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„The antioxidant capacity of subjects with Gilbert syndrome“

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List of Abbreviations

GS ........................................... Gilbert’s syndrome
FRAP ........................................ Ferric reducing ability of plasma
ORAC .......................................... Oxygen radical absorbance capacity
MDA ........................................... Malondialdehyde
ROS ........................................... Reactive oxygen species
H₂O₂ ........................................... hydrogen peroxide
O₂ ................................................. oxygen
O₂* ............................................. superoxide radical
NO* ............................................. nitric oxide
RNS ............................................. reactive nitrogen species
NO₂* ........................................... nitrogen dioxide
ONOO* ......................................... peroxynitrite
NOS₂ ........................................... nitric oxide synthase 2
NOX ............................................. nicotinamide adenine dinucleotide phosphate-oxidase
OH* ............................................. Hydroxyl radicals
R* ................................................. carbon-centred radical
RO* ............................................. alkoxyl radical
ROO* ........................................... peroxyl radical
DNA ........................................... Deoxyribonucleic acid
GSH ........................................... Glutathione
GSSG ........................................... Glutathione disulfide
4-HNE ......................................... 4-hydroxynonenal
LPO ............................................. lipid peroxidation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVD</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>HAT</td>
<td>Hydrogen atom transfer</td>
</tr>
<tr>
<td>SET</td>
<td>Single electron transfer</td>
</tr>
<tr>
<td>H</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalence antioxidant capacity assay</td>
</tr>
<tr>
<td>TOSCA</td>
<td>Total oxyradical scavenging capacity assay</td>
</tr>
<tr>
<td>PRAC</td>
<td>Cupric ions (Cu²⁺) reducing antioxidant power assay</td>
</tr>
<tr>
<td>ABTS⁺⁺</td>
<td>2,2'-Azinobis 3-ethylbenzthiazoline-6-sulphonic acid radical scavenging assay</td>
</tr>
<tr>
<td>DPPH⁺</td>
<td>2,2-Diphenyl-1-picrylhydrazyl radical scavenging</td>
</tr>
<tr>
<td>DMPD⁺⁺</td>
<td>N,N-dimethyl-p-phenylenediamine radical scavenging assay</td>
</tr>
<tr>
<td>HMOX</td>
<td>Heme oxygenase</td>
</tr>
<tr>
<td>CN</td>
<td>Crigler-Najjar</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LOO⁺</td>
<td>Lipid-peroxyl radicals</td>
</tr>
<tr>
<td>LOOH</td>
<td>Lipid hydroperoxide</td>
</tr>
<tr>
<td>UDPGT</td>
<td>Uridine 5'-diphospho (UDP)-glucuronosyltransferase</td>
</tr>
<tr>
<td>AEP</td>
<td>Appropriateness Evaluation Protocol</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamin-etraacetic-acid</td>
</tr>
<tr>
<td>AAPH</td>
<td>2,2'-azobis 2-amidinopropane dihydrochloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>CV%</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>TBA</td>
<td>thiobarbituric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>TPTZ</td>
<td>Tripyridyltriazin</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>TCH</td>
<td>total cholesterol</td>
</tr>
<tr>
<td>AHA</td>
<td>American Health Association</td>
</tr>
</tbody>
</table>
1 Introduction

This thesis was conducted within the framework of the project “Antioxidative and Antigenotoxic Potential of Bile Pigments”, facilitated by FWF-Austrian Science Fund. The research was executed at the Department of Nutritional Sciences, situated at the University of Vienna.

Bilirubin, a yellow colored bile pigment formed by the heme catabolism, was proven to exhibit a potential antioxidant activity, due to the ability to scavenge overproduced reactive oxygen species (ROS) and thus protects against oxidative stress damage, primarily increased DNA damage. In particular, lipid peroxidation, which is responsible for cell aging and the development of chronic diseases including cardiovascular diseases (CVD) and cancer is inhibited by albumin-bound bilirubin. There is a multitude of studies focused on the association between an elevated circulation of bilirubin and the prevalence of diseases resulting from oxidative damage.

Gilbert’s syndrome (GS), a relatively common condition which applies to approximately 3-10% of the general population, is a benign form of mild unconjugate hyperbilirubinemia, caused by a 70% reduction of the glucuronidation of bilirubin, catalyzed by the uridine-diphosphate-glucuronosyltransferase (UGT1A1). Based on the resulting elevated bilirubin level GS subjects have a reduced susceptibility for the formation of oxidative damage and moreover, a lower prevalence for the subsequent development of diseases.

The aim of the following research project was to investigate whether or not individuals with GS, compared to a healthy control group, do indeed show a diminished oxidative stress potential as a result of an elevated bilirubin level and therefore have minor oxidative stress damage. The antioxidant capacity was determined by the antioxidant capacity assays FRAP (Ferric reducing ability of plasma) and ORAC (Oxygen radical absorbance capacity) as well as by the measurement of MDA (Malondialdehyde), a biomarker for oxidative stress.
2 Literature Review

2.1 Oxidative stress and free radical metabolism

2.1.1 Free radicals
Free radicals are highly unstable atoms or molecules, which contain one or more unpaired electrons in its orbital and are therefore extremely reactive. Due to free radical's affinity to absorb electrons from adjacent molecules to stabilize itself, they have the tendency to proceed as a chain reaction due to the subsequent transformation of the non-radical donor to a radical. [CLARKSON and THOMSON, 2000] Various types of free radicals react in different ways and have a more potent reactive effect compared to non-radicals. [HALLIWELL et al., 1995] These chemically reactive molecules are linked to oxygen and known as reactive oxygen species (ROS) in contrast to non-radical species, for example, hydrogen peroxide (H$_2$O$_2$). Oxygen (O$_2$) exhibits two unpaired electrons and therefore, theoretically constitutes as a free radical. However, based on a specific electron arrangement, the reactivity is restricted. [HALLIWELL and GUTTERIDGE, 1990]

ROS emerge from endogenous as well as exogenous sources. However, the most common is the production of the by-product superoxide radical (O$_2^*$), and it takes place in the mitochondria of the cell. When combined, O$_2^*$ and nitric oxide (NO*) are highly reactive and generate reactive nitrogen species (RNS), such as NO*, nitrogen dioxide (NO$_2^*$) and peroxynitrite (ONOO*), due to the activity of nitric oxide synthase 2 (NOS$_2$) and NADPH oxidase (NOX). Hydroxyl radicals (OH*) are also highly reactive and attack numerous molecules in the nearby environment. Alongside OH*, gaseous NO$_2^*$ and NO* free radicals also include the carbon-centred (R*), alkoxyl (RO*), and peroxyl radical (ROO*), which all generate from lipid peroxidation. [HALLIWELL et al., 1995]

Beyond the involvement in cell signaling and homeostasis, increased levels of ROS alter lipid, protein, and DNA structures and subsequently initialize cell aging, toxic exposure, radiation damage and diseases. [DEVASAGAYAM et al. 2004] However, free radicals are involved in major physiological functions including the production of O$_2^*$ by activated phagocytic cells in order to
eliminate bacteria, fungi and viruses. [CURNUTTE and BABIOR, 1987] To avoid a damaging impact of ROS, cells feature a number of defense mechanisms to prevent or limit intracellular damage including antioxidants such as Vitamin E, ascorbic acid, glutathione and antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase. Therefore, physiological ROS levels are held at a low rate. [HARRISON et al., 2005]

2.1.2 Antioxidants

Antioxidants are defined as molecules which limit the transfer of electrons from a free radical to a non-radical, hence oxidation. Moreover, the antioxidant defense systems protect against radical damages in organisms and therefore reduce the ROS load and defuse it to less reactive products. [SIES, 1997] Antioxidants found in the human body are distinguished between extracellular and intracellular due to their location. Intracellular enzymatic antioxidants, such as superoxide dismutase, glutathione peroxidase and catalase regulate the reactive potential of substrates in the body. Extracellular antioxidants, such as albumin, transferrin and lactoferrin inhibit the radical reaction of metal ions by inducing a complex formation in the organism. [GUTTERIDGE, 1995] Non-enzymatic antioxidants are proteins, vitamins and provitamins, flavonoids, polyphenols, peptides with thiol groups, ubiquinone and uric acid. [POWERS et al., 2004] Furthermore, bilirubin, urate and albumin are able to scavenge free radicals directly. A variety of antioxidants are provided by food such as lipophilic vitamins including vitamin E, carotenoids, water-soluble vitamin C and Selenium, which affects the activity of glutathione peroxidase. [GUTTERIDGE, 1995] The following tables provide an overview of the most common antioxidants and their main function.

