression pattern in ATL cells reflects the HBZ enhancement and Tax elimination seen in early phase of infection. In addition to Tax and HBZ, p12, p13, p30, and Rex have been also described to contribute to establishing persistent viral infection *in vivo* [21].

In the chronic phase of HTLV-1 infection, proviral load (reflecting the number of infected cells) becomes stable in most infected individuals; however, there is a broad range in variation of the load among them [9]. HTLV-1 proviral load can differ by more than 1000-fold between different carriers. Previously, Tax has been considered as the most important factor that controls proviral load [30], but recent evidences suggest the importance of HBZ [22]. Further, since the variation of HTLV-1 sequence among them is very limited, host factors and their relationship with HBZ are thought to be important determinants of proviral load. This is supported by the finding that HTLV-1−infected individuals who possess major histocompatibility complex (MHC) alleles which can efficiently bind and present antigen-present peptides from HBZ have significantly lower proviral load [31]. Conditional transgenic mice that express HBZ in $CD4^+$ T cells have been also reported to develop HAM/TSP-like

skin inflammation by 18 weeks after birth, followed by T-cell lymphomas like ATL after a long latent period [32]. This reveals that HBZ is a key player in HTLV-1 pathogenesis.

Genetic alterations and epigenetic changes are recognized as mechanisms implicated in oncogenesis. In the later stage of HTLV-1 infection, HBZ works synergistically with other viral proteins to modulate host cell factors and then may permit infected cells to survive long enough to go through the alterations and changes, transforming infected T cells into ATL cells, which are characterized as aneuploid cells with flower-shaped nuclei (flower cells) (Figure 2F) [21, 33]. It has been known that somatic alterations or epigenetic variations in genes such as *p53, p16INK4,* and *p27KIP1* are seen in ATL cells and ATL patients with genetic changes in p53 and p16 have a poor prognosis [28]. HBZ has been also reported to form complexes with various transcription factors and signal transduction molecules such as CREB and c-Jun, resulting in the dysregulation of host cell-signaling pathways [22]. Therefore, it leaves no doubt that these host proteins are closely associated with ATL development; however, the exact mechanism of their association remains to be elucidated.

3. Currently Available Therapies for ATL

ATL is a subtype of peripheral T-cell lymphoma (PTCL), which is known to be extremely difficult to treat due to refractory nature to even aggressive chemotherapy regimens or relapse [34]. Among the various entities of PTCLs, ATL has the worst prognosis with 5-year overall survival of 14% [34]. In Japan, it is estimated that there are about 1.08 million HTLV-1 carriers [9] and approximately 700 to 1000 people die of this disease per year [5]. ATL is classified into four disease subtypes (acute, lymphoma, chronic, and smoldering) based on clinical features and the median survival time varies according to the disease type: acute type, 6 months; lymphoma type, 10 months; chronic type, 24 months; and smoldering type, 3 years or more [34, 35]. The acute, lymphoma, and chronic types with unfavorable prognostic factors have been regarded as aggressive ATL subtypes requiring immediate treatment, and intensive combination chemotherapy or hematopoietic stem-cell transplantation (HSCT) is generally recommended therapeutic options [36]. Additionally, several new agents against ATL are now under investigation.

The Japan Clinical Oncology Group-Lymphoma Study Group (JCOG-LSG) has been investigating the efficacy of combination chemotherapy for ATL since the early 1980s. Although the cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) regimen and CHOP-like regimens have been evaluated from the start of the investigation, the outcome of these regimens was still inferior to that in other PTCLs [4, 37, 38]. In Western countries, combination therapy with interferon-α (IFN-α) and zidovudine (AZT) has been widely used for all disease subtypes of ATL. It has been reported that the median survival time and 5-year overall survival for acute type treated with IFN-α/AZT were 9 months and 28%, respectively, whereas these values were 7 months and 0%, respectively, for lymphoma type [39]. These reports indicate that conventional chemotherapeutic agents alone yield few or no long-term remissions or potential cures in ATL patients.

Allogeneic HSCT is now considered as a promising alternative treatment that can provide long-term remission in a proportion of patients with ATL [33, 36]. Recently, a nation wide retrospective study of allogeneic HSCT for ATL in Japan has reported that median survival time and 3-year overall survival were 9.9 months and 36%, respectively [40].

Several molecules expressed on the surface of ATL cells are expected as target molecules for ATL therapy and therapeutic agents using monoclonal antibodies against them are currently under investigation [36]. Among them, monoclonal antibody against CCR4, which is a chemokine receptor, gets a lot of attention [5]. Chemokines act as signaling molecules in the migration and tissue homing of various leukocytes through triggering of chemokine receptors. CCR4 is a seven-transmembrane G-protein coupled receptor and its expression is markedly increased in ATL cells compared with normal T-cells [41]. Further, leukemia cells from about 90% of ATL patients have been reported to show CCR4 expression, being associated with skin infiltration and prognosis [42]. CCR4 plays an important pathogenetic role in ATL and has been thought as a good target for ATL therapy.

Mogamulizumab/KW-0761 is a humanized monoclonal antibody against human CCR4. Its antibody-dependent cellular cytotoxic (ADCC) activity is enhanced by a glycoengineering technology and it shows potent antitumor activity mediated by enhanced ADCC against ATL cells [5, 43]. Phase I clinical trial of mogamulizumab has been conducted in patients with CCR-positive PTCL, including ATL and assessed the safety, pharmacokinetics, recommended phase II dose, and its efficacy [44]. Subsequently, the phase II trial was conducted in 28 patients with aggressive CCR4-positive ATL [45]. Complete response rate, overall response rate, and median overall survival period were 30%, 50%, and 13.7 months, respectively, indicating the clinical efficacy of mogamulizumab.

Although mogamulizumab is expected to provide a new, promising treatment option in ATL patients, there is much room for improvement in its efficacy of therapy. Further, because most HTLV-1 carriers are asymptomatic and medical intervention to them is restricted despite the overwhelming difficulty in ATL therapy, the prevention in asymptomatic phase is very important. However, the preventive strategy is inadequate and many HTLV-1 carriers live with apprehension about developing ATL. Also, the virus is associated with HAM/TSP and uveitis [6−8]. The development of additional therapeutic and preventive strategies for ATL patients and HTLV-1 carriers is necessary.

4. Risk Factors for ATL Development

To develop new therapeutic and preventive strategies against ATL, the identification of risk factors associated with ATL development is essential. To explain the characteristics of ATL leukemgenesis, HTLV-1 infection has been reported to be associated with a variety of genetic abnormalities. In this regard, however, given asymptomatic status of most HTLV-1 carriers [9, 46, 47], it is obvious that the infection itself is not sufficient to develop ATL from the status. Risk factors for ATL development in HTLV-1 carriers have been investigated in many epidemiological and clinical studies [2, 3, 9]. Age, HTLV-1 infection in childhood, male sex, and abnormal immune system of host have been suggested as the risk factors. Also, laboratory markers such as interleukin 2 receptor may serve as ATL indicators. In general, an increased viral load has been recognized to be a very important risk factor for oncogenesis by viruses [3]. There are accumulated data indicating a relationship between an increased viral load and viral-associated malignancies. HTLV-1 proviral DNA load in the peripheral blood mononuclear cells (PBMCs) is also evaluated in some epidemiological and clinical studies to support the hypothesis that the increased load level is an important predictor of developing ATL.

A series of the Miyazaki cohort study reported that HTLV-1 proviral load level is higher in HTLV-1 carriers who developed ATL than in asymptomatic HTLV-1 carriers [48−51]. In Japan in 2002, a nationwide prospective cohort study for asymptomatic HTLV-1 carriers, the Joint Study on Predisposing Factors of ATL Development (JSPFAD), was initiated and this important finding was confirmed in a large number of subjects [9]. In the cohort of 1218 asymptomatic HTLV-1 carriers, 14 subjects progressed to overt ATL during 2002 to 2008. All of the 14 subjects were among those with the highest group of baseline proviral load (range, 4.17-28.58 copies/100 PBMCs). It was indicated that HTLV-1 carriers with higher proviral load levels belong to the high-risk group of ATL development. It is speculated that the onset of ATL is influenced by the decrease of viral load level.

As shown in Figure 2B, HTLV-1 spreads not via free viral particles but via virological synapse formed on cell-to-cell contact [24]. Therefore, the elimination of HTVL-1−infected cells from a living individual possibly leads to the decrease of viral load level. Apoptosis induction in HTVL-1−infected cells and ATL cells is potentially an effective means for the elimination.

5. Apoptosis Induction in ATL Cells by Functional Foods and Their Ingredients

ATL occurs in only a part of viral carriers (approximately 2.5% to 5%) after a long latent period (at least 20-30 years) [3, 9, 46, 47]. Considering this, a diet taken dairy may play a considerable role in the prevention of ATL in a similar manner to that of life-style-related diseases such as diabetes and cancers. Dietary patterns, foods, nutrients and other dietary constituents are closely associated with the risk for several types of cancer and it has been estimated that 35% of cancer deaths may be related to dietary factors [12]. Functional foods and their ingredients are focused as natural resources for the prevention and treatment of cancer [10, 11].

Several functional foods and their ingredients have been reported to induce apoptosis in ATL cells and HTLV-1−infected cells [52−55]. Green tea and its main ingredient, epigallocatechin-3-gallate (EGCG) (Figure 3A), induces apoptosis *in vitro* in peripheral bood T lymphocytes of ATL patients, showing with data on the decreased expression of HTLV-1 *pX* mRNA [52]. It is striking that HTLV-1 provirus load in peripheral blood lymphocytes of HTLV-1 carriers is diminished by green tea drinking [56]. Capsaicin (Figure 3B) and genistein (Figure 3C), which are ingredients of red pepper and soy, respectively, induce apoptosis by the inhibition of NF-κB pathway [53−55]. These findings support the efficiency of apoptosis induction by functional foods and their ingredients for the prevention and therapy of ATL.

6. Carnosol, Rosemary Ingredient, Induces Apoptosis in ATL Cells via Glutathione Depletion

We screened local agricultural products (1700 samples from 283 species) grown in Miyazaki prefecture of Japan for their growth-inhibitory activity against ATL cells and

HTLV-1−infected cells. It was found that carnosol (Figure 3D), which is a polyphenol contained in rosemary (*Rosmarinus officinalis*), induces apoptosis in ATL cells via glutathione depletion [13].

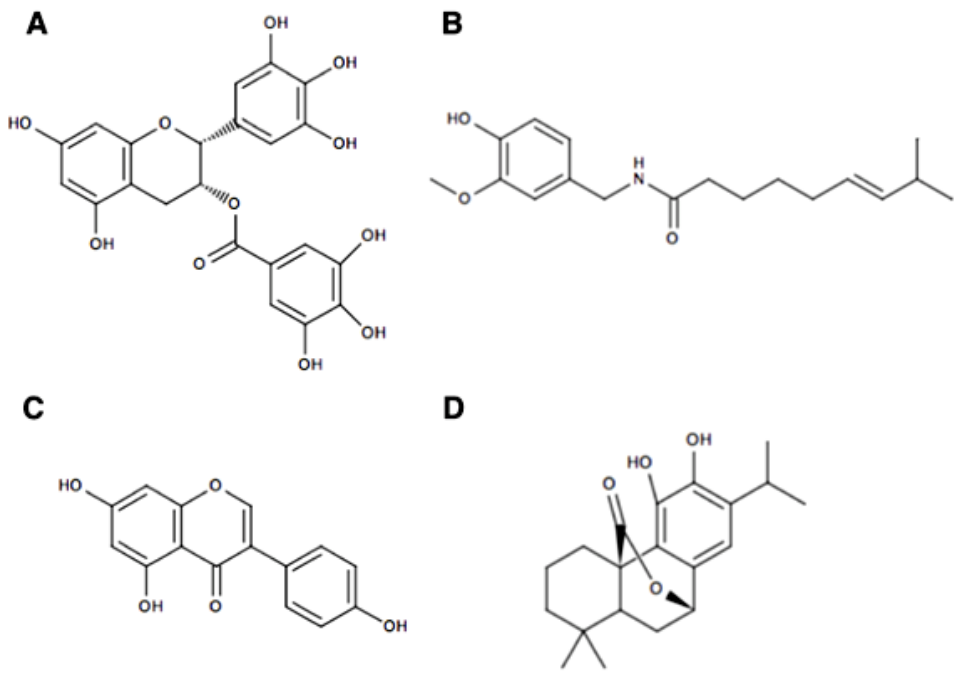

Figure 3. Chemical structure of functional food ingredients that have apoptosis-inducing activity in ATL cells. (A), EGCG; (B), capsaicin; (C), genistein; (D), carnosol.

Glutathione is required for the maintenance of redox-status and plays a central role as antioxidant in the protection against oxidative stress through the cycling of GSH (reduced form) and GSSG (oxidized form) (Figure 4A) [57, 58]. In carnosol-treated ATL cells, while amounts of both GSH and GSSG are significantly decreased, the ratio of GSH and GSSG is not affected [13]. Further, N-acetyl-L-cysteine, which is precursor of glutathione, cancels the efficiency of carnosol. From these findings, it is suggested that the apoptosis-inducing activity of carnosol in ATL cells is caused by the depletion of glutathione.

It is of interest why carnosol induces apoptosis in ATL cells. Living cells are always producing reactive oxygen species (ROS) such as H_2O_2 endogenously by the vital activity [59]. Glutathione prevents the oxidation of intracellular components as a buffer against endogenous ROS by detoxifying H_2O_2. Glutathione depletion by carnosol is suggested to increase oxidative damage to the proteins, triggering apoptotic signaling.

A possibility is the activation of intrinsic pathway of apoptosis caused by oxidative damage to mitochondria [59, 60]. A dominant role of mitochondria is the production of energy currency of the cell, adenosine 5'-triphosphate (ATP) and the ATP production process requires oxygen. Therefore, living cells are always producing ROS as by-product and it is detoxified by antioxidative systems such as GSH-GSSG cycle (Figure 4A). When glutathione is depleted from cells, undetoxyfied ROS is accumulated in the cells and then oxidatively damages mitochondria (Figure 4B), resulting in the leakage of cytochrome *c* into the cytosol

(Figure 4C). Subsequently, cytochrome *c* interacts with Apaf-1 and procaspase-9 and forms a multimeric complex called the apoptosome (Figure 4D).

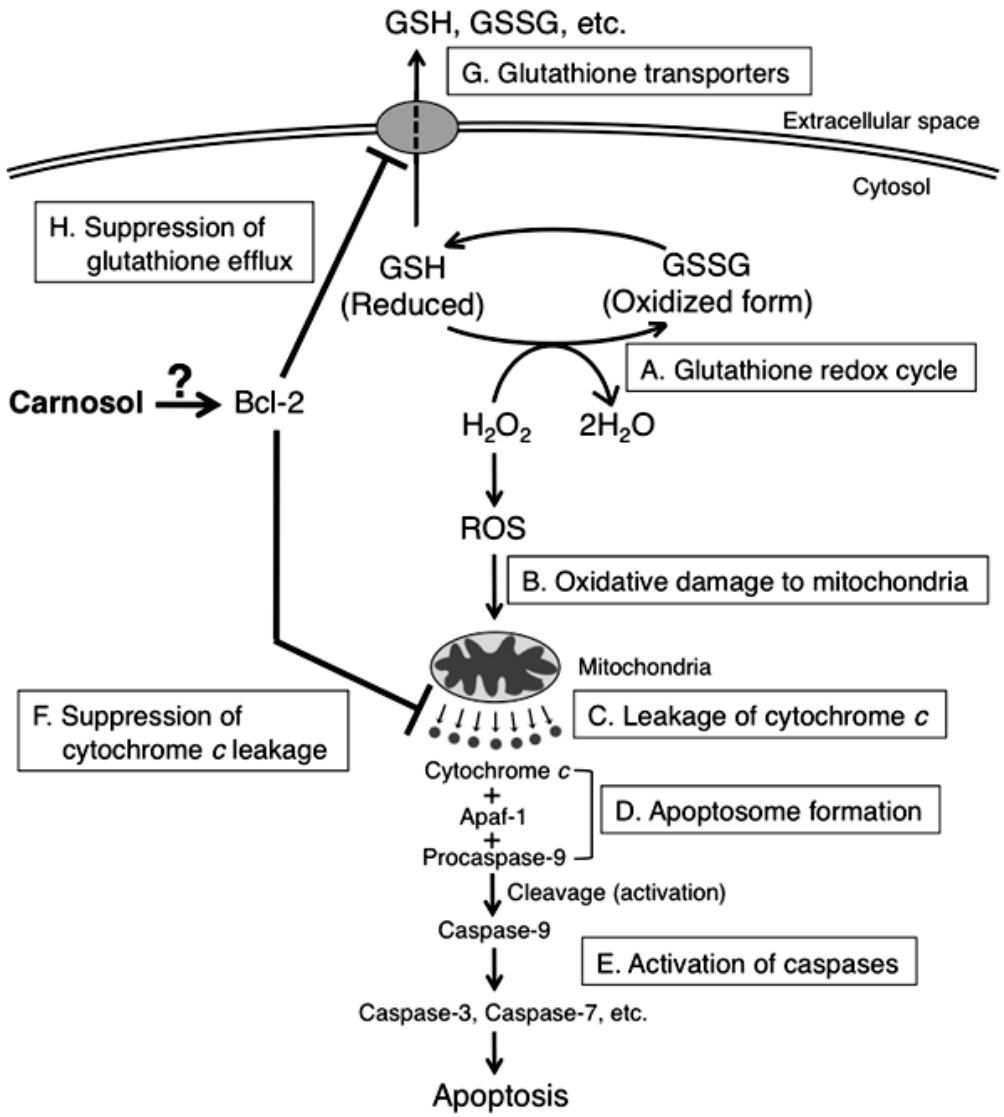

Figure 4. Apoptosis-inducing mechanism in glutathione depleted cells and action mechanism of carnosol. Glutathione functions as antioxidant in the protection against oxidative stress through the cycle of GSH (reduced form) and GSSG (oxidized form)(A). Living cells are always producing ROS such as H_2O_2 by the vital activity and it is detoxified by GSH-GSSG cycle. In glutathione depleted cells, undetoxyfied ROS is accumulated and then oxidatively damages mitochondria (B), by which cytochrome *c* leaks into cytosol (C). Cytochrome *c* interacts with Apaf-1 and procaspase-9 and forms apoptosome (D). By cleavage of procaspase-9, caspase-9, which is the activated form, activates other caspases such as caspase-3 and caspase-7 (E), leading to apoptosis. Bcl-2, an anti-apoptotic protein, has a function that suppresses the leakage of cytochrome *c* from mitochondoria (F). Glutathione efflux is regulated by glutathione transporters (G) and Bcl-2 has another function that suppresses the efflux (H). Carnosol may induces apoptosis in ATL cells by regulating the expression and function of Bcl-2.