<table>
<thead>
<tr>
<th>Extracellular Antioxidants</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>Reaction with lipid-peroxyl- radicals</td>
</tr>
<tr>
<td>Glutathione (GSH)</td>
<td>non-enzymatic reduction of H₂O₂ and ONOO*</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Reduction of S-S-bonds</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>Reaction with lipid-peroxyl- radicals</td>
</tr>
</tbody>
</table>
Albumin | Binds Iron, reaction with OH*
---|---
Transferrin | Binds Iron
Uric acid | Binds Iron- and Copper

Tab. 1: List of extracellular antioxidants (modified after MELCHER, 2010)

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Location</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>cytosol, mitochondria</td>
<td>$\text{O}_2^*$ - dismutation</td>
</tr>
<tr>
<td>Catalase</td>
<td>cytosol</td>
<td>$\text{H}_2\text{O}_2$ – reduction</td>
</tr>
<tr>
<td>Glutathione-Peroxidase</td>
<td>cytosol, mitochondria</td>
<td>$\text{H}_2\text{O}_2$ – reduction</td>
</tr>
<tr>
<td>Glutathion (GSH)</td>
<td>cytosol, mitochondria</td>
<td>substrate of GSH-Peroxidase</td>
</tr>
<tr>
<td>secondary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>cytosol</td>
<td>reduction of S-S-bonds</td>
</tr>
<tr>
<td>Glutathione-Reductase</td>
<td>cytosol</td>
<td>GSSG-reduction to GSH</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>membranes</td>
<td>scavenges lipid- ROO*- radicals</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>cytosol</td>
<td>scavenges lipid- ROO*- radicals</td>
</tr>
</tbody>
</table>

Tab. 2: List of primary and secondary antioxidants (modified after MELCHER, 2010)

The main function of primary antioxidants is the detoxification of superoxide radical and hydrogen peroxide. Secondary antioxidants have the ability to stop radical chain-reactions (bilirubin, vitamin E), obtain the reduced state of primary antioxidants (Vitamin C, GSH-reductase), and stimulate the reduction of oxidized proteins. [HALLIWELL and GUTTERIDGE 1990, BLITZER 2002]

### 2.1.3 Oxidative stress

The term “oxidative stress” has been defined as an imbalance between oxidants, such as free radicals and antioxidants, due to an overload of prooxidants caused by low alimentary intake of antioxidants, degradation of endogenous antioxidants or a rise in reactive species, due to either mental or physical stress. Oxidative stress affects DNA, lipids and proteins as well as generates impairment and destruction of membranes, enzymes and eventually cell death. Free radical damage is able to accumulate and subsequently leads to cell damage. Furthermore, it leads to diseases including cardiovascular diseases, atherosclerosis, diabetes, neurodegenerative and chronic diseases. [HALLIWELL, 1997] Various environmental and pathological factors trigger the
formation of oxidative stress such as radiation, ultraviolet radiation, atmospheric toxins, toxic chemicals and excess malnutrition. [DEVASAGAYAM et al., 2004]

Fig. 1: Oxidative Stress (modified after ELMADFA and LEITZMANN, 2004)

The influence of ROS in diseases was established by numerous studies, and presently over 70 pathologies are substantially connected with oxidative stress and its subsequent biochemical impact, such as peroxidation of lipids assessed by biomarkers including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), proteins evaluated by protein carbonyls, nucleic acids measured by oxidative DNA bases and carbohydrates determined by glycosilation products. [KUSANO and FERRARI, 2008]

2.1.4 Lipid peroxidation

Lipid peroxidation (LPO) is an extensively investigated mechanism responsible for cell aging and the development of diseases such as cardio vascular diseases (CVD) and cancer. Polyunsaturated fatty acids (PUFAs) are prone to peroxidation due to their instable structure that contains a number of double bonds which weaken the bond of the hydrogen atoms on the adjacent carbon
atom. The number of double bonds affects the susceptibility of PUFAs for peroxidation and therefore, parts are readily attacked by free radicals and become oxidized into lipid hydroperoxides. [PORTER et al., 1995] If the process is initiated, it continues as a free radical-mediated chain reaction. The termination of the reaction is affected by the oxygen concentration and the availability of antioxidants. The decomposition of hydroperoxides, which is activated by high temperatures and exposure to transition metal ions, leads to the production of secondary lipid peroxidation products including the aldehyde MDA and 4-HNE. Therefore, LPO products represent instable markers of oxidative stress with the tendency to form reactive byproducts. [GUTTERIDGE, 1995] Lipid peroxidation markers such as MDA show elevated levels in the blood and plaques of atherosclerotic patients. [RAVANDI et al., 2011]

2.1.4.1 MDA as a biomarker for oxidative stress
Malondialdehyde is among the numerous reactive aldehydes, which derive from the decomposition of lipid peroxides, the most widely used biomarker for an overall lipid peroxidation. [KHOSCHSORUR et al., 2000]

The method is based on the reaction of MDA with thiobarbituric acid (TBA), through which a MDA-TBA adduct is formed that can be assessed by fluorometry, spectrophotometry and high-performance liquid chromatography (HPLC). Due to a high reactivity with other substances such as bile pigments or other lipid peroxidation products, the specificity of this assay could be affected. However, it is a reliable method to test environmental and nutritional factors which are responsible for increased lipid peroxidation in a pathological as well as a healthy population. [WASOWICZ et al., 1993] Therefore, the TBA assay represents a simple and rapid way to determine lipid peroxidation in which MDA is derivatized, and it provides improved specificity due to HPLC-based methods in combination with fluorescence detection. [LYKKESFELDT, 2001]

2.1.5 Methods to measure the oxidative capacity
A method for the fast quantification of the capability of an antioxidant to prevent diseases is desirable. However, a total antioxidant capacity assay with only one chemical reaction is rather unlikely to provide an all-over outcome. Moreover, a
lack of a validated assays complicates the comparison of reported results from diverse research groups.

Various researchers describe antioxidant capacity differently. [HUANG et al., 2005] The quantification of the antioxidant capacity includes the cumulative action of all antioxidants present in body fluids and plasma, and moreover, yield an integrated parameter rather than the simple amount of measurable antioxidants. A complete antioxidant capacity potentially provides more biologically relevant information than the determination of individual antioxidant concentrations. The antioxidant capacity of the cell is essentially due to the enzyme system, whereas that of plasma is mainly modulated by dietary antioxidants and therefore represents a more adequate example for the in vivo balance between oxidizing species and antioxidant substances. [GHISELLI et al., 2000]

The “antioxidant capacity” determined by a particular assay describes only the chemical reactivity in terms of specific conditions applied in the examination and therefore, the results cannot be generalized as reference to the “total antioxidant activity”. [HUANG et al., 2005]

FRANKEL and MEYER [2000] suggested that one-dimensional methods to evaluate biological antioxidants are disadvantageous. To establish an appropriate method, the authors indicate that a test protocol ought to adequately (a) choose a biologically relevant substrate, (b) check various oxidation conditions, (c) measure initial as well as secondary oxidation products, (d) compare antioxidants at the identical molar concentrations of active substances and (e) quantify based on the percent inhibition, rates of hydroperoxide constitution or decomposition and the induction period.

Standardized analytical methods of antioxidant capacity would provide an instruction for the adequate implementation of assays, validate the comparison of data, serve as an aid to control potential variation within or between compounds, and lastly, provide the allocation of quality standards for regulatory issues and health claims. [PRIOR et al., 2005] Therefore, PRIOR et al. [2005]
suggested a list of requirements for the standardization of antioxidant capacity assays for routine quality control analyses: (1) the detection of the chemistry effectively present in potential implementations, (2) the use of a biologically relevant radical source, (3) a simple appliance, (4) the employment of procedures with a defined chemical mechanism and endpoint, (5) an easy excess to the equipment, (6) adequate reproducibility, (7) applicable for the measurement of lipophilic as well as hydrophilic antioxidants and utilization of diverse radical sources and (8) the adaptability to high throughput analyses.

To determine the total antioxidant capacity on the basis of a single method would provide insufficient data. Therefore, a multitude of different assays is required to assess the antioxidant status and oxidative stress appropriately including antioxidant capacity assays, the determination of isolated antioxidants and the utilization of biomarker for the measurement of oxidative stress damage. [PRIOR and CAO, 1999]

2.1.5.1 Chemical background
Antioxidant capacity assays can be roughly subdivided into two major groups: methods with (a) reactions based on hydrogen atom transfer (HAT) and (b) reactions based on single electron transfer (SET). Both assays detect the radical or oxidant scavenging capacity, instead of the prophylactic antioxidant capacity of a sample. The HAT method quantifies the hydrogen atom donating capacity compared to the SET method, which measures the reducing capacity of an antioxidant. [HUANG et al., 2005]

**HAT-based assays** consist of a synthetic free radical generator, an antioxidant and an oxidizable molecular sample and assess the competitive reaction kinetics, which leads to an evaluation deduced from the kinetic curves. [HUANG et al., 2005] HAT-based methods determine the ability of an antioxidant to quench free radicals by hydrogen (H) donation rapidly and independent of the pH-value. However, the presence of reducing molecules such as metals interferes with the reactivity. [PRIOR et al., 2005]

\[
AH + X^* \rightarrow A^* + XH
\]
The hydrogen atom transfer reaction represents a key step in the radical chain reaction and hence, HAT-based assays are more relevant to the assessment of unwanted lipid peroxidation in biological systems due to peroxyl radicals. Especially the ORAC assay provides useful information on radical chain-breaking capacity as a result of the use of peroxyl radicals as the oxidant. Therefore, the ORAC assay presently presents the method of choice for quantifying the peroxyl radical scavenging capacity of samples. [HUANG et al., 2005]

Assays based on the HAT reaction include: [GÜLCİN, 2012]

- Oxygen Radical Absorbance Capacity (ORAC)
- Trolox equivalence antioxidant capacity assay (TEAC)
- Inhibition of induced LDL oxidation
- Total oxyradical scavenging capacity assay (TOSCA)
- Crocin-bleaching assays
- Chemiluminescent assay

**SET-based assays** detect the capacity of a potential antioxidant to donate one electron and reduce compounds such as radicals, metal ions and carbonyl groups, which is expressed by a change in color. [HUANG et al., 2005] The relative reactivity correlates with the deprotonation and ionization potential of the reactive functional group. [PRIOR et al., 2005]

SET-based assays are composed of two elements in the reaction reagent: (a) the antioxidants and (b) the oxidant, which also represents the probe and absorbs an electron from the antioxidant and therefore, elicits a change in color of the sample.

oxidant (probe) + e\(^-\) (from the antioxidant) \(\rightarrow\) reduced probe + oxidized antioxidant

The intensity in the color change is proportional to the concentrations of the antioxidant, and when the color change reaches a plateau the end point of the
reaction has been achieved. An alternation of absorbance is plotted against the antioxidant concentration to provide a linear curve, which reflects the reducing capacity of the antioxidant, usually expressed in Trolox equivalence (TE). [HUANG et al., 2005]

Various SET-based assays were developed to measure the reducing capacity of antioxidants at different pH-values, which influence the reaction (acidic, neutral and basic). Acidic conditions like the ones used in the FRAP assay, may suppress the reducing capacity based on protonation of antioxidant compounds. In basic conditions however, the reducing capacity of the probe would be enhanced due to proton dissociation of phenolic compounds. [HUANG et al., 2005]

The pH dependent procedure can be quite time consuming due to the slow completion of the reaction and measures the percentage reduction of the product instead of the kinetics. [PRIOR et al., 2005] The interference of contaminants such as metals possibly leads to a debased reproducibility and consistency of results. [HUANG et al., 2005]

Assays based on the SET reaction include: [GÜLCIN, 2012]

- Ferric ion reducing antioxidant power (FRAP)
- Trolox equivalence antioxidant capacity assay (TEAC)
- Total phenolics assay by Folin-Ciocalteu reagent assay
- Total antioxidant potential assay (using a Cu^{2+}-complex as oxidant)
- Cupric ions (Cu^{2+}) reducing antioxidant power assay (PRAC)
- 2,2-Azinobis 3-ethylbenzthiazoline-6-sulphonic acid radical scavenging assay(ABTS^{**})
- 2,2-Diphenyl-1-picrylhydrazyl radical scavenging (DPPH*)
- N,N-dimethyl-p-phenylenediamine radical scavenging assay (DMPD^{**})
2.2 Bilirubin
In 1849, VIRCHOW discovered bilirubin in blood extravasates and termed it as “Hematoidin”. In 1864, the name Bilirubin was given by STADELER, who obtained a defined and pure preparation of the dark red bile pigment and subsequently it was recognized that an accumulation of bilirubin was the reason of the yellow color in jaundiced patients. In 1874, TARCHANOFF showed the direct association between pile pigments and hemoglobin, and in 1942, FISCHER and PLIENINGER synthesized Bilirubin IXa and proposed a structure, which was accepted for over 30 years. [BOSMA, 2003]

2.2.1 Structure
All bile pigments share a similar structure skeleton, however each pigment possesses a characteristic β-substituent. Bilirubin is a yellow colored bile pigment and exhibits a porphyrine structure, which consists of four covalent bound open-chain pyrrole rings. At the two pyrrolic rings on the outside are polar lactam groups (-CO-NH) bound in opposite to the inner pyrole rings, which are linked to polar propione sidechains (-COOH). The naturally found bilirubin IXa is a 4Z, 15Z isomer of the unconjugated form of bilirubin, which is formed by heme cleavage. [VITEK and OSTROW, 2009]

Fig. 2: Structure of Bilirubin IXa [McDONAGH, 1997]
2.2.2 Heme catabolism

Bilirubin, is the end product of heme catabolism and is derived from its metabolic precursor biliverdin in mammalian systems. [FARRARA et al., 1994] There are two forms of Bilirubin: direct reacting bilirubin, which is conjugated and found in the bile, as well as indirect reacting bilirubin, the unconjugated (UCB) main form in blood that only reacts in the presence of a catalyst. The direct form can be converted into the indirect form by glucuronidase, an enzyme that is involved in the degradation process of heme. [BOSMA, 2003] The bilirubin concentration in humans is based on the heme decomposition derived from hemoglobin and other hemoproteins including cytochromes, catalase and peroxidase, as well as a small pool of free heme. The production amounts to approximately 300mg per day, of which about 25% is produced from non-hemoglobin sources and 75% of aged red blood cells. [VITEK and OSTROW, 2009]

Bilirubin is produced by the action of the enzyme heme oxygenase (HO), the rate-limiting enzyme in heme catabolism, that metabolizes cyclic heme into biliverdin, carbon monoxide and ferrous iron, followed by the reduction of biliverdin to bilirubin, catalyzed by biliverdin reductase. [VITEK, 2012]

There are two catalytically active isoforms of heme oxygenase. HO-1 is in charge of reactions of acute phase instant response against oxidative stress. HO-2 is a constitutive isoenzyme, which is expressed mainly in the brain and is presumed to function as a neurotransmitter or neuromodulator. Furthermore, a third enzyme (HO-3) exists, which is believed to have no coding function and is just represented by a pseudogene. [HAYASHI et al., 2004] However, a recent study showed possible regulatory functions of pseudogenes that might indicate HO-3 also has biological potential by contributing to gene regulation. [PINK et al., 2011]
Fig. 3: Bilirubin metabolism. The generation of bilirubin due to haeme-oxygenase and biliverdin reductase. [BOSMA, 2003]