Procaspase-9 is cleaved into caspase-9, which is the activated form, and then activates other caspases such as caspase-3 and caspase-7 to orchestrate the biochemical execution steps of apoptosis by cleaving many downstream substrates (Figure 4E). In ATL cells treated with carnosol, not only glutathione depletion but also activation of caspase-3 and caspase-7 is seen [13]. Further, carnosol has been reported to downregulate Bcl-2 [61], an anti-apoptotic protein that suppresses the leakage of cytochrome *c* from mitochondria (Figure 4F) [62]. Collectively, it is suggested that carnosol induces apoptosis via the intrinsic pathway of apoptosis, although other possibilities such as extrinsic pathway and thioredoxin system remain [13, 60].

Molecular mechanism by which carnosol causes glutathione depletion is not yet understood. Based on studies on glutathione metabolism, we propose a hypothesis to explain glutathione depletion in carnosol-treated ATL cells. Glutathione depletion has been described in a number of different apoptotic systems, with several studies showing that the depletion is the result of accelerated efflux [59, 63]. In ATL cells, the depletion occurs for short time of only 3 h after carnosol treatment [13]. This rapid depletion implies the accelerated efflux of glutathione. Glutathione efflux is regulated by glutathione transporters (Figure 4G) [64, 65]. Apoptosis induced by distinct stimuli has been described to promote glutathione depletion via the activation of a plasma membrane efflux transport [64]. Carnosol may also promote the efflux of glutathione via such activation function. Regarding this, Bcl-2 has been reported to increase intracellular glutathione by suppressing the efflux as another function of anti-apoptosis (Figure 4H) [66]. It is possible that carnosol causes glutathione depletion in ATL cells via cancellation of the efflux-suppressing function by Bcl-2 downregulation.

7. Glutathione Depletion as a Possible Target for Prevention and Therapy of ATL

Investigation into the mechanism underlying glutathione depletion by carnosol may lead to the development of new therapeutic and preventive strategies for ATL. The proliferation of PBMCs derived from HTLV-1 carriers has been reported to be dependent on glutathione and suppressed by the inhibition of a glutathione transporter, ABCC1/MRP1 [67]. Carnosol has been also reported to have the anticancer activity in several cancer cells and animal models [61, 68]. Regarding carnosol toxicity, it has been shown that carnosol is not toxic to PBMCs derived from healthy human individuals [61]. Glutathione depletion by carnosol, other functional food ingredients, and other drugs is a possible target for prevention and therapy for ATL.

Conclusion

ATL is a fatal malignancy caused by infection with HTLV-1. There is at present no accepted curative therapy and the development of new therapeutic and preventive strategies is necessary. We found that carnosol, a rosemary ingredient, induces apoptosis in ATL cells via glutathione depletion. Investigation into action mechanism of carnosol suggests that glutathione depletion by carnosol triggers intrinsic apoptosis pathway. Gluthathione depletion

may be a possible target for prevention and therapy for ATL. In the future, we hope the day will come when new preventive and therapeutic strategies for ATL are used worldwide.

Acknowledgments

We dedicate this work to Mr. Fumiaki Mieno (deceased, March 19, 2013), who inspired our work in protection and exploitation of intellectual property. We thank Sachiko Tomiyama, Tokoyo Imai, Toshiro Morishita, and Makoto Kodama (Miyazaki Prefectural Industrial Support Foundation) for managing our study.

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Chapter 7

Glutathione Food Supplementation: Present Situation in Thailand

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Abstract

Glutathione is a widely mentioned biochemical molecule in present clinical biochemistry. It is no doubt that glutathione has several advantages to human bodies. Hence, there are many applications of glutathione products in present biomedicine. An interesting application is the glutathione food supplementation. In the present brief article, the authors will summarized and discussed on the present status of glutathione food supplementation in Thailand.

Introduction

Glutathione is a widely mentioned biochemical molecule in present clinical biochemistry. It is no doubt that glutathione has several advantages to human bodies [1 – 3]. Biochemically, glutathione is an antioxidant which is regularly generated intracellularly from its oxidized form by glutathione reductase activity [4]. Intracellular synthesis of glutathione occurs as a two-step reaction, which is strongly relating to rate-limiting gamma-glutamylcysteine synthetase activity [4]. In human, liver plays major role as synthesizer of circulating glutathione [4]. In addition, kidney can also salvage glutathione through the gamma-glutamyl transpeptidase reaction [4]. There are many useful cellular functions of glutathione including

"bioreductive reactions, maintenance of enzyme activity, amino acid transport, protection from harmful oxidative species, and detoxification of xenobiotics [5]".

In clinical medicine, glutathione is confirmed for its advantages in several aspects. Ballatori et al. noted that "glutathione deficiency or a decrease in the glutathione/glutathione disulfide ratio manifests itself largely through an increased susceptibility to oxidative stress, and the resulting damage is thought to be involved in diseases, such as cancer, Parkinson's disease, and Alzheimer's disease [6]". In oncology, glutathione is accepted for "modifying the cellular response to several anti-cancer treatment modalities [5]." Estrela et al. concluded that "glutathione content of cancer cells is particularly relevant in regulating mutagenic mechanisms, DNA synthesis, growth, and multidrug and radiation resistance [7]." Hence, it is no doubt that glutathione plays important roles in cancer treatment at present. Focusing on neurodegenerative diseases, Schulz et al. mentioned that "the pathogenesis of several neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, Friedreich's ataxia and amyotrophic lateral sclerosis, may involve the generation of reactive oxygen species and mitochondrial dysfunction [8]" and further extrapolated for the role of glutathione as antioxidant that can correspond to the pathogenesis of those neurodegenerative diseases [8].

Hence, there are many applications of glutathione products in present biomedicine [9]. An interesting application is the glutathione food supplementation. In the present brief article, the authors will summarized and discussed on the present status of glutathione food supplementation in Thailand.

Glutathione Food Supplementation in Thailand

There are many new food products that can be classified as food supplementations. The glutathione food supplementations are widely seen in the present day. Valencia and Hardy noted that "new nutraceutical products for nutritional support and antioxidant therapy such as glutathione require practical advice and information on the indications, methods and routes of administration, dosing (therapeutic drug monitoring), stability and physicochemical compatibility [10]." In Thailand, there are many glutathione food supplementation products. However, there is still no official scientific report on those products.

In fact, glutathione is not only available in the form of oral food product but it is also available in the form of injectable drug. There are many illegal injectable products of glutathione in Thailand and those illegal products are usually proposed for their property to act as a skin whitening inducer. In fact, glutathione in oral form is safer and there are some evidences on its property as a skin whitening inducer. Arjinpathana and Asawanonda noted that "oral glutathione administration results in a lightening of skin color in a small number of subjects [11]" and also proposed that "long-term safety has not been established and warrants more extensive clinical trials [11]."

Focusing on oral product, there is also a limitation of the allowable amount of glutathione in the product. In Thailand, according to Thai FDA, the legally allowable of glutathione is 250 mg/day. Also, it is regulated for demonstrating of the properties of glutathione in the label. The "skin whitening inducer" is not allowable to be included in the food supplementation product's label [12]. At present, there are many forms of oral glutathione

food supplementations. Some are pure glutathione whereas the others appear as combined formula. The examples of combined formula are those with herbs (such as glutathione plus ginseng) or with other nutrients (such as glutathione plus other amino acids).

Conclusion

There are many glutathione food supplementation products in Thailand. However, there is no official scientific report on its property. At present, the non - oral form of glutathione is banned in Thailand and the use of oral glutathione food supplementation is also controlled for allowable amount. There is also a good system for regulation of illegal use of glutathione in Thailand.

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Chapter 8

Possible Role of Oxidative Stress and Glutathione S-Transferase Gene Polymorphisms in Etiopathogenesis of Diabetes Mellitus and Its Chronic Complications

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Abstract

Diabetes mellitus, as a disease with dramatically increasing incidence, affects the quality of life especially due to the presence of chronic diabetic complications like neuropathy, retinopathy or nephropathy. One of the factors in complex and not fully understood etiology of diabetes and its chronic complications is oxidative stress, characterized as an imbalance between reactive oxygen species (ROS) production and the function of antioxidant mechanisms. Glutathione S-transferase (GST) represents a family of enzymes catalyzing the conjugation of glutathione with various electrophilic compounds to facilitate their excretion. GSTs detoxify some of the secondary ROS generated during oxidation of membranes or other cellular constituents, act in the detoxification of organic hydroperoxides and protect cells from peroxide-induced cell death. GST enzymes are involved in the synthesis of inflammatory mediators, leukotrienes and prostaglandins and act also in cell signalling pathway as potential regulators of apoptosis. The most researched GST enzymes are glutathione S-transferase mu 1 (GST M1) and glutathione S-transferase theta 1 (GST T1). It has been shown that individuals carrying the *null* genotype of GST have significantly reduced activity of this enzyme compared to *wild* genotype carriers. This chapter brings comprehensive review

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about the possible role of oxidative stress in etiopathogenesis of type 1 diabetes, type 2 diabetes and chronic diabetic complications. Recent studies also assume the role of GST gene polymorphisms while the information varies according to the authors, region and studied population. More knowledge about this predisposition factor may bring the basis for the possible therapeutic intervention in the future.

Introduction

Diabetes mellitus as chronic metabolic disease becomes the pandemy nowadays considering its dramatically increasing incidence worldwide. The quality of life in patients with diabetes is mainly determined by the presence of chronic diabetic complications which are not typical just for adulthood but regarding the early diabetes onset they can be found also in children and adolescents.

One of the most important risk factors for development of chronic diabetic complications is persistent hyperglycemia that induces the various metabolic processes acting in mutual interactions, like non-enzymatic glycation of proteins, polyole pathway, oxidative stress, activation of protein kinase C, activation of proinflammatory cytokines or lack of neural growth factor. Except hyperglycemia (which influence is enhanced by diabetes duration and poor compensation), relevant risk factors are genetic predisposition, immunologic, environmental or epigenetic factors. These factors may explain common clinical experience when some patients despite insufficient diabetes compensation do not suffer from any chronic complications and on the other hand, some patients with euglycemia and short diabetes duration may have some signs of chronic complications. According to Diabetes Control and Complication Trial (DCCT, 1993), important risk factors for microvascular complications are cigarette smoking and genetic susceptibility to hypertension at early stages of diabetes and poorer glycemic control, higher blood pressure and unfavorable lipid profile at later stages.

Among discussed pathways involved in the pathogenesis of chronic complications belong activation of proinflammatory cytokines and oxidative stress, as an imbalance between production of reactive oxygen species and their destruction by antioxidant mechanisms. Glutathione S-transferase (GST) enzymes are involved in the degradation of various exogenous and endogenous compounds, in the synthesis of inflammatory mediators, leukotrienes and prostaglandins and act also in cell signalling pathway as potential regulators of apoptosis. As GST enzymes detoxify some of the secondary reactive oxygen species generated during oxidation of membranes or other cellular constituents, they have also antioxidant activity. They act in the detoxification of organic hydroperoxides and protect cells from peroxide-induced cell death (Hayes, 2005). The most researched is glutathione S-transferase mu 1 (GST M1) enzyme in GST M class with its gene located in Chromosome 1p13.3 and glutathione S-transferase theta 1 (GST T1) enzyme in GST T class with its gene located in Chromosome 22q11.23. It has been shown that individuals carrying the *null* genotype of GST have significantly reduced activity of this enzyme compared to *wild* genotype carriers (Datta et al., 2010). GST M1 and T1 gene polymorphisms have been described to be associated with various pathological conditions like cardiovascular, lung, oncologic or gynecological diseases. According to already published studies, GST T1 or M1 gene polymorphisms can be regarded as risk factors for development of diabetes mellitus

(Pinheiro et al., 2013) and chronic diabetic complications (Kariž et al., 2012; Hovnik et al., 2009), however the results differ according to the authors, population and the region.

Presented chapter is focused on possible role of oxidative stress and GST gene polymorphisms in etiopathogenesis of diabetes mellitus and its chronic complications. The knowledge about pathogenetic factors contributes to individual approach to the patient and may bring the base for therapeutic intervention in future.

Diabetes Mellitus

Diabetes mellitus is chronic metabolic disease of heterogeneous etiology with hyperglycemia as the main symptom caused by insufficient action of insulin because of its absolute or relative deficiency and it is accompanied by the disorder of metabolism of sugars, lipids, proteins, water and minerals.

According to American Diabetes Association (ADA, 2012) the classification of diabetes mellitus is:

Diabetes mellitus

I. Type 1 diabetes mellitus (T1D)
II. Type 2 diabetes mellitus (T2D)
III. Specific types of diabetes
    Genetic defects of beta cells, Genetic defects of insulin action, Diseases of exocrine pancreas, Endocrinopathies, Diabetes induced by drugs and chemicals, Infections, Uncommon forms of immune-mediated diabetes, Other genetic syndromes sometimes associated with diabetes.
IV. Gestational diabetes mellitus

Impaired Fasting glucose (IFG)
Impaired Glucose Tolerance (IGT).

Type 1 diabetes (T1D), the most common form of diabetes in childhood, is characterized by variously quickly ongoing inflammation of pancreatic beta cells leading to absolute lack of insulin that determines the need of its substitution. Etiopathogenetically is in majority of cases autoimmune inflammation (type 1a diabetes) of pancreatic beta cells with destruction by T lymphocytes and auto-antibodies produced by B lymphocytes, in minority of cases the inflammation is idiopathic (type 1b diabetes). Important risk factors are genetic predisposition of individual (HLA alleles DQ2, DQ8, DR3, DR4), endogenous factors (immune system) (Jeseňák et al., 2012) and exogenous environmental factors (e.g. viral infection). T1D has typical phases: preclinical phase, phase of manifestation of diabetes, partial remission („honeymoon period“) and chronic phase with lifelong dependence on exogenous insulin. Preclinical phase precedes several months even years the manifestation of T1D. In this period of autoimmune inflammation several antibodies can be proven (IA-2 – antibodies against tyrosine phosphatase, ICA – islet cell antibodies, IAA – insulin autoantibodies, GADA – glutamic acid decarboxylase antibodies) that are positive at the time of diagnosis of T1D in

85 – 90% patients. Nowadays no intervention exists possible to prevent or delay the manifestation of T1D. Clinical symptoms appear at destruction approximately of 90% pancreatic beta cells. In some children the manifestation is gradual; contrary in other children the development of symptoms is rapid with diabetic ketoacidosis. Seasonality of manifestation is well described; the peak is in winter months. Non-urgent symptoms of T1D are polyuria, polydipsia, blurred vision, loss of weight despite increased appetite or stagnation of weight in growing children, abdominal pain, frequent urination – also during night or enuresis nocturna, vaginal candidosis, emesis, recurrent skin, urinary or other infections, higher excitability and deterioration results in school. Urgent symptoms of diabetic ketoacidosis include dehydration, recurrent vomiting, acetone odor of breath, hyperventilation (Kussmaul breathing), disorientation, unconsciousness or shock (Čiljaková et al., 2013). In laboratory findings, the diagnosis is confirmed by hyperglycemia, increased concentration of glycosylated hemoglobin (HbA1c) (it may not be present in the case of rapid onset of diabetes), metabolic disruption of water, electrolyte and acid-base balance and low even missing concentration of C peptide (except honeymoon period in T1D). Diabetic ketoacidosis may be the first manifested sign in 30% children with T1D. Its incidence is higher in patients younger than five years and in children with poor social economic status. In newly diagnosed T1D the incidence of ketoacidosis is higher in countries with lower incidence of diabetes (Rewers et al., 2008). More than half patients with T1D is diagnosed at the age less than 15 years however recently significant increase in incidence of T1D was noted in children younger than 5 years (Green et al., 2000). Incidence of T1D is very variable between various countries and ethnic groups (0.1 – 37.4 / 100 000 people) (Craig et al., 2006). Approximately in 80% children after initiation of therapy by exogenous insulin the need of insulin transiently decreases. Post-initial remission is defined as the need of exogenous insulin less than 0.5 IU/kg/day while maintenance of HbA1c less than 7% (DCCT norm). Ketoacidosis and younger age at diagnosis of T1D decrease the probability of remission onset. Its duration is very variable (few months even few years) and it is followed by chronic phase of lifelong dependence on insulin.