At the beginning of the metabolic process of bilirubin senescent or hemolyzed red blood cells degrade to hemoglobin, which is segmented to heme and globin. Heme is broken down to biliverdin IXa by oxidation, which in turn is reduced to bilirubin IXa and subsequently bound to the protein albumin, bilirubin is transported via bloodstream to the liver. [WILLIAMS et al., 2006] After reaching the liver, a basolateral organic-anion-transporter actively carries bilirubin in dissolved form in the cytoplasma by binding (itself) to specific binding proteins. Subsequently, two molecules of glucuronic acid react with UDP-glucuronosyltransferase (UGT1A1) and conjugate bilirubin to bisglucuronosyl bilirubin, which is actively secreted into bile and further transported into intestinal lumen, where it is exposed to more metabolic changes. Moreover,
there are a number of crucial enzymatic steps essential for the homeostasis of bilirubin, which presumably influences the risk of metabolic diseases, such as cardiovascular diseases, diabetes, metabolic syndrome, arterial hypertension and obesity. [VITEK, 2012]

2.2.3 Physiological concentrations of bilirubin
Many factors influence the serum bilirubin level, including gender, fasting, smoking, drug intake, race and age, which in turn affect the biological impact of bilirubin on the human body. [VITEK and SCHWERTNER, 2008] The average physiological concentration of bilirubin in healthy subjects ranges from 5-17µmol/l. Considerably higher rates could be reached under diseased conditions, such as jaundice. [ASAD et al., 2001] Patients with GS exhibit a mild hyperbilirubinaemia with levels between 17,1 – 85µmol/l in contrast to the more severe variants, Crigler-Najjar (CN) 2 with 120 – 340µmol/l and CN1 with 340-685µmol/l. [GILBERT and LEREBOULLET 1901, ARIAS 1962, CRIGLER 1952]

2.2.4 Relevance of bilirubin as an antioxidant
Antioxidants as well as toxic properties have been attributed to bilirubin [ASAD et al., 2001], and it was known for a long time to be a potentially toxic metabolite, especially for the central nervous system. Nevertheless, it might provide numerous beneficial effects for the human body, as data from the last few decades have proven [VITEK, 2012] Its antioxidant activity is predominantly due to its ability to disable free radical species such as peroxide radicals and superoxide anion. [ASAD et al., 2001]

Due to the lipophilic nature of bilirubin, the molecule provides specific protective activity against lipid soluble metabolites. [STOCKER and AMES 1987, STOCKER 1990] Exogenous bilirubin has the ability to influence the lipid peroxidation in the heart and kidney and has a protective effect on thymus cells against apoptosis. [DUDNIK et al. 2001] Unconjugated as well as conjugated bilirubin safeguards low-density lipoprotein (LDL) against peroxidative attack of scavenging peroxyl radicals. [WU et al., 1996]
Bilirubin exhibits a constitution of conjugated double bonds in combination with a pair of reactive hydrogen atoms in all present forms including free, albumin-bound and conjugated bilirubin. The reactive atoms dispense a hydrogen (H), especially to lipid-peroxyl radicals (LOO*) and subsequently form lipid hydroperoxide (LOOH) and bilirubin radicals. Due to the structure of bilirubin, the molecule is prepared to donate electrons to plasma oxidants to form its C-10 methylene group. Therefore albumin-bound bilirubin has a major influence on the antioxidant activity in plasma and protects LDL particles from plasma oxidants. [STOCKER, 2004] In contrast to free bilirubin, the albumin-bound molecule forms oxidized biliverdin, which is reduced by the potent redox cycle of biliverdin reductase. [NEUZIL and STOCKER, 1994, BARANO et al., 2002]

Moreover, bilirubin has been proven to be more effective at protecting lipids from oxidation than water-soluble antioxidants, like glutathione, with its main function of protecting proteins from oxidative damage. [SED Lak et al., 2009] A study confirmed the cytoprotective effect of bilirubin in the brain, more specifically, the combination of bilirubin and GSH have complimentary cytoprotective qualities. Bilirubin accounts for the prevention of lipid peroxidation and could therefore be of great importance for the stability of membrane proteins due to their hydrophobic environment whereas GSH mainly protects against the oxidation of water-soluble proteins, hence diverse substances in the cytosol. GSH is able to protect against lipid peroxidation as well, with an excessively smaller potency of preventing oxidative degradation of lipids than bilirubin. [SED Lak et al., 2009] Free radical mediated cell lysis is inhibited at significantly lower bilirubin concentrations, much more than other potent antioxidants including ascorbate or Trolox. [ASAD et al., 2001] A minimal level of bilirubin (10µM) is able to protect against 10000 times higher concentrations of hydrogen peroxide. [DORE et al., 1999] Compared to the vitamin E analog Trolox, a lipid-soluble antioxidant, bilirubin has been shown to be nearly 30 times more effective in preventing in vitro LDL oxidation. [WU et al., 1994] Furthermore, serum bilirubin has been indicated to be a considerable contributor to the total antioxidant capacity in blood. [FREi et al., 1988] Bilirubin has also been demonstrated to have anti-inflammatory properties. [VITEK and
Therefore, among other functions, bilirubin is able to scavenge overproduced reactive oxygen species, activate anti-inflammatory actions and has direct effects upon cell signaling. [VITEK et al., 2007a]

2.3 Inherited forms of unconjugated hyperbilirubinemia

There are three grades of inherited unconjugated hyperbilirubinemia known in the human body, which all show a normal morphological liver and other hepatic functions that are unaffected, as well.

2.3.1 Morbus Gilbert

The least severe form, Gilbert syndrome, was first introduced in 1901, by GILBERT and LEREBOULET. [1901] It is the most common hereditary cause of increased bilirubin. This condition is found in 3 – 10 % of the general population, with a more frequent occurrence in males (12.4%) than in females (4.8%). [RADU & ATSMON, 2001] A harmless jaundice caused by elevated levels of unconjugated bilirubin in the bloodstream is the main symptom and requires no treatment. It is characterized by unconjugated hyperbilirubinemia that occurs in the absence of bilirubinuria, hemolytic anemia or liver malfunction. [SAMPietro et al., 1999] Gilbert syndrome does not lead to chronic liver dysfunction or fibrosis and is therefore considered benign.

GS is characterized by a decrease in hepatic bilirubin UDPGT activity (>50%) and describes a heterogeneous group of disorders. [FELSher BF, 1973] Although a congenital defect, GS seldomly becomes clinically apparent until after puberty due to hormonal (steroids) suppression of the hepatic bilirubin clearance. [ODELL, 1980] The disorder is based on a reduction in bilirubin glucuronidation catalyzed by the UDP-glucuronosyltransferase (UGT1A1). [BOSMA et al. 1995, BOSMA 2003] Due to diminished activity of the conjugation enzyme, which is solely responsible for bilirubin glucuronidation, the conjugation of bilirubin is reduced, which is associated with an enhanced production of monoconjugated bilirubin. [DELGIUDICE et al., 1999]
Fig. 4: Ribbon diagram of the predicted 3D structure of UGT1A1. The purple ribbons are α-helices, and the long arrow indicates the β-sheet. In codon 71 in the α-helix of the mutated UGT1A1, the dotted arrow indicates the replacement of arginine for glycine. [LEE et al., 2010]

BOSMA et al. [1995] and MONAGHAN et al. [1996] proved a polymorphism in the promoter region of the gene for UDPGT1 which seemed to occur in most patients with Gilbert Syndrome. The UGT1A1 gene and its TATA box, a phenoborbital responsive enhancer module in its regulatory region, [RITTER at al., 1992] is determined on chromosome 2q37 and encodes nine functional proteins. [URAWA et al., 2006]

The wild type of TATA box exhibits six recurrences of TA and is conventionally written as A(TA)6TAA. [RITTER et al., 1992] In subjects with Gilbert Syndrome, a variant promoter for the gene encoding bilirubin UDP-glucuronosyltransferase (UDPGT) contains a two-base-pair addition (TA) in the TATAA unit, upstream of the first exon of UGT1A1, showing seven rather than the usual six repeats. [BOSMA et al., 1995] The additional dinucleotide has been indicated to decrease the expression of the UDPGT1 gene, and the bilirubin conjugation in Gilbert Syndrome is the result. This leads to a 70 to 80% reduction of bilirubin
glucuronidation in homozygous UGT1A1*28 carriers. [BOSMA et al. 1995, BOSMA 2003]

Fig. 5: Nucleotide sequence of the BGT gene promoter. [TRIOCHE et al., 1999]