Type 2 diabetes (T2D), the most common form of diabetes in adulthood and in whole population, is characterized by insulin resistance (impaired insulin action in target tissues) and impaired insulin secretion. In the etiopathogenesis combination of lifestyle (obesity, lack of physical activity, poor diet, smoking, stress, lack of sleep) and genetic factors play role. Insulin resistance, which occurs primarily in the muscles, fat tissue and liver, gradually increases demands on insulin secretion, thus leads to compensatory hyperinsulinism, later to disruption of glucose homeostasis and eventually to manifestation of T2D. Insulin resistance, caused by structural or functional change of insulin receptor and / or by failure of post-receptor mechanisms, causes the disturbances in glucose metabolism. Lack of insulin inhibition on glucose production leads to increase liver gluconeogenesis, to increase lipolysis in the liver and fat tissue with elevated concentration of free fatty acids. Glucose influx to the tissues, especially to the muscles, is insufficient so the production of glycogen is decreased. At the beginning of T2D, basal secretion of insulin may be increased even with fasting hyperinsulinism. With the continuation of disorder, insulin secretion decreases due to secondary defect of pancreatic beta cells probably as the consequence of long lasting hyperglycemia (glucose toxicity), chronic increase of free fatty acids, lipotoxicity or storage of amylin. T2D can manifest at any age but most commonly after 40 years, however diabetes

can run discreetly long time before clinical manifestation. Therefore T2D can be diagnosed accidentally and some patients can have signs of microangiopathic (even macroangiopathic) complications at the time of diagnosis or T2D can manifest as hyperosmolar hyperglycemic (nonketogenic) coma. Clinical symptoms are the same as above described non-urgent symptoms of T1D and some patients can have other signs of metabolic syndrome (central obesity, hypertension, dyslipidemia).

Monogenic diabetes is group of hereditary forms of diabetes caused by mutations of certain autosomal dominant gene leading to disruption in insulin production or action. It has some typical symptoms: diabetes diagnosed in the first six months of life, family history of diabetes, mild fasting hyperglycemia (5.6 – 8.5 mmol/l) or diabetes associated with extra-pancreatic manifestation. In majority of cases the diagnosis can be set based on DNA testing. These clinical units include neonatal diabetes (permanent and transient) and MODY diabetes (maturity onset diabetes of the young) type 1 – 11. Some cases are possibly treated by peroral antidiabetics.

More rare causes of diabetes include genetic syndromes associated with diabetes, cystic fibrosis related diabetes, pancreatopathy or endocrinopathies.

Diabetes mellitus according to the recommendation of American Diabetes Association can be diagnosed based on one of three criteria (ADA, 2012):

1. The presence of typical symptoms of diabetes (polyuria, polydipsia, weight loss) and concurrently glycemia > 11.1 mmol/l at any time during the day.
2. In absence of clinical symptoms, fasting glycemia (without energy intake minimally during last 8 hours) > 7.0 mmol/l (necessary to confirm by repeated measure).

Glycemia in the 120^{th} minute of oral glucose tolerance test > 11.1 mmol/l (at intake 75 g glucose or 1.25 g/kg of weight by standard method).

Treatment of diabetes if complex and depends on diabetes type. Management of T1D includes exogenous supplementation of insulin (subcutaneously, in ketoacidosis intravenously), healthy lifestyle and physical activity. The insulin treatment in children is from the beginning by intensified insulin regimen as the prevention of onset of chronic complications. The principle of treatment is application of short-acting insulin (bolus, postprandial) just before the main meals and of long-acting insulin (basal) in the evening (or if necessary, twice daily). In the last decades insulin analogues are used which have better facilities like more quickly onset of effect (ultra-fast analogues) and longer duration of effect (protracted analogues) acquired by replacement of certain amino acid. Patients with insufficient compensation are indicated to treatment by insulin pump that as yet the best mimics the natural production of insulin by the pancreas. Eating of patient with diabetes should be regularly (six times daily), individually with counting carbohydrates and with sufficient amount of various nutrients, fiber, vitamins and probiotic cultures. Patient with diabetes should not be exempt from physical education, contrary it is desirable to promote a physical activity. The management of T2D focuses on lifestyle intervention – physical activity, proper diet, quit smoking, later on antidiabetic drugs or insulin therapy. There are several classes of anti-diabetic medications – metformin (biguanide class) as the first choice, then sulfonylureas, nonsulfonylurea secretagogues, alpha glucosidase inhibitors,

thiazolidinediones, glucagon-like peptide-1 agonists and dipeptidyl peptidase-4 inhibitors. Oral medication can be used alone or insulin can be given. Most patients do not initially need insulin therapy. If necessary, long-acting insulin is usually given at night (or twice daily) and in case of insufficiency also postprandial short-acting insulin can be added.

For the control of the treatment, self-monitoring of glycemia by glucometer is important, also monitoring of glycosuria, glycosylated hemoglobin measurement every three months, monitoring of blood pressure and serum lipids. Once a year it is appropriate to complete ophthalmological, neurological examination and measurement of level of microalbuminuria for early detection of potential diabetic complications.

Complications of Diabetes Mellitus

Patients with diabetes are compromised by acute and chronic diabetic complications. Acute complications – hypoglycemia, diabetic ketoacidosis, hyperglycemic hyperosmolar coma and lactate acidosis may directly threaten the life of patients. Chronic complications may worse the quality of life and with their progression may also lead to life-threatening situations.

Chronic diabetic complications are not typical just for adults but in relation to early diabetes onset can be found also in children and adolescents. Chronic complications, that by deficiency of adequate treatment can reduce life expectancy by 25 – 50%, are divided into microvascular (neuropathy, nephropathy, retinopathy) and macrovascular complications (ischemic disease of heart, brain and limbs). The presence of chronic complications increases with diabetes duration however several years before clinical manifestation are notable functional and structural changes those early detection is the aim of screening examination of patients with diabetes.

Diabetes duration and compensation are important but not the only risk factors in pathogenesis of chronic complications. In clinical practice some patients despite short diabetes duration and good compensation have some signs of chronic complications and conversely some patients with poor compensation and long diabetes duration do not suffer from any complications.

This points to other factors in pathogenesis like genetic predisposition, immunologic, environmental or epigenetic influences. Examination of gene polymorphisms is new approach in diagnostics of chronic diabetic complications and may give a basis for individually customized therapy in future.

Regular screening of diabetic retinopathy should be started in children older than 11 years with diabetes duration of two years or more, or in younger children with diabetes duration of five years and more.

Examination by direct ophthalmoscopy should be realized once a year or more frequently in the case of high risk of worsening or loss of vision. As the prevention of nephropathy it is recommended to examine blood pressure and microalbuminuria once a year (Donaghue et al., 2007). Regarding possible false positive results (physical activity, period) mainly in adolescents it is necessary to repeat the positive results. In patients older than 12 years, blood pressure should be measured annually and serum lipid profile every five years as the screening of macrovascular complications (Table 1).

Table 1. Recommendation for screening of chronic diabetic complications (Donaghue et al., 2009)

	Recommended screening frequency	Recommended methods	Therapy
Retinopathy	Annually from age 11 years with diabetes duration > 2 years or from 9 years with diabetes duration ≥ 5 years	Fundus photography or mydriatic ophtalmoscopy	Improvement of diabetes compensation, laser therapy
Nephropathy	Annually from age 11 years with diabetes duration > 2 years or from age 9 years with diabetes duration ≥ 5 years	Concentration of albumin in the first morning sample or ratio albumin / creatinine	Improvement of diabetes compensation, ACE inhibitors (or sartans), decrease of blood pressure
Neuropathy	Consensus was not reached	Personal history, physical examination	Improvement of diabetes compensation
Macrovascular complications	After age 12 years	Serum lipid profile every 5 years, blood pressure annually	Improvement of diabetes compensation, decrease of blood pressure, statins

Diabetic Neuropathy

Diabetic neuropathy, nerve disorders associated with diabetes mellitus, is the most frequent chronic diabetic complication and common reason to visit a doctor. It can be present even at the time of diagnosis of T1D or T2D and it occurs in about 40 – 90% of diabetic patients after ten years of diabetes duration. Especially children can be affected by its sub-clinical form – without visible symptoms but detectable by special diagnostic methods. Diabetic neuropathy is characterized by variety of symptoms (paresthesia, dysaesthesia, fault of vibration or thermal sensitivity, impaired proprioception, pain) as diffuse or focal disorders of peripheral somatic or autonomic nerve fibers can be present. It may lead to invalidism due to severe pain, muscle weakness or nerve paresis. Regarding the variability of manifestation of diabetic neuropathy several classifications exist. Classification of diabetic neuropathy according to (Rybka, 2007; Schroner, 2006) is following (Table 2):

Table 2. Classification of diabetic neuropathy

<table>
<tr><td colspan="4">Sub-clinical neuropathy</td></tr>
<tr><td rowspan="8">Clinical neuropathy</td><td rowspan="4">Symmetrical</td><td>Distal symmetrical neuropathy</td><td>Sensory
Motor
Mixed</td></tr>
<tr><td colspan="2">Autonomic neuropathy</td></tr>
<tr><td colspan="2">Proximal symmetrical neuropathy</td></tr>
<tr><td colspan="2">Acute painful neuropathy</td></tr>
<tr><td rowspan="4">Asymmetrical</td><td colspan="2">Cranial neuropahy</td></tr>
<tr><td colspan="2">Peripheral mononeuropathy</td></tr>
<tr><td colspan="2">Radiculopathy</td></tr>
<tr><td colspan="2">Asymmetrical proximal motor neuropathy</td></tr>
<tr><td colspan="3">Mixed</td></tr>
</table>

Currently, also modified Thomas classification is recommended (Freeman, 2005):

1. Symmetrical neuropathy
a) Distal senzory-motor neuropathy
b) Autonomic neuropathy
c) Acute painful neuropathy
d) Hyperglycemic neuropathy
e) Insulin-induced neuropathy
f) Proximal diabetic neuropathy (lumbosacral radiculoplexopathy)
g) Chronic inflammatory demyelinating polyradiculoneuropathy

2. Asymmetrical (focal) neuropathy
a) Cranial neuropathy
b) Thoraco-abdominal neuropathy
c) Focal neuropathy of limbs
d) Proximal motor neuropathy (diabetic amyotrophy)

Regarding the range of autonomic nervous system, disorders of various organ systems may be expressed:

- cardiovascular system: rest tachycardia, orthostatic hypotension, increased arrhythmogenesis of myocardium, cardiomyopathy, circulation instability, decreased tolerance of physical activity and heat, edema of lower limbs
- gastrointestinal system: dysphagia, odynophagia, gastroparesis, nausea, diarrhea, constipation, fecal incontinence
- urogenital system: atonia of vesica urinaria, urine retention, common urinary tract infection, erectile dysfunction, painless while pressing testes
- respiratory system: decreased lung functions, decreased cough reflex sensitivity, decreased basal bronchial tone, decreased ventilatory response to hypercapnia, increased susceptibility to infections
- sudomotoric system: feet anhidrosis, increased sweating of upper part of the body, sweating after food and at the night
- endocrine system: asymptomatic hypoglycemia, hormonal contraregulation disorder
- other: pupillary reflex disorder.

Diagnostic range of diabetic neuropathy extends as it responds to ascending demands. However, conventional investigations (patient´s history, physical and neurological examination) could verify clinically manifesting forms of neuropathy. Nowadays huge impact is put on early diagnostics of clinically silent forms that has led to development of many new possibilities like electrophysiologic examinations, quantitative examination of sensitive functions, heart rate variability (the gold standard in diagnostics of cardiovascular autonomic neuropathy) (Havlíčeková et al., 2009), cough reflex sensitivity (Čiljaková et al., 2009) or electrodermal activity. For examination of specific organs may serve electrogastrography, spirometry, diffuse lung capacity for carbon monoxide or colour skin tests with changing colours depending on sweat amount to diagnose sudomotor neuropathy. New non-invasive

examination for early neuropathy detection is corneal confocal microscopy which quantifies the pathology of small nerve fibers (their density, morphology or branching) in cornea, the most dense innervated part of human body.

Diabetic Retinopathy

Diabetic retinopathy (DR), typical microvascular diabetic complication, primarily affects retinal vessels in diabetic patients. Nowadays, it is the most common cause of blindness in developed countries among adults. Blindness can be caused also by other eye disorders like secondary glaucoma, cataract or macular degeneration. The prevalence of DR is noticed in 2 – 7% of diabetic patients after two years, in 50% of patients after ten years and in 75% of patients after twenty and more years of diabetes duration. It progresses from mild non-proliferative abnormalities characterized by increased vascular permeability through moderate and severe non-proliferative retinopathy characterized by vascular closure up to proliferative retinopathy with typical new blood vessels growth. DR can be clinically classified into these forms (Table 3):

Table 3. Clinical stages of diabetic retinopathy (DR) (Rybka, 2007)

	Eye background
Non-proliferative DR	
Mild	Microaneurysmas, microhemorrhages, intraretinal hemorrhage, venous abnormalities
Moderate	Vessel changes at macula area, hard exudates, „cotton“ exudates
Severe	Intraretinal microvascular abnormalities (IRMA), retinal ischemia
Proliferative DR	
Beginning	Retinal or papillary neovascularisation
High risk	Traction amotio of retina, intravital hemorrhage
Diabetic maculopathy	Macular edema

DR is the best available for objective examination of all microvascular complications. In diagnostics, eye background examination (color fundus photography or ophtalmoscopy) can detect manifesting forms of diabetic retinopathy. Slit lamp examination can investigate the spatial relations between retina and vitreous.

Examination of pupillary reflex latention is possible by classic investigation and also by infrared reflex pupillography. Other possible methods are vision examination, fluorescein angiography showing retinal circulation, Amsler grid identifying what parts of visual field are damaged or eye ultrasonography used in the cases of vitreous hemorrhage or cataract. Fluorophotometry enables to measure posterior vitreous penetration ratio as the parameter reflecting the blood-retinal barrier permeability. New diagnostic tool for diabetic retinopathy and macular degeneration using LED technology is in development.

Diabetic Nephropathy

Diabetic nephropathy is characterized by glomerular, tubular and mesangial damage accompanied by basement membrane thickening, mesangial expansion and hyalinisation of glomerular inter-capillary connective tissue. In clinical practice, progressive kidney disease with proteinuria, hypertension and gradual decrease in renal functions is typical (Table 4). Manifest nephropathy is presented in 30 – 35% of patients with diabetes duration over 15 – 20 years.

Table 4. Clinical stages of diabetic nephropathy (Rybka, 2007) (GF, glomerular filtration)

	characteristics	**Urinary findings**
Latent stage (hyper-filtration-hypertrophic)	increase GF about 10 – 40%, ultrasound hypertrophic of kidneys, slightly enlargement of basal membrane	transitory microalbuminuria 30 – 100 mg/day, resp. 20 – 70 µg/min
Incipient diabetic nephropathy	decrease GF, common hypertension (mainly diastolic), progression in enlargement of basal membrane	permanent microalbuminuria 30 – 300 mg/day, resp. 20 – 200 µg/min
Manifest diabetic nephropathy	Next decrease GF, hypertension progression, sclerotisation of many glomerules	proteinuria >300 mg/day, resp. >200 µg/min
Chronic renal insufficiency even kidney failure	Terminal phase, uremia, dialysis necessary	proteinuria >0,5 g/day, serum creatinine > 200 µmol/l

Diagnostic tests for diabetic nephropathy include urine sediment, protein urine test, microalbuminuria, urine and blood creatinine, blood urea and nitrogen. Microalbuminuria is usually set from 12-hour night urine sample while good glycemia compensation, normal blood pressure and without excessive physical activity. The result is considered as positive when at least two from three urine samples are positive, investigated at the period 3 – 6 months with the one month distance interval. Investigation of metalloproteinase-9 in blood can detect earlier cases of kidney damage. Possible indicator of tubulopathy is N-acetyl-beta-D-glucoseaminidase (NAG). Its positivity in urine precedes the positivity of microalbuminuria in diabetic nephropathy. In the early stage, filtered albumin does not pass into the urine due to increased reabsorbtion in the proximal tubule and only NAG is present. Next, the reabsorption capacity of tubules is exceeded and microalbuminuria together with NAG is positive, in the advanced stages of nephropathy the positivity of NAG is caused by glomerular basement membrane damage as well as by tubular cells destruction. NAG is set as a ratio to creatinine in the urine (upper border is 0,25 U/mmol of creatinine). Other biomarkers involved in renal tubular damage (kidney injury molecule 1, KIM-1; neutrophil gelatinase-associated lipocalin, NGAL; liver-type fatty acid binding protein, L-FABP), changes of extracellular matrix (collagen IV, matrix metalloproteinases, fibronectin), markers of oxidative stress (8-hydroxy-deoxyguanosine) or microinflammation (TNF-α, transforming growth factor β, orosomucoid, monocyte chemoattractant protein-1) are also searched (Holzscheiter et al., 2014; Genovese et al., 2014).