Between the length of the TATAA element and UGT1A1 expression, an inverse correlation was detected. The homozygosis for the variant TATAA element alone is not sufficient for the manifestation of the Gilbert phenotype, and other factors also contribute to the serum bilirubin levels. [BOSMA et al. 1995] This phenotype is for instance seen less often in women, most likely due to the lower daily bilirubin production. [KADAKOL et al., 2001]

2.3.1.1 GS in ethnic groups

Between different populations a discrepancy of the genetic background of GS occurs. Clinical GS in Caucasians is characterized by a reduced UCT1A transcription caused by A(TA)7TAA polymorphism with a frequency of the UGT1A1*28 allele between 35 and 40%. [BOSMA et al., 1995] Hence, the homozygous mutation occurs in 11-16% of the cases. [MONAGHAN et al., 1996] The majority of Caucasians and Africans with Gilbert Syndrome has a homozygous TA insertion in the promoter region of the UGT1A1 gene, which is believed to be the main cause of this condition. Though less common in people
of an African origin, two additional polymorphisms of the TATAA unit have been observed, a shorter sequence A(TA)5TAA of the UGT1A1*36 gene and a longer sequence A(TA)8TAA of the UGT1A1*37 gene, which leads to increased transcriptional activity. [EHMER et al., 2008] Some populations show a minor commonness for the UGT1A1*28 allele and therefore develop other mutations causing GS. [HALL et al., 1999] The phenotype G71R allele, which was not found among a European population, commonly occurs in Japanese and Asian individuals, more specifically in jaundiced newborns. Due to a homozygous and heterozygous G71R mutation the expression of bilirubin UGT is 40-70% decreased. [YAMAMOTO et al., 1998] Furthermore, among an Asian population, elevated serum bilirubin levels where detected, elicited by the Y486D mutation, although the frequency of the G71R and Y486D alleles containing these mutations are unknown. [SATO et al., 1996]

2.3.1.2 GS in neonates
Physiological neonatal jaundice manifests during the first 5 days of life, in about 50% of neonates and primarily occurs from unconjugated bilirubin. [CHOWDHURY et al., 1995] A delayed maturation of bilirubin uridine diphosphate-glucuronosyltransferase (UGT1A1) reduced erythrocyte lifespan, but an increased load of bilirubin to the liver trigger elevated bilirubin levels in newborns. Breast-fed infants develop a higher plasma bilirubin level of 256-410µmol/L in the space of the first 10-19 days. [OSBORN et al., 1984]

GS is speculated to influence the development of neonatal hyperbilirubinemia, which is defined by an increased total bilirubin plasma level above 223µMol/L. [MAISELS, 1988] Many incidences of GS occur in neonates and are suggested to amplify the development of neonatal jaundice within the first 2 days of life. [BANCROFT et al. 1998] KAPLAN et al. [1997] demonstrated that neonates with a G-6-PD-deficiency in combination with the variant UDPGT1 promoter have a higher risk to develop hyperbilirubinemia. If just one condition is present, the G-6-PD-deficiency or the presence of the variant allele alone did not raise the risk of hyperbilirubinemia.
The risk for neonates to develop a serious hyperbilirubinemia is highly dependent on the serum bilirubin level at discharge. Nevertheless, of the discharged newborns with an increased risk, only approximately 8% develop a major hyperbilirubinemia. [KEREN et al., 2008] The combination of clinical and laboratory data are needed to identify infants with an affinity for elevated bilirubin levels. [HANCHARD et al., 2011]

For European, North American and African infants with a prolonged neonatal jaundice, BURCHELL and HUME [1999] advise a genetic screening test for GS detecting a possible TATA box mutation of UGT1AA. In Japanese neonates, the Gly71 Arg mutation should be determined.

### 2.3.1.3 Symptoms and Treatment

Gilbert’s syndrome is a benign condition causing mild, unconjugated hyperbilirubinemia manifested by episodes of mild intermittent jaundice and an absence of overt hemolysis or liver disease. [WATSON and GOLLAN, 1989]

The morbidity and mortality are not significantly affected, and no major dysfunctions of the drug metabolism were detected. However, the risk of drug toxicity may be increased due to concurrent genetic deficiencies in xenobiotic pathways. [DeMORAIS et al. 1992] Thus, a clinical screening for GS is advised despite the non-existence of a specific treatment. [BOSMA, 2003] A recent study suggests that GS might pose a potential risk factor for drug toxicity. As a common condition in GS, a haplotype of multiple genetic variants at the UGT1A gene locus changes the responsiveness and the expression of a human UGT1A1 significantly. Moreover, the study redefined the functional and molecular basis of GS as a condition with a complex genotype and is possibly able to modify the metabolism extensively and is therefore, a potential risk factor for infection and drug therapy. [EHMER et al., 2012]

In another study, GS probably influences the prevalence of cholelithiasis and might exhibit a higher possibility for gallstones at a relatively young age. [ORIGA et al., 2009]
ASTOFILI et al. [2011] investigated the relation between GS and breast cancer. The affected function of the UGT1A1 enzyme of GS patients causes a level of degradation of 4-OH-estrogens, which represent estrogen metabolites and acts cancerogenous. It is suggested that the risk for breast cancer rises because of the polymorphism UGT1A1*28 in GS subjects as well as a potential exposure to increased exogenous hormones. To date, no studies exist that compare the level of jaundice in breast cancer patients to healthy individuals, and relations between GS and breast cancer are insufficiently evaluated. Therefore, the correlation between GS and breast cancer is simply a theory and needs to be affirmed by further clinical studies.

2.3.1.4 Methods to diagnose GS

2.3.1.4.1 The “caloric restriction” or “starving test”
This procedure was developed by Agustine Gilbert in 1906, and is characterized by two days of fasting (max. 400kcal per day) and a subsequent elevation of unconjugated serum bilirubin up to 100 %. A distinct difference between GS and healthy individuals should be given, though the discrimination was insufficient if patients with acute hepatitis or liver cirrhosis were compared to GS subjects. [THOMSEN et al., 1989] The caloric restriction test requires a hospital admission during the 2-day testing phase to monitor the caloric intake and the blood glucose levels and is for this reason extremely time consuming. [TEICH et al., 2008]

2.3.1.4.2 The nicotinic acid provocation test
GS can be diagnose if a mild, fluctuating unconjugated hyperbilirubinemia is present without persisting hemolysis, and a regular outcome of other liver function tests is performed. After an overnight fast, an intravenous (50mg) or oral (170-300mg) administration of nicotinic acid (niacin) induces an increase of unconjugated serum bilirubin, which is subsequently measured every 30-60 minutes for about 5 hours. GS Subjects show a more potent rise and a delayed clearance in comparison to healthy individuals. [FROMKE and MILLER, 1972] Due to the positive outcome for individuals with GS and chronic liver disease, the nicotinic acid test does not qualify for a differentiation between those 2
groups. [DICKEY et al., 1991] In other studies, both the nicotinic acid test and the starving test were compared and reported a more potent increase of bilirubin in the former method. [DAVIDSON et al., 1975]

2.3.1.4.3 Overnight Rifampin Test
The Rifampin Test is a highly sensitive and specific procedure. The substance competes for the excretory pathways in liver cells and further induces cytochrome P-450 isoenzymes. [HALLAL et al., 2006] An oral dose of 900g Rifampin is given to both fasting as well as nonfasting adults, which causes a much higher rise of total serum bilirubin in GS patients than in healthy subjects. After fasting and the subsequent oral application, the bilirubin concentration increases > 1,9 mg/dl after 2-6 hours. Without fasting, the level rises > 1,5 mg/dl after 4-6 hours. Due to the extreme increase in GS in comparison to healthy individuals, the method is able to differentiate between these two groups. [MURTHY et al., 2001] A rise of serum bilirubin > 2,4mg/dl provides a 93,8% sensitivity and 93% specificity compared to the increase of unconjugated bilirubin > 1,3mg/dl with 100% sensitivity and specificity for the diagnosis of GS. Hence, the method represents a useful diagnosis of GS if those cut-off levels are used. [MOUSAVI et al., 2005] THALLAL et al. and MURTHY et al. did not report possible adverse effects such as heartburn, vomiting or hepatitis due to Rifampin, which may induce hemolysis. Additionally, severe hypersensitivity reactions including the Stevens-Johnson Syndrome, toxic epidermal necrolysis and intravascular coagulation, which represent more serious complications and seldomly occur after the administration of Rifampin, were documented. [TEICH et al., 2008]

2.3.1.4.4 Fractionation via thin-layer chromatography
In the diagnosis of GS, the following method provides an exact measurement of conjugated and unconjugated bilirubin levels. In GS, approximately 6% of the total serum bilirubin is conjugated, compared to the higher amount of 17% in healthy subjects and 28% in patients with chronic hepatitis. For this particular method fasting is not required because it does not affect the percentage of
conjugated bilirubin, despite an increased total serum bilirubin level. [SIEG et al., 1990]

2.3.1.4.5 Genetic testing
Genetic testing is a valuable method, especially for the investigation of prolonged jaundice in neonatal cases. GS is an important criterion to detect a prolonged neonatal jaundice because of its contribution to hyperbilirubinemia. [LAFORGIA et al. 2002] Furthermore, for standard therapy with certain drugs (Atazanavir and Irinotecan), the genotyping for the (TA)7 variant of the UGT1A1 promoter region is more commonly used as a predictive factor. The gold standard method for genotyping the UGT1A1*28 mutation is characterized by the time-consuming direct sequencing of the UGT1A1 promoter region. [EHMER et al., 2008]

2.3.2 Crigler-Najjar-Syndrom
Two additional and more severe variations of pathologic hyperbilirubinemia were named after their discoverers, Crigler and Najjar, and are autosomal recessive disorders. [ARIAS, 1962]

Type I described first by CRIGLER and NAJJAR in 1952, is a rare form with approximately 170 described cases worldwide until 1999. [JANSEN, 1999] It represents an acute form of indirect hyperbilirubinemia with increased bilirubin levels of 15-50mg/dl and tends to result in death within the first few months in infants. This form is characterized through the nearly complete absence of UDP-glucuronosyltransferase and the significantly low enzyme activity below 1%. [CRIGLER and NAJJAR, 1952] The therapy includes phototherapy, blood transfusions, plasmapheresis and liver transplantation. [FOX et al., 1998] A study with gunn-rats investigated the phenotype of Crigler-Najjar (CN) I to develop a new approach of therapy. Instead of observing liver cells, which seldomly multiply, fibroblasts are used as a unit of measure for the transfection in combination with UDP-glucuronosyltransferase and were subsequently implanted in rats. After the completion of the procedure, the bilirubin level decreased to the standard value of healthy subjects. However, after 6 weeks all rats developed peritoneal tumors. [SEPPEN et al., 1997]
In 1962, the less lethal type CN II was distinguished from type I and subsequently described by ARIAS and CRIGLER. The serum bilirubin level of type II patients is elevated to 10-20mg/dl, and the enzyme activity is determined at 1-10%, which represents an indirect hyperbilirubinemia. For a differential diagnosis of type I and Type II, Phenobarbital can be administrated, which induces an enzyme induction of the UDP-glucuronosyltransferase and yields a 30% decrease of the bilirubin level in type II. [ARIAS, 1962]

2.3.3 GS and Cardiovascular diseases
CVD are the main source for death in developed countries and include atherosclerosis, coronary heart disease, stroke and hypertension. Atherosclerosis is characterized by a thickening and stiffening of arteries due to fatty plaques and mineral accumulation, which leads to an insufficient supply of blood in the myocardium. [RAVANI et al., 2011] Cardiovascular tissue injury has been suggested to be a result of oxidative stress in cardiac and vascular myocytes. [DHALLA et al., 2000] Furthermore, a significant deposit of iron in atherosclerotic lesions indicates that the iron catalyzed formation of free radicals possibly influence the development process of atherosclerosis. [YUAN and LI, 2003]

A negative association between the prevalence of CVD and an elevated circulation of bilirubin was shown in a number of studies. [VITEK et al., 2002 and LIN et. al, 2006] Moreover, a negative link and dose-response between serum bilirubin and the atherosclerotic process in humans is speculated. [NOVOTNY and VITEK, 2002] A strong association with a decreased risk of CVD is exhibited by homozygous UGT1A1*28 allele carriers with high serum bilirubin concentrations. [Lin et al., 2006]

GS patients have a minor susceptibility of serum oxidation, regulated by the relationship between the oxidizability of serum and the serum bilirubin concentration, which possibly indicates a potential mechanism for the diminished prevalence of CVD in GS. [BLUMER et al., 2008] CVD exhibit increased oxidation of circulating lipids related to a liability of plasma to copper afflicted oxidation. Furthermore, CVD and hyperlipidemia patients have an
elevated rate of plasma oxidation, due to the lower concentrations of lipophilic antioxidants. [KONTUSH et al., 1997]

BLUMER et al. reported a beneficial lipid profile in GS patients due to a higher ratio of HDL:LDL in plasma compared to a healthy group, independent of the physical activity level and alimentary intake of saturated fat. A difference of 13,3µmol/l in circulating bilirubin between GS and healthy subjects, which yields to a 13,6% amelioration in the liability of plasma to oxidation was presented. [BLUMER et al., 2008]

A study showed a significantly lower spectrum of atherogenic lipoprotein in patients with GS, due to a negative correlation between unconjugated bilirubin and small dense LDL as well as triacylglycerides. This inverse relationship constituted an approximately three times lower presence of atherogenic lipoprotein spectrum in GS subjects than in the healthy control group. Therefore, individuals with GS may have a decreased risk of atherosclerosis because of the amplifying effect of reduced small dense LDL levels on the protective antiatherogenic effect of hyperbilirubinemia. [OCADLIK et al., 2011]

A population study revealed a significantly reduced risk for the development of CVD given that the bilirubin level was increased by 17µmol/l. [HOPKINS et al., 1996] In a group of 15,000 men, the correlation between CVD and bilirubin indicated that each 1,0µmol/l increase in serum bilirubin diminished the potential CVD risk approximately 6,5%. [NOVOTNY and VITEK, 2003] With 2%, male patients with GS show a significantly decreased prevalence of ischemic heart disease compared to healthy individuals with approximately 12%. [VITEK et al., 2002]
3 Materials and Methods

3.1 Study design
This current study aims to establish the genotoxic effect of Bilirubin regarding subjects with GS compared to a healthy control group. The main objective is to identify affected metabolites, which are associated with GS and in addition, to also generate hypotheses for further investigations. This research was conducted as a part of the project entitled “The physiological relevance of bile pigments”, (FWF Austrian Science Fund; project no. P21162), which was executed at the Department of Nutritional Sciences, University of Vienna.

3.1.1 Required criteria for subjects

Inclusion criteria:

 ✓ age 20 – 80 years
 ✓ activity of y-glutamyltranspeptidase < 100 IU
 ✓ activity of alanine-aminotransferase < 100 IU
 ✓ activity of aspartate-aminotransferase < 100 IU

Subjects with GS:

 ✓ total serum bilirubin concentration > 20.52 µmol/L (>1.2 mg/dl)
 ✓ unconjugated serum bilirubin concentration > 17.1 µmol/L (>1 mg/dl)

Healthy control subjects:

 ✓ total serum bilirubin concentration < 20.52 µmol/L (<1.2 mg/dl)
 ✓ unconjugated serum bilirubin concentration < 17.1 µmol/L (<1 mg/dl)
Exclusion criteria:

- younger than 20 and older than 80 years
- currently smoking > one cigarette per day
- alcohol consumption > 7 beverages per week
- excessive exercise > 10 hours per week
- medication that affects the liver metabolism (e.g. Rifampicin, Probenecid)
- antioxidant supplementation in the past 4 weeks prior the blood sampling
- a history of diseases like cardiovascular diseases, liver diseases (e.g. hepatitis B or C), haemolysis, cholelithiasis, cancer (past or present), chronic kidney diseases and diabetes mellitus
- persons with transplants

3.1.2 Classification of the participants

![Diagram showing the classification of participants]
3.1.3 Subject recruitment
Subjects were recruited by newspaper, Internet advertisements and postings on notice boards of public establishments (universities, hospitals, pharmacies), as well as by clinical staff of the General hospital of Vienna. All chosen participants who met the inclusion criteria, have given their informed written consent. A total number of 104 subjects was recruited, out of which 76 fulfilled the required inclusion criteria and were allocated into a GS group (n= 38) or a control group. (n= 38)

The participants had to keep 2 appointments in order to complete the study. On the first appointed day, potential subjects had to provide a small blood sample and complete a questionnaire. Subjects who met the inclusion criteria were then invited for a second examination day. On the day preceding the second examination the subjects were supposed to maintain a low caloric diet with 400 kcal in total until 5 p.m., followed by an overnight fast. On the following morning, venous blood was sampled by trained medical staff at the General hospital of Vienna. Furthermore, cell samples from both the buccal mucosa and urine were taken.