Macroangiopathic Complications

Macroangiopathic complications of diabetes include coronary artery disease (CAD), myocardial infarction, cerebrovascular diseases and peripheral vascular disease (limb ischemia). Prevalence of CAD in diabetic patients is around 30% and death because of cardiovascular diseases is the main cause of death in subjects with diabetes. Diabetes mellitus is three times more frequent in patients with stroke and the mortality of diabetics with cerebrovascular accident is three times higher. In T2D patients, macrovascular complications are more frequent than microvascular and in T1D patients, cardiovascular risk is enormously increased in the fourth decade of life.

As hypertension and dyslipidemia are main risk factors for atherosclerotic changes, their early identification belongs to basic approach in *diagnostics.* Clinically manifested macrovascular complications can be diagnosed by electroencephalography, coronarography, echocardiography, carotid ultrasound, contrast CT or MRI of brain, clinical examination of limbs (positional tests, ankle brachial index), ultrasound of limb arteries, digital subtraction angiography, CT angiography or MRI angiography.

Etiopathogenesis of Chronic Diabetic Complications

The etiopathogenesis of diabetic complications is complex and up to now not completely understood. Long lasting hyperglycemia triggers the variety of pathways – non-enzymatic glycation of proteins, oxidative stress, polyole pathway and sorbitol production, activation of the transcriptional factor nuclear factor-kappa B, increased production of protein kinase C, increased generation of proinflammatory cytokines, decrease production of vasodilatation products (nitric oxide, prostaglandins), decrease of myoinositol origin or change in Na^+K^+ATP-ase activity. These pathomechanisms may effect directly the target cells (like neurocytes, retinal cells, glomerules) or through the endothelial damage may cause the microangiopathy of capillaries supplying various organ systems with their subsequent disorder. Because of common clinical experience that diabetes duration and compensation do not always correlate with onset of chronic diabetic complications, other factors have been considered as important in pathogenesis – genetic, immunologic, environmental or epigenetic factors (Villeneuve et al., 2010). Important risk factors for microvascular complications are cigarette smoking and genetic susceptibility to hypertension at early stages of diabetes and poorer glycemic control, higher blood pressure and unfavorable lipid profile at later stages (DCCT Research Group, 1993).

An important role of diabetes *compensation* in development of chronic diabetic complications was proved by multicenter randomized study Diabetes Control and Complication Trial (DCCT) between 1983 and 1993 (DCCT Research Group, 1993). 1441 patients with type 1 diabetes (diabetes duration 1 – 15 years) were enrolled to the study, divided into two groups according to the treatment by either conventional or intensified insulin regimen. Children younger than 13 years did not participate but 195 adolescents were enrolled. The prevalence of micro- and also macrovascular complications was significantly less frequent in the group treated intensively compared to the conventionally treated group. The most of the patients consequently continued in the next study Epidemiology of Diabetes Interventions and Complications (EDIC) where all of the subjects underwent intensified

insulin treatment. After 4 years of EDIC study, the prevalence of chronic complications was still higher in the group initially treated by conventional regimen despite actual good compensation. In the subgroup of adolescents, the intensified therapy led to decrease of the risk of retinopathy by 53%, neuropathy by 60% and microalbuminuria by 60%. In the next following of subjects in EDIC study, these differences were enhanced – patients treated from the beginning by intensified regimen had lower prevalence of retinopathy by 74% and of microalbuminuria by 48% compared to the patients treated conventionally in the first phase. This study showed "the effect of metabolic memory" where each period of worsened compensation can negatively influence the prognosis of the diabetic patient. Some studies with lower number of patients did not confirm the association between diabetes compensation and chronic complications (Javorka et al., 2005).

Diabetes *duration* is also suggested as an important risk factor for development of chronic complications. The significant correlation between diabetes duration and autonomic neuropathy (based on at least one pathological result in two cardiovascular tests – heart rate variability, systolic blood pressure decrease in orthostasis) was confirmed in the biggest study focusing on chronic diabetic complications EURODIAB IDDM Complication Study (EURODIAB IDDM Complication study group, 1994) where 3250 patients with type 1 diabetes at the age 15 – 60 years took part. Similar correlation was not confirmed in some other studies (Scaramuzza et al., 1998) probably caused by different methods and lower number of subjects.

According to the recent information, not only chronic hyperglycemia but also *glycemic variability* may contribute to development of chronic diabetic complications. Acute hyperglycemia may increase circulating cytokines more than continuous hyperglycemia (Esposito et al., 2002). Acute blood glucose fluctuations can induce chronic inflammation, increase oxidative stress markers (Chang et al., 2012) and decrease blood antioxidant - glutathione (Tsai et al., 2012). The Italian study (35 adults with T1D, 33 adults with T2D) showed that except diabetes duration, that most significantly correlated with diabetic retinopathy, also glucose variability may have role as a risk factor of diabetic retinopathy, particularly in the case of acute fluctuations and acute hyperglycemia (Sartore et al., 2013).

Other risk factors were identified – *increase in diastolic blood pressure* ≥ 90 mmHg, *increased triglycerides* > 1,7 mmol/l, *decrease of serum HDL cholesterol* < 1,0 mmol/l, *microalbuminuria* > 20 µg/min, *higher body mass index*, presence of *retinopathy*, *smoking* and the period of *puberty* (Donaghue et al., 2009). The diabetes compensation in prepubertal period has lower impact on complication development compared to the period after gonadarche (Maguire et al., 2005). Many adolescents with type 1 diabetes have frequently worsened compensation due to endocrine changes in puberty leading to increase of insulin resistance (insulin like growth factor IGF-1, sexual hormones) (Court et al., 2008) but also due to excessive food intake, lack of physical activity, neglect of insulin treatment and specific behavior (alcohol, smoking, drugs, contraceptives).

Despite long lasting hyperglycemia, another significant risk factor is *genetic predisposition* of the subject due to gene polymorphisms of genes encoding various enzymes involved in the pathogenesis of origin and development of chronic complications. Probably other factors exist – immunologic, environmental, epigenetic, etc., which are not clear at present.

Treatment of Chronic Diabetic Complications

Treatment of chronic complications is complex and exceeds the purpose of this chapter. It primarily involves the improvement of metabolic control of diabetes (tight control of glycemia, lipid profile and blood pressure). Patients with type 1 diabetes are treated by intensified insulin regimen and in patients with type 2 diabetes, administration of selected per-oral anti-diabetics or insulin therapy is considered. Physical activity, life style modification, quit smoking and dietary management also contribute to adequate compensation and to a delay of the onset of chronic complications. Actually used management of diabetic neuropathy includes supportive (thioctic acid, vitamins, antioxidants) and symptomatic treatment (e.g. magnetotherapy, painkillers, beta blockers) while other therapeutic possibilities are experimental, till now (inhibitors of aldose reductase, selective inhibitor of protein kinase C beta, C peptide substitution, drugs preventing formation of AGEPs). Proliferative retinopathy (and also macular edema) can be effectively treated by laser photocoagulation of retina, vitrectomy (surgical removal of the vitreous gel) may be used in the case of severe bleeding or retinal detachment. Possibilities of medicament treatment include intravitreal injections of anti-VEGF (vascular endothelial growth factor), oral fenofibrate or anti-hypertensive therapy. In diabetic nephropathy, restriction of protein and salt intake and normalization of blood pressure is recommended. For initial treatment angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers are used, later calcium channel blockers, diuretics or beta-blockers can be added. Use of anti-platelet agents is effective for secondary prevention of diabetic macroangiopathic complications, HMG-CoA reductase inhibitors (statins) are effective for primary and secondary prevention of coronary artery disease and cerebral infarction. Treatment of coronary artery disease and cerebral infarction is the same as in non-diabetic patients. Possibilities in the management of limb ischemia depend on the stage of disease and include muscle training, medications (anti-platelet therapy, infusion of prostaglandins), percutaneous transluminar angioplasty, dermoepidermal grafts (Strelka et al., 2013), infection treatment, local therapy of ulceration which is to prevent limb amputation.

Current possibilities and perspectives in the management focus on pathophysiological pathways with effort to intervene in them so the knowledge about etiopathogenesis of diabetic complications may help to improve the therapeutic approach in future.

Oxidative Stress

Oxidative stress is the result of imbalance between the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and their destruction by antioxidant mechanisms.

Reactive Oxygen and Nitrogen Species

Oxidation – reduction (redox) reactions are vital for respiration, metabolism and energy supply. Endogenous ROS are generated by partial reduction of oxygen by multiple

mechanisms in mitochondria, peroxisomes, endoplasmic reticulum and in the cell membranes. The main source of production of ROS are mitochondria which can convert energy from nutrients into a usable form, adenosine triphosphate (ATP). This process called oxidative phosphorylation involves transport of protons across the inner mitochondrial membrane by means of the electron transport chain – a series of proteins that transfer electrons via redox reactions with gradually increasing electrochemical proton gradient. In the last reaction of this chain, electron reacts with oxygen molecule to give water, however in about 0.1 – 2 % of electrons passing through the chain, oxygen is instead incompletely reduced to generate the superoxide radical (.O2-). Important enzymes able to produce superoxide are NADPH oxidase, xanthine oxidase and cytochrome P450. Superoxide can interact with other molecules, promote production of other ROS like hydrogen peroxide (H2O2), hydroxyl radical (OH-) or hydroperoxyl radical (HOO.), inactivate specific enzymes or initiate further chain reactions. Peroxyl radical has special significance because of its involvement in lipid peroxidation. The hydroxyl radical is extremely reactive, immediately removes electrons from any molecule turning this molecule into a free radical and thus propagating chain reaction. Hydrogen peroxide has lower activity however due to longer time for traveling into the nucleus of the cell, it is more harmful for macromolecules such as DNA. Lipid peroxidation, oxidative degradation of lipids resulting in damage of cell membranes and cells, consists of three major steps. In initiation, peroxyl (HOO.) or hydroxyl radical (OH-) react with unsaturated fatty acid to generate water and fatty acid radical (R.). In propagation reaction, fatty acid radical reacts readily with oxygen (O2) to create peroxyl-fatty acid radical (ROO.). This radical is also unstable and reacts with another free fatty acid to produce new fatty acid radical and lipid peroxide (ROOH). The propagation process continues and can consume valuable polyunsaturated fat and create quantity of lipid peroxide. The radical reaction can stop (termination phase) when two radicals react together to generate an inactive product. The end products of lipid peroxidation are reactive aldehydes such as malondialdehyde, potentially mutagenic compound, or 4-hydroxynonenal which is suggested to play role in cell signal transduction and gene expression. In addition to endogenous ROS, also exogenous ROS exist which can be produced form tobacco, smoke, pollutants, drugs, xenobiotics or radiation.

RNS are molecules generated from the reaction of superoxide and nitric oxide (NO) to form peroxynitrite (ONOO-). This reaction depletes the bio-activity of NO, an important endothelial mediator contributing to vessel homeostasis by inhibiting vascular smooth muscle contraction, aggregation of platelets and leukocytes adhesion to endothelium. Peroxynitrite itself can react with many biological substrates like lipids, thiols, amino acids or nucleotides. It can modify metalloproteins such as hemoglobin, myoglobin and cytochrome c by oxidizing ferrous heme into its ferric form. Peroxynitrite can cause cysteine oxidation, indirectly can lead to tyrosine nitration what contributes to damage of proteins, change in catalytic activities of enzymes, impairment of cytoskeletal organization and cell signal transduction.

To determine the quantity of oxidative stress, the establishment of the products of lipid peroxidation, nitro-tyrosine, carbonyl, malondialdehyde, substances reacting with an thiobarbituric acid or organic hydroperoxides is used.

Under physiological conditions ROS perform several functions such as catalytic oxidation of endogenous and exogenous substrates by cytochrome P450, destruction of microorganisms in phagocytes and reduction of oxygen into water in the respiratory chain. ROS also act as signaling molecules, they can induce the expression of many genes and

trigger apoptosis. On the other hand, the excessive production of ROS may lead to damage of proteins, carbohydrates or nucleic acids and if ROS-mediated damage is too much, cell undergoes apoptosis. Similarly, RNS can inactivate enzymes, lead to poly-ADP-ribosylation, mitochondrial dysfunction, impaired stress signaling and protein nitration. Isoprostanes are prostaglandin-like compounds arisen from peroxidation of essential fatty acids (mainly arachidonic acid) through catalyzation by ROS without the direct action of cyclooxygenase enzymes. Oxidative stress, as the consequence of destabilization between ROS / RNS and antioxidants, may be involved in the pathogenesis of many diseases like cancer, cardiovascular, neurological, pulmonary, metabolic and endocrine disorders.

Antioxidant Mechanisms

To protect tissues from oxidative damage, endogenous and exogenous antioxidant systems are involved. They are divided to *enzymatic antioxidant mechanisms* – glutathione S-transferase, superoxide dismutase, catalase, uncoupling proteins, paraoxonase and *non-enzymatic mechanisms* – vitamin C, E, carotenoids, coenzyme Q_{10}, uric acid, bilirubin, trace elements such as zinc, manganese, copper and nickel. According to the place of action antioxidants may be divided into primary, secondary and tertiary. Primary antioxidants act in the prevention of formation of new ROS, like superoxide dismutase (SOD), catalase, glutathione peroxidase and metal-binding proteins (ferritin, ceruloplasmin). Secondary antioxidants can scavenge reactive species, thereby avoiding the further chain reactions, such as vitamin E, vitamin C, beta-carotene, bilirubin, albumin and uric acid. Tertial antioxidants can repair bio-molecules that were damaged by ROS. To this group enzymes repairing DNA belong. Cumulative capacity of all antioxidants in serum is represented by total antioxidant status (TAS).

Non-Enzymatic Antioxidant Systems

As non-enzymatic antioxidants in humans several hydrophilic and hydrophobic compounds are available. Hydrophilic substrates are conjugated bilirubin, uric acid, glutathione, polyunsaturated fatty acids and vitamin C; among hydrophobic compounds belong coenzyme Q_{10} (ubiquinone), vitamin E, beta-caroten and other carotenoids.

Bilirubin, a bile pigment, is the end product of heme catabolism regarded as potentially cytotoxic compound soluble in fats (as unconjugated). It is associated with neonatal jaundice with possibility to lead to irreversible brain damage. On the other hand, in micromolar concentration it acts as a scavenger of the chain-carrying peroxyl radicals. Unconjugated as well as conjugated bilirubin can protect LDL-cholesterol from lipid peroxidation against peroxyl radicals (Wu et al., 1996). The metabolism of bilirubin is associated with enzyme hemoxygenase (HO) that for NADPH consumption cleaves heme to form carbon monoxide (CO), iron and biliverdin. Biliverdin is consequently converted into bilirubin by biliverdin reductase. Till now, three isoforms of HO were described: inducible isoform (HO-1) and constitutive izoforms (HO-2, HO-3). HO-1 is inducibile izoenzyme that may be activated by inflammatory cytokines (IL-1, IL-6, TNF-α, IFN-γ), by oxidants (superoxide, peroxynitrite, hydrogen peroxide, ozone), bacterial toxins, viruses and by hyperglycemia. HO-2 is

constitutive enzyme expressed in most tissues mainly in brain and testes. Izoform HO-3 was found in brain, liver and kidneys.

Uric acid is generated by metabolism of purine nucleosides from xanthine and hypoxanthine by enzyme xanthine oxidase and it is consequently excreted in the urine. Uric acid is regarded as paradoxical molecule as it may have function either as antioxidant (primarily in plasma) or as pro-oxidant (primarily within the cells) (Sautin and Johnson, 2010). Uric acid is able to scavenge ROS and prevent lipid peroxidation. Important sites where this antioxidant effect of uric acid has been proposed are in central nervous system protecting neuronal cells and in plasma protecting erythrocytes from oxidative stress. While chronic increase of uric acid may be associated with increased risk of stroke and cardiovascular diseases (Storhaug et al., 2013), acute elevation of uric acid may provide antioxidant protection. Uric acid is suggested to decrease acute activation of proinflammatory cells and decrease bio-availability of nitric oxide in the endothelial cells (and thus prevent formation of peroxynitrite). For its ability to scavenge superoxide, the presence of ascorbic acid and thiols is required. Uric acid can scavange the carbon-centered and peroxyl radicals in the hydrophilic medium (such as plasma) however cannot scavenge lipophilic radicals within lipid membranes. On the other hand, uric acid may act as pro-oxidant by forming ROS in reaction with other oxidants, predominantly in lipids. Concurrently, hydrophobic environment (in lipids) is unfavorable for antioxidant function of uric acid. High uric acid can directly induce insulin resistance by inhibiting insulin receptor substrate 1 and Akt insulin signalling (Zhu et al., 2014).

Glutathione is a tripeptide (L-gamma-Glutamyl-L-cysteinyl-glycine) in which the thiol group of cysteine serves as proton donor and is responsible for biological antioxidant activity of glutathione. Glutathione is able to reduce disulfide bonds formed within cytoplasmic proteins to cysteines and to donate proton to other unstable molecules like ROS. In this reaction, glutathione (GSH) itself becomes reactive but easily reacts with another reactive GSH to give oxidized form, glutathione disulfide (GSSG). This oxidized glutathione can be reduced back by enzyme glutathione reductase using NADPH. In healthy cells, more than 90% of total glutathione exists in the reduced form and less than 10% is in oxidized disulfide form. The ratio of reduced to oxidized glutathione is often used to measure oxidative stress and cellular toxicity. Glutathione is the major endogenous antioxidant that can directly react with ROS and can maintain exogenous antioxidants (vitamin C and E) in their reduce forms. It has also function in regulation of nitric oxide cycle, in synthesis and repair of DNA, in protein, prostaglandin and leukotriene synthesis, in amino acid transport, in iron metabolism and in detoxification. It is an inhibitor of melanin synthesis and serves as cofactor for enzyme glutathione peroxidase. According to recent information, among compounds that promote the synthesis of glutathione belong alpha lipoic acid and vitamin D (Jain and Micinski, 2013); sylimarin helps to prevent the depletion of glutathione in the liver. Dietary sources of glutathione are fresh fruits and vegetables (asparagus, potatoes, peppers, carrots, onion, cabbage, cauliflower, broccoli, avocados, spinach, garlic, tomatoes, grapefruit, apples, oranges, melon), fresh uncooked meats, and in moderate amounts in dairy products and eggs. Selenium, an important cofactor for the glutathione peroxidase (enzyme form of glutathione), is found in cereals, oats, walnuts, legumes, tuna, beef, poultry, cheese and eggs.

Polyunsaturated fatty acids. The susceptibility of fatty acids to oxidation is suggested to be directly dependent of their degree of unsaturation. Fatty acid miceles were able directly scavenge superoxide in an unsaturation – dependent manner, while the most effective fatty

acid was eicosapentaenoic acid. Supplementation of human aortic endothelial cells with polyunsaturated acids of the omega-3 series resulted in lower formation of ROS compared to the cells supplemented with saturates, monounsaturates or polyunsaturates of the omega-6 series (Richard et al., 2008). Linoleic acid has been promoted for its cholesterol lowering effect however it has been shown that dietary linoleic acid favors oxidative modification of LDL cholesterol and increases aggregation of platelets. On the other hand, alpha-linolenic acid intake is associated with lowering of blood pressure and inhibitory effects on platelets aggregation (Simopoulos, 2004). Polyunsaturated fatty acids can modulate the inflammatory response and seem to have beneficial effect in patients with chronic inflammatory diseases including diabetes mellitus (Guadarrama-Lopez et al., 2014). Dietary sourced rich in polyunsaturated fatty acids are plant-based oils (solybean oil, corn oil, sunflower oil) and fish (salmon, mackerel, herring, trout).

Melatonin is both fat- and water-soluble hormone produced mainly by pineal gland (also by retina and gastrointestinal system). It is involved in the circadian rhythms of several biological functions, especially sleep and its production is inhibited by the light. Melatonin is also powerful antioxidant as it can directly scavenge ROS and RNS (such as superoxide, hydroxyl radical and nitric oxide), it stimulates the action of other antioxidants, increases the efficiency of mitochondrial oxidative phosphorylation and reduces electron leakage (thus lowering generation of superoxide) and it can protect nuclear and mitochondrial DNA (Reiter et al., 2003). Melatonin has also immunomodulatory effect, anti-aging properties, it influences intestinal motility and acts as antagonist of pituitary gonadothropins. In many countries it is available in tablet form (on prescription or as dietary supplement) and seems to be effective in the treatment of delayed sleep phase disorder.

Vitamin E (chemically methylated phenols) exists in eight different forms: alpha-tocopherol, beta-tocopherol, gamma-tocopherol, delta-tocopherol and four types of tocotrienols (α, β, γ, δ), which differ in chemical structure (number and position of methyl groups on the chromanol ring) and biological activity. Alpha-tocopherol is the form of vitamin E preferably absorbed in humans. Vitamin E has positive effects on the formation of sex organs, increases fertility and protects cell membranes (especially in nervous, respiratory system and in erythrocytes) against peroxidation of polyunsaturated acids. Tocopherol, as donor of hydrogen atom of the phenolic group, can intercept the peroxyl radical ($ROO^{\cdot}$) more quickly than polyunsaturated fatty acid thus preventing lipid peroxidation. Formed phenoxy radical may react with vitamin C, coenzyme Q, reduced glutathione or other free radical with consequently formed oxidized tocopherol which is eliminated in the biles. Vitamin E also acts as an anticoagulant, contribute to membrane stability and inhibits the protein kinase C. The main source of vitamin E is represented by vegetable oils (wheat germ, sunflower, almond, olive, peanut oils), nuts (almond, hazelnut, peanut), seeds, whole grains, then butter, milk, tomatoes, asparagus, greens and carrots. Deficiency of vitamin E may cause neuromuscular disorders, anemia (due to oxidative damage of erythrocytes) and fertility problems, excessive amounts of vitamin E may lead to bleeding.

Vitamin C (L-ascorbic acid), an essential nutrient, is a cofactor for several enzymatic reactions. It is important in the metabolism of hydroxylysine and hydroxyprolin, amino acids necessary for the synthesis of collagen that is necessary in wound-healing and in preventing bleeding from capillaries. It is involved in the synthesis of carnitine, neurotransmitters, in the synthesis and catabolism of tyrosine. Vitamin C promotes iron absorption, acts as natural antihistamine, stimulates the leukocytes formation, development of bones, teeth and cartilage.

It acts as reducing agent, donating electrons to various enzymatic and also non-enzymatic reactions. Ability to reduce tocopheryl radical is involved in the antioxidant defense of the cells. Oxidized forms of vitamin C can be reduced by glutathione and NADPH-dependent enzymatic mechanisms so the presence of glutathione helps to maintain vitamin C in a reduce form. Deficiency of vitamin C leads to scurvy (bleeding from mucous membranes, spongy gums, bruises because of capillary fragility, fatigue) and chronic hypovitaminosis C may lead to atherosclerosis and increased risk of cardiovascular diseases. As vitamin C is water soluble and is excreted to the urine, overdose is not toxic. Dietary sources of vitamin C include a variety of fruits (citruses, berries), vegetables (chilli pepper, tomatoes, potatoes, greens) and plants (rose hip).

Coenzyme Q_{10} (ubiquinone) is lipophilic vitamin-like substance present primarily in the inner membrane of mitochondria and also in membranes of endoplasmic reticulum, peroxisomes, lysosomes and vesicles. As it is a component of the electron transport chain, it participates in aerobic respiration producing cell energy in the form ATP. Three redox states of coenzyme Q_{10} exist – ubiquinone (fully oxidized), ubisemiquinone (semiquinone) and ubiquinol (fully reduced). Fully oxidized form has the function in electron transport chain while fully reduced form plays role as antioxidant. Coenzyme Q_{10} inhibits the initiation and the propagation of lipid peroxidation as well as protein oxidation. It regenerates vitamin E and prevents the oxidation of DNA bases mainly in mitochondrial DNA. Circulating coenzyme Q_{10} in LDL cholesterol prevents the oxidation of LDL. There are two sources of coenzyme Q_{10} – endogenous and exogenous. In the multistep biosynthesis also HMG Co-A reductase plays role, so the treatment with statins lead to decreased production of coenzyme Q_{10} (causing myopathy). Among rich source of dietary coenzyme Q_{10} belong meat and fish, less amount is found in vegetables (parsley, broccoli) and some fruit (avocado).

Carotene is fat soluble orange pigment necessary for photosynthesis, found in carrot, many other fruits, plants and vegetables (e.g. sweet potatoes, tomatoes, pepper, pumpkin, chanterelle, strawberries, apricots, oranges, apples, orange cantaloupe melon, spinach, broccoli, greens) and also in lower concentration in milk-fat, eggs and butter. It is chemically tetraterpen (unsaturated hydrocarbon, $C_{40}H_x$), synthesized from eight isoprene units, while beta-carotene (red-orange pigment) has beta rings at both ends of the molecule. Antioxidant activity of carotenoids results from their ability to scavenge peroxyl radicals. Moreover, they can reduce ferric ions which play role in catalyzing LDL oxidation leading to atherosclerosis. Beta-carotene is a precursor for vitamin A through the enzyme beta-carotene 15,15´-monooxygenase found in liver and small intestine. This reaction is regulated by the status of vitamin A so beta-carotene is very safe source of vitamin A and increase intake of beta-carotene does not lead to hypervitaminosis A. Excessive consumption of beta-carotene can lead to benign carotenodermia (orange skin) however chronic high intake of synthetic beta-carotene supplements has been associated with increase risk of lung cancer in smokers, prostate cancer or intracerebral hemorrhage.

Flavonoids are polyphenolic plant secondary metabolites, sometimes reffered as vitamin P. They can be classified into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Several hundreds of flavonoids have been identified till now and among the most known belong resveratrol, silymarine, luteolin, baicalein, and scutellarein. Many of flavonoids have antioxidant function as they can inhibit ROS and RNS. Flavonoids have also been proposed to inhibit pro-inflammatory activity of enzymes involved in production of reactive species such as cyclooxygenase, lipoxygenase or inducible nitric

oxide synthase. They can modify intracellular signaling in immune cells and seem to have also anti-microbial (antibacterial, antiviral and antifungal), anti-cancer, anti-platelet and anti-allergic activity. In several studies have been shown that flavonoids can reduce the risk of atherosclerosis and hypertension, improve endothelial and capillary function, regulate carbohydrate metabolism, modify blood lipid concentrations and reduce the risk of cancer. Sources rich in flavonoids include parsley, onions, berries, bananas, citrus fruit, aronia, sea-buckthorns, dark chocolate and beverages (red wine, black and green tea, beer, coffee).

Enzymatic Antioxidant Systems

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide to give oxygen and hydrogen peroxide. This reaction includes following steps:

$$M^{(n+1)+} - SOD + O_2^- \rightarrow M^{n+} - SOD + O_2$$
$$M^{n+} - SOD + O_2^- + 2H^+ \rightarrow M^{(n+1)+} - SOD + H_2O_2,$$

where M is metal cation e.g. Cu (n=1), Mn (n=2), Fe (n=2), Ni (n=2).

Three isoforms of SOD are known. SOD1 (21q22.1) is homodimer localized in the cytoplasm and in the mitochondrial intermembrane space, SOD2 (6q25.3) is tetramer in mitochondria and SOD3 (4p15.3 – p15.1) is tetramer localized extracellularly. SOD1 and SOD3 contain copper and zinc, SOD2 containts manganese (MnSOD2). As superoxide is one the main ROS in the organism causing further chain reactions, SOD serves as the key enzyme of antioxidant mechanism.

Catalase (11p13) is enzyme localized in peroxisomes with the function in degradation of hydrogen peroxide into water and oxygen. As hydrogen peroxide is harmful compound leading to damage of cellular components, catalase belong to important antioxidant defense mechanisms. Catalase is tetramer containing porphyrin heme group (iron cation) with very high specific activity. It can also catalyze the oxidation of several metabolites and toxins (e.g. formaldehyde, formic acid, phenols, acetaldehyde, alcohols) using hydrogen peroxide.

Glutathione peroxidase (Gpx) is the family of enzymes with peroxidase activity that can reduce lipid hyprogenperoxides into corresponding alcoholes and reduce hydrogen peroxide into water and oxygen. Till now, eight isoforms have been identified which differ in chemical structure, cellular localization and substrate specificity. Gpx-1 (3p21.3) is the most studied ubiquitous intracellular cytoplasmic enzyme whose preferred substrate is hydrogen peroxide. Gpx enzymes, whose antioxidant activity depends on the presence of selenium, mainly catalyze the reaction in which two molecules of reduced glutathione (GSH) react with hydrogen peroxide to form oxidized disulfide glutathione (GSSG) and water. Next, glutathione reductase (flavoprotein, 8p21.1) reduces the oxidized glutathione by use of NADPH.

Paraoxonase (PON) (7q21.3) is a group of enzymes associated with HDL cholesterol which plays role in hydrolysis of organophosphates and has also antioxidant potential. Three genotype forms of paraoxonases (PON) have been described till now. PON1 is synthesized in the liver and transported together with HDL into plasma. Its function is to prevent oxidation of LDL cholesterol thus reducing the risk of atherosclerosis. PON1 gene is activated by

PPAR-γ and inflammatory cytokines and LDL cholesterol serum level influence plasma concentration of PON1. PON2 is membrane-bound ubiquitously expressed enzyme (brain, liver, kidney, testes, other) which protects cells against oxidative damage. PON3 has different substrate specificity as PON1, it can also inhibit the oxidation of LDL cholesterol and primarily is expressed in the liver (in low concentration also in kidneys). Its concentration is not influenced by inflammatory factors and oxidized lipids.

Uncoupling proteins (UCP1, 2, 3) are proteins in the inner mitochondrial membrane which are important in oxidative phosphorylation process, thermogenesis and also in protection against reactive oxygen and nitrogen species. UCP proteins reduce inner membrane potential through dispersion of protein gradient over mitochondrial membrane and so mitochondrial production of reactive oxygen species is decreased. Function and localization of UCP 1, 2 and 3 is shown in Table 5.

Table 5. Localization and function of uncoupling proteins

	Localization	**Function**
UCP1 (4q28-q31)	Brown fat	Production of heat, reducing energy performance through distraction of proton gradient without generating ATP
UCP2 (11q13)	Spleen, thymus, beta cells of pancreas, heart, lung, white and brown fat, stomach, testes, macrophages, brain, kidneys, liver and muscles	Termogenesis, antioxidant function, neuroprotection, export of fatty acids from mitochondria
UCP3 (11q13.4)	Striated muscles, heart	Termogenesis, neuroprotection

Metallothionein (MT) is a family of cystein-rich proteins which are localized in the membrane of the Golgi apparatus. Through the thiol group of its cysteine residues MT enzymes have the capacity to bind physiological metals (zinc, copper, selenium) as well as xenobiotic heavy metals (cadmium, mercury, arsenic, silver). They are primarily synthesized in the liver and kidneys, they are involved in regulation of physiological metals (zinc and copper) and may provide protection against metal toxicity and also against oxidative stress. Cysteine residues can capture ROS like superoxide and hydroxyl radicals while cysteine is oxidized to cystine and bound metal ions are liberated. In humans, four main isoenzymes exist – MT1, MT2, MT3 and MT4.

Beta-carotene 15,15´-monooxygenase is an enzyme which catalyzes the cleavage reaction of beta-carotene using oxygen to generate two molecules of retinaldehyde. The enzyme is present in liver and small intestine and is enhanced by the presence of bile salts and thyroxine.

Glutathione S-transferase is a superfamily of enzymes with detoxification and antioxidant functions, which are also involved in cell signaling, regulation of gene expression and in synthesis and degradation of some important bio-molecules. Their meaning is described in separate section below.

Glutathione S-Transferase

Glutathione S-transferase (GST) represents huge superfamily of isoenzymes with a variety of functions. Three major families of GSTs exist – cytosolic, mitochondrial (both soluble) and microsomal (membrane-associated proteins in eicosanoid and glutathione metabolism, MAPEG).

The best known is their detoxification function as GSTs catalyze the conjugation of reduced glutathione (GSH) with non-polar substances containing an electrophilic carbon, nitrogen or sulphur atom resulting in less reactive compounds to be better eliminated. Substrates for the reaction are chemical carcinogens, environmental pollutants, drugs and anti-tumorous substances. The broad substrate specificity of GSTs allows them to protect cells against a range of toxic chemicals, however this GST activity can be deleterious in certain cases, as it can contribute to chemoresistance against anti-neoplastic agents in the treatment of cancer or the arisen metabolite can be more toxic than the primary molecule. Regarding their function within oxidative stress, GSTs detoxify also endogenous unsaturated aldehydes, epoxides and hydrogen peroxides arisen as the secondary metabolites generated during oxidation of membranes or other cellular constituents and thus protect cells from peroxide-induced cell death. GSTs are also involved in the biosynthesis of eicosanoids, prostaglandins, testosterone and progesterone and in degradation of aromatic amino acids phenylalanine and tyrosine. They act in cell signalling pathway as potential regulators of apoptosis. GSTs can antagonize expression of genes trans-activated by the peroxisome proliferator-activated receptor gamma (PPARgamma) and may enhance gene expression driven by nuclear factor-kappaB (NF-κB) (Hayes et al., 2005).