3.1.4 Blood preparation
Blood samples were taken from test persons and collected in tubes containing ethylene-diamin-etetraacetic-acid (EDTA) or Heparin. Afterwards, the samples were centrifuged at 4°C and 2400g for ten minutes. The serum was separated and stored for further use at -80°C.
3.2 ORAC – Oxygen Radical Absorbance Capacity

The Oxygen Radical Absorbance Capacity Assay is a widely accepted standard tool for the measurement of antioxidative activity in the nutraceutical, pharmaceutical and food industries. [HUANG et al., 2002b] It is a relatively simple method, though sensitive and reliable of quantitating the oxygen radical absorbing capacity (ORAC) of antioxidants in human serum. [CAO et al., 1993] HUANG et al. [2002b and 2002a] adjusted the method to a fully automated high-throughput system with a microplate fluorescence reader in a 96-well dimension and modified the procedure to further permit the identification of the lipophilic, hydrophilic and total antioxidant capacity of a compound. The peroxyl radical generator 2,2'-azobis 2-amidinopropane dihydrochloride (AAPH) was applied for measuring the “total antioxidant capacity” of hydrophilic and lipophylic substances in protein free plasma. [PRIOR et al., 2003] Due to the standardization of the ORAC assay in combination with the use of a common calibrator, the method can be performed on many different compounds and foods. This allowed the formation of a database for an easy comparison of antioxidative capabilities of many different materials. [WU et al., 2004]

3.2.1 The Principle of ORAC

The ORAC assay is based on the oxidation of a fluorescent sample of peroxyl radicals through a hydrogen atom transfer process. The Assay is based on the inhibition of peroxyl-radical-induced oxidation and initiated by thermal decomposition of azo-compounds (AAPH). [PRIOR et al., 2003] AAPH generates molecular nitrogen and two carbon radicals. It can cause either a stable compound, or in combination with molecular oxygen, it can cause peroxyl radicals. [OU et al., 2001] These radicals decrease the intensity of the colored fluorescein and can be measured fluorimetric. If the radical generator is confronted with antioxidants, depending on their antioxidant capacity, they absorb the present peroxyl radicals and the decrease of the pigment intensity is decelerated. [PRIOR et al., 2003] The sample antioxidant capacity correlates to the fluorescence decay curve and is described as area under the curve (AUC), which is used to quantify the total peroxyl radical antioxidant activity in a sample. To compare the water-soluble vitamin E analog Trolox is used for the
antioxidant standard curve. [OU et al. 2001, HUANG et al. 2002b] ORAC is the only method that provides a complete assessment in which the inhibition time and inhibition degree is combined into a single quantity as the reaction goes to completion. [OU et al., 2001]

![Principle of the ORAC assay](HUANG et al., 2002b)

The ORAC assay according to HUANG et al. [2002b] and PRIOR et al. [2003] was previously adjusted to FLUOstar Optima, a microplate fluorescence reader with a 96-well format at our department. [STADLMAYER, 2009] Our research is based on this altered procedure with further modifications including the use of a lower dilution factor (1:160 instead of 1:620) and half of the amount of AAPH and buffer solution (426.42mg dissolved in 5ml buffer, heated to 37°C).
### 3.2.2 Reagents and Instruments

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Chemical Formula</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein disodium salt</td>
<td>C_{20}H_{10}Na_{2}O_{5}</td>
<td>fluka Chemica (CH)</td>
</tr>
<tr>
<td>Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)</td>
<td>C_{14}H_{18}O_{4}</td>
<td></td>
</tr>
<tr>
<td>Phosphate buffer (pH 7,4)</td>
<td>Na_{2}HPO_{4}</td>
<td></td>
</tr>
<tr>
<td>2,2'-Azobis (2-aminopropane)dihydrochloride (AAPH)</td>
<td>C_{8}H_{18}N_{6} * 2 HCl</td>
<td>Aldrich (D)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Apparatus</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL 600 Microplate Fluorescence Reader: FLUOstar Optima</td>
<td>Bio-Tek Instruments</td>
</tr>
<tr>
<td>96-well black polystyrene microplates</td>
<td>Greiner Bio-one Ltd. (D)</td>
</tr>
<tr>
<td>Pipettes (500µl, 1000µl, 1200µl, 5000µl)</td>
<td>Eppendorf</td>
</tr>
</tbody>
</table>

Tab. 3: Reagents and Instruments for the ORAC test

Phosphate buffer 75mmol/L, pH 7,4: In 1 L bi-distilled H_{2}O, there was 10,96g Na_{2}HPO_{4} and 10,21g KH_{2}PO_{4} dissolved. Due to the pH sensitivity of fluorescein, the pH was adjusted to 7,4 with KOH. The pH-value is important for the function of fluorescein.

AAPH Solution (97%): 426,42mg AAPH was dissolved shortly before measuring into 5ml preheated (37°C) phosphate buffer, to provide a concentration of 315 mmol/L.
Fluorescein disodium stock solution: For a concentration of $4.19 \times 10^{-3}$, 0.158mg fluorescein disodium was dissolved in 100ml phosphate buffer.

Fluorescein disodium working solution: To obtain a final concentration of $8.16 \times 10^{-5}$mmol/l 487.5µl of fluorescein disodium, stock solution was dissolved into 25ml phosphate buffer. The amount was sufficient for 4 measurements.

Trolox® (Standard): For the stock solution 62.58mg of Trolox was mixed with 100ml of PBS buffer for a concentration of 2500µmol/l and placed in an ultrasonic bath until completely dissolved. 500µl of the Trolox stock solution was filled in cups and frozen at -80°C for further use. To establish a standard line the solution was diluted to concentrations of 6.25, 12.5, 50, 75 and 100µmol/l.

Sample Plasma: Before analyzing, the samples were defrosted, centrifuged and diluted with phosphate buffer at the ratio of 1:160.

Reference Plasma (Control): To ensure accurate performance, the same plasma from a healthy female subject was used for every measurement and prepared exactly the same way as the plasma sample (dilution factor 1:160).

The Phosphate buffer, Fluorescein, and the Standard solution were prepared in advance, filled in opaque bottles and kept at 4°C. The Trolox® standard solution, the AAPH solution and the fluorescein working solution were made daily and secured properly to protect from daylight.
3.2.3 Instrument settings

<table>
<thead>
<tr>
<th>Test name</th>
<th>ORAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode</td>
<td>Fluorescence, Plate Mode</td>
</tr>
<tr>
<td>Microplate</td>
<td>Greiner black 96-well</td>
</tr>
<tr>
<td>No of cycles</td>
<td>35</td>
</tr>
<tr>
<td>No of flashes per well and cycle</td>
<td>10</td>
</tr>
<tr>
<td>Cycle time</td>
<td>60</td>
</tr>
</tbody>
</table>
| Filter and Integration | Extinctions filter: 485nm  
|                         | Emissions filter: 520nm            |
| Incubation  | On, 37°C           |
| Shaking options | Orbital, 1s, before first cycle |
| Gain       | Adjusted automatically |