Cytosolic GSTs in mammals are dimeric with subunits of 199 – 244 amino acids. According to substrate specificity, chemical affinity, structure, sequence and kinetic behavior, seven classes of cytosolic GSTs have been identified with few isoenzymes in each class – Alpha (A1 – A5), Mu (M1 – M5), Pi (P1), Sigma (S1), Theta T1 – T2), Omega (O1 – O2) and Zeta (Z1). Mitochondrial GST is represented only by class Kappa GST (K1). These isoenzymes are dimeric and comprise subunits of 226 amino acids. Most enzymes of MAPEG family are involved in the production of eicosanoids. Six human MAPEGs have been identified and belong to subgroups I, II and IV.

Huge inter-individual variability exists in expression of these genes that is the cause of change of their activity even completely loss of activity. The most researched is glutathione S-transferase mu 1 (GST M1) enzyme in GST M class with its gene located in Chromosome 1p13.3, glutathione S-transferase theta 1 (GST T1) enzyme in GST T class with its gene located in Chromosome 22q11.23 and glutathione S-transferase pi 1 (GST P1) with its gene located in Chromosome 11q13. It has been shown that individuals carrying the *null* genotype of GST M1 and T1 have significantly reduced activity of the enzyme compared to *wild* (sometimes referred as *present*) genotype carriers (Datta et al., 2010). Glutathione S-transferase P1 *Val/Val* genotype has significantly reduced plasma GST activity compared to *Ile/Ile* and *Ile/Val* genotypes (Karam et al., 2012). Likewise, differences in tissue expression exist. Expression of GST P1 is higher in liver, ovaries and lung, GST T1 expression seems to be higher in liver, kidney and lung and the expression of GST M1 is higher in liver, kidney, lung, stomach and gonads compared to other organs (Knight et al., 2007). The expression of certain GST isoforms is also changed during carcinogenesis.

Gene polymorphisms of GST T1 and M1 have been described to be associated with various diseases. Meta-analysis of 12 studies (a total of 2040 cases and 2462 controls) showed that *null* genotypes of GST M1 and T1 were associated with higher risk of essential hypertension, while no association was found in GST P1 polymorphisms (Eslami & Sahebkar, 2014). In prospective Italian study with 231 patients with T2D (average age 66.1 ± 9.7 years) was found that coronary artery disease (confirmed in 80% patients) positively correlated with male sex, smoking and the presence of GST T1 *null* genotype. Patients with GST M1 *null* / T1 *null* genotype had higher risk of atherosclerosis of concurrently three coronary arteries (Manfredi et al., 2009). Meta-analysis of 19 studies (a total of 8020 patients and 11,501 controls) referred that GST M1 *null* / T1 *null* genotype as well as isolated occurrence of *null* allele represented the risk factor for coronary heart disease (Wang J et al., 2010). Different results were brought by the study of Indian authors who found that the frequency of GST T1 *null* genotype was significantly lower in 197 patients with angiographically confirmed ischemic heart disease compared to 198 healthy individuals (Girisha et al., 2004). Similarly, GST M1 *null* genotype was significantly rarer in patients with previous acute myocardial infarction (Wilson et al., 2000). According to Danish authors, GST T1 and M1 polymorphisms were not associated with risk of ischemic vascular disease or with markers of inflammation (Norskov et al., 2011). Regarding ischemic cerebrovascular disease, GST T1 and M1 genotypes were not associated with this risk (Um et al., 2006). In population of adult male smokers at the age 20 – 59 years was found that GST T1 *null* genotype was associated with reduced concentration of HDL cholesterol and increased concentration of conjugated diens (Lee et al., 2010). According to other studies *null* genotypes of both GST T1 and M1 are associated with higher triglyceridemia and lower concentration of HDL cholesterol in both sexes (Maciel et al., 2009) or just in women (Saadat M, 2007). In adult patients with coronaroangiographically proved stenosis of coronary artery was described significantly increased concentration of LDL particules in subjects with GST T1 *null* genotype compared to wild genotype. In the case of GST M1 similar relation was not confirmed (Ueno et al., 2009).

GST gene polymorphisms have been described also in relation with bronchial asthma. Some studies claim that GST T1 *null* genotype is associated with bronchial asthma in children (Babusikova et al., 2009). According to another studies the prevalence of GST M1 *null* genotype was significantly higher and prevalence of GST P1 *Val/Val* genotype was significantly lower in asthmatic children and lung function was decreased in patients with GST M1 *null* and GST P1 *Ile/Ile* genotype (Karam et al., 2012). Meta-analysis of 14 studies (2292 adults with asthma and 5718 healthy controls) proved that GST M1 *null* and T1 *null* genotype represented increased risk of asthma especially in non-smokers (Saadat M & Ansari-Lari, 2007).

Association between GST polymorphisms and primary open angle glaucoma was investigated in meta-analysis of 14 studies (1711 cases and 1537 controls) studying GST M1 genotypes and 10 studies (1306 cases and 1114 controls) regarding GST T1 genotypes. It has been shown that in overall population GST M1 *null* and T1 *null* genotypes did not significantly increased the risk of glaucoma. The results by ethnicity showed that the association between GST M1 *null* genotype and risk for primary open angle glaucoma was statistically significant in East Asians but not in Caucasians and Latin Americans. No significant association was confirmed between GST T1 genotype and risk of glaucoma in either ethnic population (Lu et al., 2013). In relation to endometriosis meta-analysis of 14

studies about GST M1 genotype (with enrolled 1539 patients and 1805 controls) and 9 studies about GST T1 genotype (746 patients and 834 controls) was done. After correction of bias no difference in prevalence of endometriosis in women with GST M1 *null* genotype compared of GST M1 *wild* genotype was found however GST T1 *null* genotype was associated with higher risk of endometriosis (Guo, 2005).

Many of above results are conflicting probably in relation to number of enrolled subjects, regional and race differences. If an association between GST gene polymorphisms and the risk of certain disease was demonstrated, in majority of studies this increase of the risk was slightly (odds ratio between 1.2 and 2.0). Monitored diseases are polygenic so their etiopathogenesis is complex involving individual genetic predisposition, environmental, immunologic and lifestyle factors. Knowledge about contributing of GST polymorphisms to this pathogenesis may give base to individual approach to the patient and perhaps "customized therapy" in the future.

According to meta-analysis including 1085 cases and 2396 controls, individuals with at least one *null* genotype of GST M1 and T1 had higher susceptibility to hepatocellular carcinoma however probably there was no direct interactive effect of GST M1 and T1 genotypes in the risk of hepatocellular carcinoma (Sui et al., 2014). Another meta-analysis (a total of 33 studies with 4232 cases and 6601 controls) found that *null* genotype of GST M1 and GST T1 increased the risk of hepatocellular carcinoma. When stratified by ethnicity, significant results were observed among East Asians and Indians while no evidence of significant associations was found among Caucasian and African population. In the gene – gene interaction analysis, individuals with combined deletion in both genes were at higher risk for hepatocellular carcinoma (Shen et al., 2014). Meta-analysis of six studies (with a total of 1843 participants) observed that there was no association between GST P1 *Ile105Val* polymorphisms and risk of hepatocellular carcinoma. Subgroup analysis by ethnicity showed no association in Asians however *Val/Val* GST P1 genotype was associated with decreased risk of carcinoma in European compared to *Ile/Ile* and *Ile/Val* (Zhao et al., 2013).

Meta-analysis dealing with association between GST M1 gene polymorphisms and risk of colorectal cancer (a total of 17 studies, 5907 cases and 9726 controls) showed that GST M1 *null* genotype significantly increased the risk of colorectal cancer in Asians (Cai et al., 2014). Regarding GST P1 *Ile105Val* polymorphisms, the meta-analysis including 29 studies (8160 cases and 10,450 controls) found no evidence of significant association with colorectal cancer (Tan et al., 2013). In 755 patients with colorectal cancer, homozygote carriers of GST M1 (*wild* genotype) had significantly poorer survival after treatment with oxaliplatin than patients not treated with oxaliplatin when compared to non-carriers of GST M1. Neither GST P1 *Ile105Val* nor GST T1 polymorphisms were significantly associated with colorectal cancer survival (Kap et al., 2014). *Null* genotypes of GST T1 and M1 polymorphisms were associated with susceptibility to nasopharyngeal cancer risk as was found in meta-analysis of 15 studies (2226 cases and 3339 controls) (Wei et al., 2013). Similarly, few meta-analyses showed that *null* genotypes of GST T1 and M1 polymorphisms were associated with increased risk of gastric cancer (Saadat M, 2006), bladder cancer (Jiang et al., 2011; Gong et al., 2012) and acute leukemia (Tang ZH et al., 2014) compared to *wild* genotypes.

According to large meta-analysis (55 studies, 15,140 patients and 16,662 controls), a significant association was found between GST T1 *null* genotype and lung cancer risk in the overall populations, however when stratified by ethnicity GST T1 *null* genotype increased this risk in Asians but not in Caucasians and Africans (Yang H et al., 2014). GST P1

Ile105Val polymorphisms were not associated with the susceptibility of lung adenocarcinomas (Zhong et al., 2013). Similarly, lack of association was showed between GST M3 A/B gene polymorphisms and susceptibility to lung cancer (Feng et al., 2012). Regarding GST M1 polymorphisms, some studies claim that GST M1 *null* genotype increased the risk of lung cancer (Liu et al., 2012), however another studies did not confirm this association (Dzian et al., 2012). The chemotherapeutic effect on patients carrying GST M1 *null* genotype was better compared to patients with GST M1 *wild* genotype when platinum drugs were administered (Li et al., 2011).

Relation between GST gene polymorphisms and various carcinomas is complex as it can be influenced by few factors. First, GST enzymes have antioxidant activity and play role in the detoxification of electrophilic carcinogenes, so reduced function of GST may contribute to carcinoma development or worse course of the disease. On the other hand, increased activity of GST enzymes may be responsible for resistance to chemotherapy and thus treatment failure. It follows that for clarifying of those associations further studies are needed.

Oxidative Stress and Diabetes Mellitus

One of the discussed pathomechanisms in the etiology and course of diabetes mellitus is oxidative stress, as an imbalance between production of ROS / RNS and activity of antioxidant systems. ROS are liberated by activated macrophages and T lymphocytes and are induced by proinflammatory cytokines involved in autoimmune process of pancreatic beta cells. Increased level of ROS causes oxidative modification of proteins and nucleic acids and lipid peroxidation. These changes may result in alteration in biological functions of all biomolecules, in metabolism of all tissues and cell damage including beta cells.

In several studies in patients with diabetes higher concentration of products of oxidative stress was described such as increased products reacting with thiobarbituric acid (Krzystek-Korpacka et al., 2008), increased concentration of fructosamine and products of lipid peroxidation (Kostolanská et al., 2009) or higher concentration of malondialdehyde and products with carbonyl groups especially in diabetic patients with hyperfiltration of kidneys (Hernandez-Marco et al., 2009). Similarly, American authors in patients with T1D with average age 20 years found elevated level of malondialdehyde, as the product of lipid peroxidation however the excretion of 8-hydroxydeoxyguanozine, that reflects the oxidative damage of DNA, was not significantly increased (Hoeldke et al., 2009). According to the study comparing the activity of 8-iso-prostaglandin F2 alpha, as a marker of oxidative stress, between children with T1D and healthy controls, significantly increased serum activity of this isoprostane was confirmed in T1D subjects. This marker also positively correlated with values of glycosylated hemoglobin (Rachisan et al., 2014). Patients with T1D, especially with poor glycemic compensation exhibited significantly lower concentration of serum zinc, increased levels of serum copper, increased blood and urine malondialdehyde and 8-hydroxy-2-deoxyguanosine compared to healthy subjects (Lin et al., 2014).

In diabetic patients also changed activity of non-enzymatic and enzymatic antioxidant mechanisms was described**.** Children and adolescents with T1D in Belgian population had significantly lower concentration of vitamin C and reduced activity of glutathione peroxidase in erythrocytes and these concentrations negatively correlated with age of the patient and with

diabetes duration (Ndahimana et al., 1996). On the other hand, some authors do not confirm the relation between diabetes and reduced antioxidant mechanisms. For example, the Belgian authors did not find significant difference in TAC, concentration of vitamins A, E and in oxidized LDL particles in children with T1D (average age 15 years and average diabetes duration 5 years) compared to healthy controls (Willems et al., 1998). Iranian authors studied the influence of dietary intake of vitamin C, vitamin E or their combination on glycemic control parameters and antioxidant enzymes (superoxide dismutase and glutathione peroxidase) in 170 patients with T2D. After three months, fasting plasma glucose and glycosylated hemoglobin were significantly decreased in all three supplementation groups compared to placebo group. Concentration of SOD and GSH enzymes were significantly increased after consumption of vitamins in supplementation groups (Rafighi et al., 2013). Similarly, 123 Indian T2D patients were assigned to receive either standard care or with additional dietary therapy – two low-calorie fruit per day for three months. Dietary intervention resulted in significant reduction of malondialdehyde, plasma glucose, glycosylated hemoglobin and increase in serum concentration of vitamin C and reduced glutathione (Hegde et al., 2013). Meta-analysis including 14 studies with a total of 714 subjects with T2D evaluated the influence of vitamin E supplementation on glycemic control. Increased vitamin E supplementation did not result in significant reduction of fasting glucose, glycosylated hemoglobin and fasting insulin however subgroup analyses revealed a significant reduction in glycosylated hemoglobin and fasting insulin in patients with low baseline vitamin E status (Xu R et al., 2014).

Disorders of melatonin might be also involved in the etiopathogenesis of diabetes. In non-diabetic young women higher nocturnal melatonin secretion was associated with lower insulin concentration and lower insulin resistance (McMullan, Curhan et al., 2013). In further research, the authors identified 370 women who developed T2D and matched 370 controls and found that lower melatonin secretion was independently associated with a higher risk of development of type 2 diabetes (McMullan, Schernhammer et al., 2013). A meta-analysis of 23 studies involving 172,963 subjects showed that gene polymorphisms (rs10830963) of MTNR1B gene encoding melatonin MT2 receptor (G protein-coupled receptor) may be associated with impaired glucose regulation and development of T2D (Xia et al., 2012). These associations may be related to antioxidant function of melatonin or lower concentrations of melatonin may lead to sleep disorders and these can result in decrease of insulin sensitivity (Perfect et al., 2012).

Coenzyme Q_{10} provides the energy for vital cellular functions and acts as antioxidant, as well. In an open label pilot study, 9 subjects with T2D received orally ubiquinol 200 mg / day for 12 weeks and showed significant decrease of glycosylated hemoglobin however no significant changes in blood pressure, lipid profile, oxidative stress and inflammatory markers were found. In five healthy volunteers receiving the same dose of ubiquinol for 4 weeks, the insulinogenic index and ratio of proinsulin to insulin were significantly improved (Mezawa et al., 2012). In a randomized double-blind placebo-controlled trial, 64 T2D patients were randomly assigned to receive either 200 mg coenzyme Q_{10} daily or placebo. After 12 weeks of treatment, concentrations of glycosylated hemoglobin, total and LDL cholesterol were significantly decreased in the group treated by coenzyme Q_{10} (Kolahdouz Mohammadi et al., 2013).

According to Chinese study, serum concentration of phospholipid omega-3 polyunsaturated fatty acids (PUFA) was significantly decreased in patients with type 2

diabetes and non-alcoholic fatty liver disease and negatively correlated with insulin resistance (Lou et al., 2014). A protein enriched low glycemic index diet supplemented with omega-3 PUFA for 24 weeks in 30 obese patients with T2D resulted in significantly decrease of glycosylated hemoglobin, ultrasensitive CRP and waist circumference (Moosheer et el., 2014). However according to double-blind randomized placebo-controlled clinical trial including 37 diabetic patients with non-alcoholic steatohepatitis, receiving of PUFA (2160 mg eicosapentaenoic acid and 1440 mg docosahexaenoic acid) for 48 weeks did not lead to significant change in glucose control, liver enzymes or liver histology and these data provide no support for PUFA in non-alcoholic steatohepatitis (Dasarathy et al., 2014).

Activity of antioxidant enzymes can be increased as a consequence of increased oxidative stress as was found in the case of superoxide dismutase in T1D patients compared to healthy controls (Lin et al., 2014). Interesting work is the study of Serbian authors whose aim group were children with T1D in various phase of diabetes: in initial phase, in post-initial remission and in chronic course. According to their results the activity of catalase was significantly increased in all phases of diabetes compared to healthy controls. The highest activity of catalase was in early phase of diabetes then gradually decreased and its lowest activity was in chronic phase of diabetes. High activity of catalase was in positive correlation with concentration of glycosylated hemoglobin and so with insufficient diabetes compensation (Zivic et al., 2008). Activity of paraoxonase (PON), enzyme with antioxidant potential acting in the prevention of oxidation of LDL cholesterol, was significantly decreased in diabetic patients compared to healthy controls however no significant difference was found in PON gene polymorphisms in relation of T1D and diabetic complications (Ikeda et al., 1998). According to another study, paraoxonase-1 activity and myeloperoxidase activity (as a marker of inflammation) were significantly increased and total antioxidant capacity was significantly decreased in T1D patients compared to healthy controls. Activity of PON-1 negatively correlated with total antioxidant capacity. The authors concluded that increased PON activity may inefficiently compensate for the high level of chronic inflammation and low antioxidant capacity in patients with T1D (Savu et al., 2014).

Gene Polymorphisms of Antioxidant Enzymes and Diabetes Mellitus

Gene polymorphisms of antioxidant enzymes may play role in the etiology of type 1 as well as type 2 diabetes. Human leucocyte antigene (HLA) variants class II (mainly HLA-DQA1, HLA-DQB1 and HLA-DRB1) are the most important genes representing approximately 45% of genetic predisposition to type 1 diabetes. Variable number of tandem repeats (VNTR polymorphisms) of insulin gene (INS) represent around 10% of genetic predisposition to T1D and a lot of other genes, especially associated with the regulation of immune system, have been identified (CTLA-4, FOXP3, CCR5, IL2RA, PTPN22, ITPR3, OAS1, SUMO4). As oxidative stress and low efficiency of antioxidant mechanisms may at least partially contribute to development of T1D, certain gene polymorphisms of antioxidant enzymes may increase the risk of T1D. In Danish Study Group a difference in the manganese superoxide dismutase pattern was observed between sporadic cases of type 1 diabetes and healthy controls (Pociot et al., 1994). According to the Japanese authors, the frequency of *A* allele of the *G/A* single nucleotid polymorphism of the gene for thioredoxin (an antioxidative and antiapoptotic protein) was the highest in patients with T1D when compared to the

controls (Ikegami et al., 2008). Russian authors found strong association between *T1667T* and *C(-262)T* markers of the catalase gene with susceptibility to type 1 diabetes (Christiakov et al., 2004) however in another large case-control study (USA and UK population) no evidence for a major effect of *C1167T* or *C(-262)T* on T1D susceptibility was shown (Pask et al., 2006). Nuclear factor kappa B (NF-κB) is a transcriptional factor influencing the expression of many genes and playing role also in oxidative stress and inflammatory processes. Regarding highly polymorphic (CA) dinucleotide repeat microsatelite in the regulatory region of NF-κB, the presence of *A10* allele (138bp) was significantly more frequent and the presence of *A14* allele (146bp) was significantly less frequent in T1D patients compared to healthy subjects (Hegazy et al., 2001). However other studies did not find association between NF-κB1 gene polymorphisms and the risk of T1D (Martinez et al., 2006). The results may vary according to population, methods or number of enrolled subjects.

Type 2 diabetes is polygenic multifactorial disorder where life style, environmental triggers and genetic predisposition play role. Oxidative stress has been implicated in pancreatic beta-cell damage, insulin resistance and vascular function in diabetic patients and the dysfunction of antioxidant enzymes may be associated with the pathogenesis of T2D. Some genes have been identified to increase the risk of obesity and T2D (PPAR-γ, TCF7L2, PC-1, PGC-1α, IRS-2) and other possible etiology factors are gene polymorphisms of antioxidant enzymes. The study assessing the association between gene polymorphisms of SOD3 (two missense mutations - *Ala40Thr (GCG>ACG)* and *Arg213Gly (CGG>GGG)*, and a silent mutation, *Leu53Leu (CTG>TTG)*) and insulin resistance and the susceptibility to T2D in Japanese adults. In *Ala40Thr* variant, the frequency of *Thr* allele and the number of subjects with *Thr* allele were higher in T2D patients compared to non-diabetic subjects. Patients with *Thr* allele showed earlier age at diagnosis of diabetes, higher prevalence of hypertension and lower insulin sensitivity compared to those without the allele (Tamai et al., 2006). In the meta-analysis of 23 studies was shown significant association between UCP2 *Ala55Val* and UCP3 *-55C/T* polymorphisms and increased susceptibility for T2D in Asians however no significant association of the UCP1 *-3826A/G*, UCP2 *-866G/A* and *Ins/Del* with T2D was observed (de Souza et al., 2013).

Information about association between glutathione S-transferase gene polymorphisms and diabetes mellitus are controversial and vary probably according to the number of enrolled subjects, geographical or methodical differences. Much more studies have been done in adult population with T2D compared to children with T1D. In an endogamous population from north India (321 patients with T2D vs. 309 healthy controls) the proportion of individual genotypes - GST T1 *null*, GST M1 *null* and GST P1 *Val/Val* was higher in diabetic group. Double combination GST T1 *null* / M1 *null* represented significant 2.9- fold increased risk of diabetes. Combination of *null* genotypes at GST T1 and GST M1 loci and *Val/Val* genotype of GST P1 locus showed the highest odds ratio – 9.64 (Mastana et al., 2013). Similarly, GST M1 *null* genotype, GST T1 *null* genotype and *Ile/Val* and *Val/Val* genotypes of GST P1 were associated with increased risk of T2D in patients from the Southern part of India (512 patients with T2D and 270 healthy controls) (Ramprasath et al., 2011). In Brazil population (120 patients with T2D and 147 healthy controls) GST T1 *null* genotype conferred 3.2-fold increased risk to T2D however no association was found between GST M1 polymorphisms and T2D risk. Moreover, GST T1 *null* genotype was associated with higher concentration of triglycerides and VLDL cholesterol and GST M1 *null* genotype was associated with increased concentration of fasting glucose, glycosylated hemoglobin and hypertension, so *null*

genotypes of both isoenzymes may contribute to the clinical course of T2D (Pinheiro et al., 2013). In Egyptian T2D patients (100 cases and 100 controls) was found that GST T1 *null* and GST M1 *null* genotypes, alone or combined, were associated with increased risk of diabetes. Patients with GST T1 *null* genotype had higher concentration of triglycerides and VLDL cholesterol and patients with GST M1 *null* genotype had significantly higher level of glycosylated hemoglobin and higher diastolic blood pressure (Amer et al., 2011). The same collective of authors presented that in 112 patients with T2D was found higher frequency of *Val* allele in exon 5 of GST P1 compared to 188 healthy controls and the presence of *Ile/Val* GST P1 was significantly more common in T2D patients (Amer et al., 2012). The authors from southern Iran found that the frequency of GST M1 *null* genotype and combination GST T1 *null* / M1 *null* was significantly higher in patients with T2D compared to healthy controls and represented increased risk for T2D however the frequency of individual GST T1 and GST P1 polymorphisms did not significantly differ between subgroups (Moasser et al., 2012). According to Turkish authors dealing with 127 patients with T2D and 127 healthy controls, the risk of T2D was significantly increased with GST M1 *null* genotype while GST T1 *null* genotype represented increased risk for T2D only in the combination with other genes (Gonul et al., 2012). In the North Indian population (100 patients with T2D and 200 healthy controls) was found that GST M1 *null* and GST P1 *Ile/Val* genotype were associated with T2D while no association was confirmed in GST T1 polymorphisms. When considering multiple association, GST M1 *null*, T1 *present* and P1 *Ile/Ile* demonstrated and increase in T2D risk (Bid et al., 2010). The only study dealing with GST A1 polymorphisms, from Japanese authors, found that the frequency of GST A1 *B allele was higher in patients with T2D (n=63) compared to healthy subjects (n=405) and the risk for T2D among the GST A1 *B carriers was significantly increased by current smoking status. In this study GST P1 genotype did not affect the risk of diabetes (Oniki et al., 2008). The same collective of authors demonstrated that GST T1 *null* and dual GST T1 *null* / GST M1 *null* genotypes are independent risk factors for T2D development and this risk is further increased with current smoking (Hori et al., 2007). In Chinese population, GST T1 *null* genotype conferred the increased risk for T2D while no association was found between GST M1 polymorphisms and risk of T2D. Patients with GST T1 *null* and also with GST M1 *null* genotype had higher levels of LDL cholesterol, apolipoprotein B and lipoprotein A (Wang G et al., 2006). The meta-analysis of 9 studies (a total of 1354 cases with T2D and 1666 controls) confirmed the significant association between T2D and GST T1 *null* as well as GST M1 *null* genotype while no significant association was found in GST P1 gene polymorphisms and T2D (Tang et al., 2013). Similarly, another meta-analysis of 11 studies (a total of 2577 cases with T2D and 4572 controls) suggests *null* genotypes of GST T1 and M1 and dual *null* genotypes are associated with increased risk of diabetes (Zhang J et al., 2013).

Information of GST polymorphisms and T1D is much less. In Swedish population at the age 0 – 35 years (639 T1D patients and 474 healthy controls) was found that GST T1 and GST M1 polymorphisms are not associated with T1D risk in this population as a whole. In the subgroup of subjects at the age 14 – 20 years, GST M1 *wild* genotype was associated with increased risk for T1D development and GST M1 *null* genotype was regarded as protective. Relation between GST T1 polymorphism and T1D at any age was not confirmed (Bekris et al., 2005). In our previously published study in Slovak children and adolescents (116 patients with T1D and 47 healthy controls) we found that GST T1 *null* genotype was more frequent in diabetics compared to healthy controls and represented 2.1-fold increased risk of T1D. The

combination GST T1 *null* / M1 *wild* was significantly more frequent in patients with diabetes and represented 2.9-fold increased risk of T1D (Vojtková et al., 2013).

To sum up, gene polymorphisms of GST enzymes are potential risk factors for T2D development however the findings of available studies are not uniform. According to the most of researches and meta-analyses, GST T1 *null* and GST M1 *null* genotypes approximately two-fold increase the risk of T2D. It can be explained by increased oxidative stress that is involved in the pathogenesis of T2D as *null* genotypes of GST T1 and M1 are associated with decreased activity of GST enzymes which have also antioxidant activity. The association between GST polymorphisms and T1D has been the subject of interest just in two studies, till now. GST M1 *present* genotype seems to be risk factor for T1D development however it should be elucidated by further studies. The question, why GST M1 *wild* genotype can be unfavorable, may be explained by few speculative possibilities. First, the absence of GST may up-regulate other antioxidant genes like superoxide dismutase (Otto-Knap et al., 2003). Second, GST enzymes are normally involved in the synthesis of inflammatory mediators, leukotrienes and prostaglandins (Hayes et al., 2005), so lack of GST activity may lead to decrease in the inflammatory response and to protection against T1D. Third, an unknown compound may be metabolized by GST into a toxic form, so *null* genotype would be protective, such as dihaloalkanes are bio-activated by increased activity of GST T1 into more genotoxic metabolites (Sherratt et al., 1998) or GST pi knockout mice are protected against acetaminophen toxicity as acetaminophen is not activated into its toxic metabolite (Henderson et al., 2000).

Oxidative Stress and Chronic Diabetic Complications

Etiopathogenesis of chronic diabetic complications is complex and not fully understood, till now. Long lasting hyperglycemia (enhanced with diabetes duration and poor compensation) in the background of genetic predisposition induces many mutually interacting metabolic pathways like non-enzymatic glycation of proteins, polyol pathway, oxidative stress, activation of proinflammatory cytokines, protein kinase C or activation of nuclear factor kappa B.

Biochemical and metabolic changes in diabetes duration cause the increase of reactive oxygen species (ROS) origin and usually also change of antioxidant systems activity. ROS, especially superoxide anion can cause damage of endothelial cells leading to diabetic microangiopathy. In hyperglycemia condition endothelial cells are exposed to major glucose turnover, from glycolysis through pyruvate decarboxylasis into Krebs cycle with consequence of higher transport of electrones though mitochondrial enzymes. Electron-overloaded mitochondria produces significant amount of superoxide anions that lead into nitric oxide decrease, DNA damage, increased formation of AGEP´s, increased expression of the receptor for AGEP´S, overactivity of hexosamine pathway, activation of protein kinase C and even activation of polyol pathway. These processes act in mutual interactions and many components of mentioned pathways can increase the ROS generation. ROS can activate pleiotropic transcription factor, nuclear factor kappa B (NF-κB) which can cause multiple pathological changes in gene expression (Giacco & Brownlee, 2011).

Oxidative stress may be one of the pathways in the pathogenesis of diabetic neuropathy, structural and functional damage of nerve cells associated with diabetes mellitus, which has been the subject of research in fewer studies. Hyperglycemia induces various enzymatic and non-enzymatic reactions leading to increase of oxidative stress. Overproduction of ROS can lead to oxygenation of macromolecules, damage of membranes, enzymatic systems, proteins, lipids, DNA and to decrease of antioxidant ability of cells (Rybka, 2007). Oxidative stress can directly damage neurocyte myelin, macromolecules and membranes of nerve cells and induces their apoptosis. Considering the blood supply of nerves through vasa nervorum, also dysfunction of endothelial cells contributes to diabetic neuropathy.

Diabetic nephropathy is characterized as alteration of glomerular, tubular and mesangial cells associated with thickening of basal membrane, expansion of mesangium and hyalinisation of glomerular intercapillary connective tissue that is clinically manifested as progressive kidney disease with proteinuria, hypertension and gradual decline of renal functions (Rybka, 2007). Hyperglycemia induces oxidative stress in mesangial cells that have dominant role in the development of diabetic nephropathy. Oxidative stress increases the expression of tissue growth factor β1 (TGF-β1) and fibronectin that promote the expansion of extracellular matrix thus contribute to thickening of glomerular basal membrane and renal damage. ROS due to their chemical reactivity may oxygenate and alter proteins, lipids, carbohydrates and DNA in all cells including endothelial, tubular, epithelial and mesangial cells. Under condition of hyperglycemia-induced oxidative stress, activation of signal transduction cascades and stimulation of transcription of profibrotic genes in renal cells occur. Overproduction of reactive oxygen species alters the mesangial cells, activates processes leading to apoptosis and increases the activity of protein kinase C (PKC) in mesangial and glomerular cells (Hernandez-Marco et al., 2009). PKC acts in cell signalling while the isoforms PKC-β1 and 2 worsen renal blood flow, increase the capillary leak, induce the production of extracellular matrix and activate proinflammatory cytokines, so they contribute to microvascular injury. PKC can also stimulate the production of ROS. These changes together with non-enzymatic glycation of proteins lead to renal hyper-perfusion, hyper-filtration, accumulation of extracelular matrix, vasoconstriction of renal vessels, reconstruction of renal structure and even to nephrosclerosis (Ha et al., 2008).

Diabetic retinopathy is microvascular disease primarily involving retinal vessels of diabetic patients. Long lasting hyperglycemia initiates various metabolic processes leading to damage of pericytes, thickening of endothelial basal membrane of capillaries, changes of osmotic gradient, changes of retinal pigment epithelium, increase of perfusion pressure, greater exudation, weakening the walls of capillaries and formation of microaneurysms. Consequently, alteration of hemoretinal barrier occurs and protein and lipid exudates appear. There are occlusions of capillaries and districts without retinal perfusion. Hypoxia is a stimulus for the formation of new blood vessels in the retina, which is the most serious abnormality od diabetic retinopathy. Retinal microvasculature is composed of two types of cells, endothelial cells and pericytes. Pericyte dysfunction belong to typical early signs of diabetic retinopathy with subsequent dysfunction and apoptosis of endothelial cells. Advanced glycation end products (AGEPs) induced by hyperglycemia together with ROS induce nuclear factor NF-κB and apoptosis of endothelial cells and pericytes. AGEPs and oxidative stress are involved also in the thickening of the retinal basal membrane, the formation of acellular capillaries and microaneurysms (Yamagishi et al., 2008).

Diabetic macrovascular complications include coronary artery disease, cerebral artery disease and peripheral artery disease while the central pathomechanism is atherosclerosis resulting form chronic inflammation and injury of arterial wall. Oxidized lipids from LDL particles, promoted by angiotensin II, accumulate in the endothelial cell of arteries which are infiltrated by monocytes. Monocytes subsequently differentiate into macrophages which accumulate oxidized lipids thus forming foam cells. Foam cells stimulate macrophage proliferation and attraction of T lymphocytes which induce smooth muscle proliferation and accumulation of collagen. Rupture of lipid-rich atherosclerotic lesion with a fibrous cap causes to acute vascular infarction (Fowler, 2008). In T2D, main pathomechanism leading to atherosclerosis is insulin resistance which seems to be independent predictor as it promotes atherogenesis also in the absence of hyperglycemia. Insulin resistance in adipocytes leads to release of free fatty acids and their increased oxidation in aortic endothelial cells causes increased production of superoxide by mitochondrial electron transport chain. Then, superoxide leads to mitochondrial DNA damage, activates a variety of proinflammatory signals and inactivates anti-atherogenic enzymes - prostacyclin synthase and endothelial nitric oxide synthase (eNOS). In T1D, insulin resistance is not a major abnormality. According to the DCCT/EDIC trial, intensive treatment of T1D, with decrease of glycosylated hemoglobin, was associated with a 42% risk reduction in all cardiovascular events and with a 57% risk reduction of nonfatal myocardial infarction, stroke or death from cardiovascular disease (Nathan et al., 2005). On the other hand, intensive insulin therapy in diabetic patients can cause pro-atherogenic effects through overstimulation of insulin signaling pathways not affected by insulin resistance such as production of vasoconstrictor endothelin-1, increased expression of cellular adhesion molecules and overdrive of MAP kinase leading to cellular growth, proliferation of vascular smooth muscle cells and expression of angiotensinogen (Giacco & Brownlee, 2011).

In condition of chronic diabetic complications, increased markers of oxidative stress have been described. Sudomotoric sweating disorder was found in subjects with recent-onset (less than 2 years) T1D. Urinary malondialdehyde excretion, a measure of lipid peroxidation, and serum nitric oxide correlated negatively with total sweat, what refer to association of oxidative stress and sympathetic dysfunction in early T1D (Hoeldke et al., 2011). A prospective study (a total of 89 diabetic patients with average age 54 years) was designed to investigate if the plasma levels of biomarkers of oxidative stress (superoxide anion, hypochlorous acid, peroxynitrite, 8-iso-prostaglandin F2α, vitamin E / lipid ratio and vitamin C) predict the progression of diabetic neuropathy and mortality over six years. Increased superoxide generation was associated with a decline in median sensory nerve conduction velocity and deterioration in heart rate variability at rest over six years. Low vitamin E / lipid ratio tended to predict a decline in peroneal motor nerve conduction velocity, an increase in malleolar vibration perception thresholds and plasma superoxide generation was associated with an increased risk of mortality (Ziegler et al., 2014). An important role of peroxynitrite and protein nitration in the pathogenesis of diabetic neuropathy was shown in experimental study in mice. Treatment with the peroxynitrite decomposition catalyst and protein nitration inhibitor for four weeks led to partially correction of sensory nerve conduction slowing and small sensory nerve fiber dysfunction. Only treatment with peroxynitrite decomposition catalyst led to correction of motor nerve conduction deficit and increase in intraepidermal nerve fiber density (Stanniichuk et al., 2014). The model of diabetic endothelial dysfunction was studied in high-fat diet fed streptozocin rats. Supplementation by alpha linolenic acid led

to reduced formation of superoxide and peroxynitrite in diabetic vascular segments. Interestingly, alpha linolenic acid intake enhanced endothelial nitric oxide synthase (eNOS) but inhibited inducible nitric oxide synthase (iNOS) whereby attenuates oxidative / nitrative stress (Zhang W et al., 2013). Carotid intima media thickness is a non invasive marker of sub-clinical atherosclerosis. In children with T1D carotid intima media thickness significantly positively correlated with serum nitric oxide and negatively with total antioxidant capacity what may reflect the role of oxidative stress in the development of atherosclerosis in young diabetic subjects (El Samahy et al., 2013).

Concentration of vitamin C was reduced in T1D children and adolescents with nephropathy what may be explained by increased renal excretion of this vitamin. Activity of glutathione peroxidase was reduced in diabetic patients with neuropathy what may suggest the importance of oxidative stress in the etiopathogenesis of chronic complications. Plasmatic level of vitamin E was increased in diabetic subjects with insufficient glycemic control and increased level of plasmatic lipids that may be explained by higher transport capacity of vitamin E (Ndahimana et al., 1996). Serbian authors describe lower total antioxidant capacity of serum (TAC) in adults with diabetes and concurrently with distal symmetric polyneuropathy compared to healthy individuals. Correlation of TAC with glycemia, diabetes duration or with the stage of functional damage of nervous system was not found (Dordevič et al., 2008). Japanese authors analyzed association between fruit intake (rich for vitamin C, E, carotene, retinol equivalent, dietary fiber, potassium and sodium) and incident diabetic retinopathy in 978 patients with T2D. Risk for diabetic retinopathy declined with increased intake of fruit and vegetables, vitamin C and carotene (Tanaka et al., 2013). Similarly, treatment with ascorbic acid blocked acute hyperglycemic impairment of endothelial function (measured by the forearm blood flow reactive hyperemic response to 5 minutes of upper arm occlusion) in eight adolescents with T1D (Hoffman et al., 2012). Current medical research focus also on novel compounds with antioxidant function and many of them have been shown to be beneficial in animal models. Cardioprotective effect of sodium ferulate, ROS scavenger, has been shown in streptozocin-induced diabetic rats (Xu et al., 2012). Antioxidant mimetics with selen-manganese complexes can possess superoxide dismutase, catalase and glutathione peroxidase activity and decrease the level of lipid peroxidation products (Hosakote et al., 2012). Seleno-organic glutathione peroxidase mimetic, M-hydroxy ebselen, attenuated diabetic nephropathy and diabetes-associated atherosclerosis in mice (Tan et al., 2013) and superoxide dismutase mimetic, tempol, ameliorated the early retinal changes in diabetic rats (Rosales et al., 2010). Novel copper-zinc superoxide dismutase mimetic D34 seems to have antihyperglycemic and neuroprotective effects (Wang C et al., 2011).

Gene Polymorphisms of Antioxidant Enzymes and Chronic Diabetic Complications

Superoxide dismutase (SOD) catalyzes the conversion of superoxide into oxygen and hydrogen peroxide, which is split by catalase into water and oxygen. In the international studies (SURGENE, Genesis and GENEDIAB) seven single nucleotide polymorphisms in region SOD1 was followed in 1285 patients with T1D. *T* alelle of rs1041740 was associated with reduced glomerular filtration and with higher prevalence of incipient and manifested nephropathy. Similarly, *G* allele of rs17880135 was associated with the presence of incipient

and manifested nephropathy (Mohammedi et al., 2011). In the six year prospective study DIABHYCAR seven single nucleotide polymorphisms in SOD1 was monitored in 3744 European patients with diabetes and the association between rs1041740 and microalbuminuria was found. Three variants (rs9974610, rs10432782, rs1041740) were associated with increased risk of death from cardiovascular causes (sudden death, myocardial infarction and stroke) (Neves et al., 2012). In MnSOD2 gene T→C substitution results in a substitution of valine for alanine at position nine (*Val9Ala*). Variant *Ala* MnSOD2 has alpha helical structure, is easier imported into mitochondria and reaches higher mitochondrial activity. *Val* MnSOD2 variant has partially beta-sheet secondary structure, is partly retained in the inner mitochondrial membrane and partly degraded in proteasomes. In addition, its mRNA is rapidly degraded so *Val* MnSOD2 variant has reduced enzyme activity (Sutton et al., 2005). According to Egyptian and Russian authors (El Masry et al., 2005; Zotova et al., 2003) the frequency of *Ala/Ala* genotype of enzyme MnSOD2 is significantly lower in patients with diabetic neuropathy and contrary *Val/Val* genotype is significantly more frequent in patients without neuropathy. In Slovenian T1D patients, *Val/Val* genotype MnSOD2 (*Val16Ala*, rs 4880) polymorphism was significantly more frequent in patients with diabetic retinopathy (Hovnik et al., 2009). In Mexican T2D patients, individuals with *TT* genotype of SOD2 (*Val16Ala*) had significantly higher risk of macroalbuminuria compared to patients with *CC* genotype (Ascencio-Montiel et al., 2013). A meta-analysis involving seventeen articles found that *C* allele of *C47T* polymorphism (*Val16Ala*) in SOD2 gene had protective effect on risk diabetic microvascular complications, diabetic nephropathy and diabetic retinopathy (Tian et al., 2011). Regarding gene SOD3, *Arg/Arg* genotype was significantly more common in patients with neuropathy (Zotova et al., 2004).

Catalase is enzyme localized in peroxisomes, functionally able to degrade the hydrogen peroxide into water and oxygen. In the case of *C1167T* catalase genotype, the prevalence of *C* allele was higher and the prevalence of *T* allele was lower in patients with diabetic neuropathy compared to the patients without neuropathy (Strokov et al., 2003). According to another study (Christiakov et al., 2006) *262TT* catalase genotype was associated with higher activity of this antioxidant enzyme in erythrocytes compared to *262CC* genotype. These results assume the protective role of *262TT* catalase genotype against rapid development of diabetic neuropathy. On the other hand, no significant association was found between *C262T* polymorphisms of catalase and the risk of diabetic retinopathy (Hovnik et al., 2009).

Glutathione peroxidase (Gpx) is the family of enzymes with peroxidase activity that can reduce lipid hyprogenperoxides into corresponding alcoholes and reduce hydrogen peroxide into water and oxygen. Of the eight previously described Gpx is the most studied ubiquitous intracellular enzyme Gpx-1 (3p21.3). In codon 198 (rs1050450), *T* allele is associated with reduced enzyme activity of this enzyme compared to *C* allele. According to British authors *T* allele of Gpx-1 was associated with increased risk of diabetic neuropathy (Tang et al., 2012).

Uncoupling proteins (UCP1, 2, 3) are important in oxidative phosphorylation, thermogenesis and in protection against reactive oxygen and nitrogen species. In relation to diabetic complications, *G866A* UCP2 gene polymorphisms and *C55T* UCP3 gene polymorphisms were found to be associated with lower risk of development of neuropathy in patients with T1D (Rudolfsky et al., 2006). Brazilian authors investigated *3826A/G* polymorphism of UCP1 gene in patients with T1D and the carriers of *G* allele were significantly at higher risk of diabetic retinopathy (Brondani et al., 2012). In healthy young

Japanese men, the association between heart rate variability and polymorphisms UCP2 (*Ins/Del* in exon8) and UCP3 (*C55T*) was confirmed (Matsunaga et al., 2009).

As *paraoxonase* enzymes act in prevention of oxidized LDL formation and thus in prevention of atherogenous plaques they can reduce the risk of atherosclerosis and coronary artery disease. Serum paraoxonase activities were decreased in non-diabetic patients with coronary artery calcification (Kaya et al., 2013) and low paraoxonase-1 activity was better atherosclerotic risk predictor than low HDL cholesterol in T2D patients (Patra et al., 2013). According to the PREDICT study (a total of 589 patients with T2D), PON1 activity negatively correlated with insulin resistance and triglycerides, however no association was found between coronary artery calcification and PON1 activity, concentration or genotype (Mackness et al., 2012). In T2D patients with macrovascular complications, significantly lower activity of paraoxonase-1 and significantly higher concentration of malondialdehyde, oxidized LDL cholesterol, monocyte chemoattractant protein-1 and vascular cellular adhesion molecule-1 was found compared to diabetic patients without macrovascular complications (Sozer et al., 2014). Meta-analysis including a total of 10 studies involving 2877 patients and 3246 controls elucidated association of PON1 and PON2 gene polymorphisms with the risk of diabetic nephropathy and retinopathy. Four functional variants were evaluated – *Q192R* (rs662) and *L55M* (rs854560) in PON1; and *S311C* (rs7493) and *A148G* (rs12026) in PON2. According to the results, PON1 *L55M* gene polymorphisms were significantly associated with diabetic retinopathy with no evidence of between-study heterogeneity. No association was found between PON1 *Q192R* as well as PON2 gene polymorphisms and the risk of diabetic retinopathy or nephropathy (Wang J et al., 2013).

Metallothionein (MT) is family of enzymes with function in energy metabolism and with antioxidant activity. Chinese authors studied seven single nucleotid polymorphisms in MT genes (rs8052394 and rs11076161 in MT1A gene, rs8052334, rs964372, and rs7191779 in MT1B gene, rs708274 in MT1E gene, and rs10636 in MT2A gene). According to their results, type 2 diabetes with neuropathy was positively associated with rs10636 and rs11076161 (Yang L et al., 2008).

Clarifying the association between glutathione S-transferase gene polymorphisms and diabetic complications has been the objective of many already published studies. No significant correlation was found between GST M1 / T1 gene polymorphisms and diabetic sensory-motor neuropathy in adult Russian patients with T1D (216 with diabetic neuropathy and 250 without neuropathy) (Christiakov et al., 2006). In our already published pilot study in Slovak adolescents with T1D (19 with cardiovascular autonomic neuropathy and 27 without neuropathy) was found that GST T1 *present* and combination GST T1 *present* / M1 *null* genotype can be considered as risk factor for development of cardiovascular autonomic neuropathy (Vojtková et al., 2013).

In Slovenian population of adults with T2D (206 with myocardial infarction and 257 without it) was found that GST M1 *null* / T1 *null* haplotype was associated with increased risk for coronary artery disease (CAD) and myocardial infarction while no similar association was found regarding GST P1 gene polymorphisms (Karič et al., 2012). Similarly, in 287 subjects with T2D was found that GST T1 *null* and combination GST T1 *null* / M1 *null* genotype might be potential determinants of susceptibility to advanced carotid atherosclerosis (Santl Letonja et al., 2012). Also Italian authors following 231 patients with T2D (thereof 184 with CAD) confirmed that smoking habits and GST T1 *null* genotype significantly correlated with the increasing extent of coronary atherosclerosis. Patients with both GST T1 *null* / M1

null genotype had the highest risk for three-vessel CAD (Manfredi et al., 2009). GST T1 *null* genotype was associated with increased LDL cholesterol and triglycerides in T2D patients and this genotype was more frequent in T2D patients with CAD than T2D patients without CAD (Ramprasath et al., 2011).

According to Slovenian authors who followed 604 patients with T2D (284 with retinopathy and 320 without retinopathy with diabetes duration at least 10 years) the carriers of GST T1 *null* genotype had twofold increased risk for diabetic retinopathy and GST M1 *null* genotype was associated with lower frequency of retinopathy (Cilenšek et al., 2012). Similarly, in 124 young adults with T1D, GST M1 *wild* genotype represented the risk factor for diabetic retinopathy (Hovnik et al., 2009) while no association was found between GST M1 gene polymorphisms and diabetic nephropathy.

According to the study following three group of patients (50 subjects in each): diabetic patients without chronic kidney disease (CKD), diabetic subjects with CKD and non-diabetic CKD subjects, GST T1 *null* genotype was associated with chronic kidney disease in diabetic as well as in non-diabetic patients disregarding GST M1 genotype (Datta et al., 2010). Similarly, GST T1 *null* genotype represented the risk factor for end stage renal disease in diabetic patients however GST M1 polymorphisms did not influence this disease (Yang Y et al., 2004). No association was found between GST M1 gene polymorphisms and diabetic nephropathy in Japanese patients with T2D (Fujita et al., 2000). In our previously published study was found that GST T1 *null* genotype was associated with microalbuminuria and incipient nephropathy in children with T1D (Vojtková et al., 2013).

Diabetes duration and compensation are important but not the only factors influencing the development of diabetic complications. In their complex etiology also genetic predisposition plays role and gene polymorphisms of GST enzymes seem to participate. According to present information, most of the studies claim that GST T1 *null* genotype might be a risk factor for impaired lipid profile, coronary artery disease and diabetic nephropathy, while GST M1 *wild* genotype seems to increase a risk of diabetic retinopathy. The reasons why these polymorphisms can be unfavorable are explained above (in the paragraph about GST gene polymorphisms and diabetes mellitus). The question why GST T1 / M1 polymorphisms are associated with increased risk of complications in specific organ system (e.g. GST T1 – nephropathy, GST M1 – retinopathy) has not been answered and it may be related to higher expression of these genes in particular organs. Certainly, these associations represent large unexplored field and should be clarified by further studies.

Conclusion

Oxidative stress, as an imbalance between production of reactive oxygen / nitrogen species and function of antioxidant mechanisms, seems to be involved in many pathological conditions such as diabetes mellitus and its chronic complications.

In patients with type 1 as well as type 2 diabetes, increased markers of oxidative stress have been found. Concentration of antioxidant mechanisms is also usually changed in diabetic patients – either increased as the result of enhancement of antioxidant defense or decreased as a consequence of exhaustion of antioxidant activity. Glutathione S-transferase belongs to important antioxidant enzymes with few other functions as in detoxification or in

synthesis of proinflammatory mediators. The most researched isoenzymes, GST M1 and T1, occur in two polymorphisms – *wild* and *null*, while *null* genotype is associated with significantly lower activity of appropriate enzyme. GST gene polymorphisms seem to be associated with increased risk of diabetes or its chronic complications and in most studies, *null* genotype of GST T1 or M1 represents the risk factor what may be explained by decreased antioxidant activity. On the other hand, some studies claim that *wild* genotype is associated with increased risk of diabetes or chronic diabetic complications what may be related to other functions of GST enzymes or with up- and down-regulation of other antioxidant enzymes. It follows that the field of research is still not completely explored and further studies are necessary to clarify mentioned relations.

The knowledge about etiopathogenic factors is essential as it enables possible intervention in future. Yet, patients with diabetes are recommended, in addition to insulin therapy and proper physical activity, to receive adequate dietary intake of fruit and vegetables or to use antioxidants as vitamin C and E. Further studies are necessary to confirm or refute the usefulness of other possible supplements (coenzyme Q10, melatonin) to improve glycemic control and thus contribute to better course of diabetes and to prevention or delayed onset of chronic diabetic complications.

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