Tab. 4: Basic Instrument settings ORAC

First, the FLUOstar Optima had to be activated, the appropriate settings adjusted and the incubation enabled (37°C). The instrument is able to measure absorbance as well as fluorescence with different measurement adapters. Therefore, it is of the utmost importance to check if the correct one is used. 5ml of phosphate buffer was kept in a 37°C warm water bath, and 426,42mg AAPH was provided for later use. Six solutions were prepared for the standard curve from 6,25 to 100µmol/l. The plasma was diluted with phosphate buffer in test tubes in a ratio of 1:160 and vortexed. After finishing the solutions, the reagents were transferred into the wells of the microplate with electronic Eppendorf pipettes based on the schemata in table 5.
<table>
<thead>
<tr>
<th>Reagents / well</th>
<th>Blank (µl)</th>
<th>Control (µl)</th>
<th>Standard (µl)</th>
<th>Sample (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trolox®</td>
<td></td>
<td></td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Sample Plasma</td>
<td></td>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Reference Plasma</td>
<td></td>
<td></td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Fluorescein solution</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

**Incubation of the microplate for 10 minutes at 37°C in FLUOstar Optima**

| AAPH solution 37°C | 25 | 25 | 25 | 25 |

Tab. 5: Pipetting schemata (max. volume 350µl/well) for ORAC

The blank, standards, controls and samples were analyzed in duplicate via electronic Eppendorf pipette. All steps of the schemata were followed, beginning with 25µl blank in each well of column 1A to 1B. 25µl of control plasma/well was pipetted in column 1C to 1F. The standard solutions were transferred in column 1G to 2G, beginning with 6.25µmol up to 100µmol. In columns 3A to 6H, each plasma sample was inserted twofold. In every well 150µl fluorescein working solution was added, and the half filled microplate was immediately inserted into the FLUOstar Optima. Without measuring, a test program was adjusted to incubate for 10 minutes at 37°C and shaken for 3 minutes. After the incubation, the recently dissolved AAPH that was preheated in the phosphate buffer was transferred into each well (25µl). The plate was reloaded into the microplate reader, and the fluorescence was measured every minute for 35 minutes in adjusted conditions (pH 7.4, 37°C). An evaluation software collected the data points during this period, compared them with the data of the standard (Trolox) and expressed them as µmol of Trolox equivalents TE/1. The calculation of the data is described in section 1.4.
3.2.4 Work plan for the ORAC assay

In order to maintain the total analyzing time of 35 minutes, 6 columns were filled per run (half a plate). The columns were first incubated for 10 minutes, thereafter the AAPH solution was added and measured for a further 35 minutes.
For ideal timing, two microplates were used simultaneously. When the first plate was measured, the 6 first columns of a second one were then prepared. When the first half of the second plate was in the reader, the second half of the first plate was arranged. Each plate was completely filled, though only used once.

3.2.5 Processing of data
By applying the formula of the area under the curve into a Microsoft Excel spreadsheet, the data were analyzed. By subtracting the AUC of the blank from that of the sample, the Net AUC was obtained. [OU et al., 2001]

a. Calculation of the AUC and the Net AUC for samples (plasma) and standard (Trolox)

\[
\text{AUC} = 0.5 + \frac{f_1}{f_0} + \cdots + \frac{f_i}{f_0} + \cdots + \frac{f_{34}}{f_0} + 0.5\left(\frac{f_{35}}{f_0}\right)
\]

\[f_0 = \text{initial fluorescence reading at 0 min.}\]
\[f_i = \text{fluorescence measure at time } i.\]

Fig. 8: Illustration of Net AUC (ORAC)
a. Standard curve
The curve was obtained by plotting the concentrations 6.25, 12.5, 50, 75 and 100 [µmol/l] of Trolox against the corresponding Net AUC.

\[ \text{ORAC value} = \frac{(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}})}{(\text{AUC}_{\text{trolox}} - \text{AUC}_{\text{blank}})} \times (\text{molarity of trolox}) \]

b. Calculation of ORAC values via Trolox regression equation
The relative ORAC value (Trolox equivalents) was calculated and expressed as µmol Trolox equivalent/l and the dilution factor of the plasma (1:160) was taken into account.

3.2.6 Quality assurance
Over a period of two days, reference plasma was determined twofold. The table below shows the standard deviation (SD) and the coefficient of variation for intra- and interday.
Table 8: Accuracy of the ORAC analyzing

<table>
<thead>
<tr>
<th></th>
<th>Orac (µmol TE/l)</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 Intraday</td>
<td>8677,92</td>
<td>214,34</td>
<td>2,47</td>
</tr>
<tr>
<td>Day 2 Intraday</td>
<td>9826,44</td>
<td>279,08</td>
<td>2,84</td>
</tr>
<tr>
<td>Interday mean</td>
<td>9252,18</td>
<td>812,13</td>
<td>8,78</td>
</tr>
<tr>
<td>Interday total</td>
<td>9498,29</td>
<td>595,34</td>
<td>6,27</td>
</tr>
</tbody>
</table>

3.3 FRAP – Ferric Reducing Ability of Plasma

3.3.1 The Principle of FRAP
The FRAP assay, which was developed by BENZIE and STRAIN (1996), represents a simple and reliable method to determine the antioxidant capacity of biological fluids. The essential mechanism of this procedure is the reduction of Fe³⁺ in consequence of the impact of antioxidant substances as well as due to the Fe³⁺/Fe²⁺-Tripyridyltriazin (TPTZ)-redoxsystem, which is the subsequent development of a blue colored Iron(II)-complex.

The spectrometric measurement of Fe²⁺ was defined at a low pH-value of 3,6 and at a wavelength of 593nm. [BENZIE and STRAIN, 1996] This method was...
adopted and modified for prior analysis at the Department of Nutritional Sciences. [VALENTINI, 2009]

The reaction time was determined at 6 minutes [BENZIE and STRAIN, 1996], though the wavelength was altered to 540nm in relation to the filter availability of the used apparatus. Furthermore, to increase the throughput rate of the samples, 96-well microplates were used. [TSAO et al., 2003]

The Ferric Reducing Ability of Plasma is a common technique for food extracts and pure substances that led to the newly coined term “Ferric Reducing Antioxidant Power Assay”.

### 3.3.2 Reagents and Instruments

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Chemical Formula</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acetate trihydrate</td>
<td>CH$_3$COONa · 3H$_2$O</td>
<td>Fluka Chemica (CH)</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>C$_2$H$_4$O$_2$</td>
<td></td>
</tr>
<tr>
<td>6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)</td>
<td>C$<em>{14}$H$</em>{18}$O$_4$</td>
<td></td>
</tr>
<tr>
<td>2-amino, 4, 6-dichloro-s-triazine (TPTZ)</td>
<td>C$_3$H$_2$Cl$_2$N$_4$</td>
<td></td>
</tr>
<tr>
<td>Ferrous(II)-sulphate-heptahydrate</td>
<td>FeSO$_4$ · 7H$_2$O</td>
<td></td>
</tr>
<tr>
<td>Iron(III)-chloride-hexahydrate</td>
<td>FeCl$_3$ · 6H$_2$O</td>
<td>Riedl de Haen (D)</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>HCl</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Apparatus</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL 600 Microplate Fluorescence Reader: FLUOStar Optima</td>
<td>Bio-Tek Instruments</td>
</tr>
<tr>
<td>96-well microplates</td>
<td>Sterlin (UK)</td>
</tr>
<tr>
<td>Pipettes (500µl, 1000µl, 1200µl, 5000µl)</td>
<td>Eppendorf</td>
</tr>
</tbody>
</table>

Tab. 9: Reagents and Instruments for the FRAP assay
Acetate buffer 300mmol/L, pH 3.6: In 1 liter bi-distilled H₂O, 3.1g C₂H₃NaO₂ * 3 H₂O and 16ml concentrated acetic acid was dissolved.

TPTZ - solution (10mmol/l in 40 mmol/l HCl): 585µl HCl and 31,2mg 2.4.6-Tripyridyl-s-triazin was dissolved in 100ml distilled water.

FeCl₃ * 6 H₂O - solution(20 mmol/l): 54.1mg Iron(III)-chloride was dissolved into 100ml distilled water.

Frap - solution: 25ml acetate buffer, 2,5ml Iron(III)-chloride and 2,5ml TPTZ-solution were compounded. (10:1:1) The solution was newly prepared daily and stored in a bathwater at 37°C.

FeSO₄ * 7 H₂O - solution (2000 mmol/l): In 100ml distilled water, 55,6mg Iron(II)-sulfate was dissolved to obtain a stock solution, which was further diluted into the concentrations of 50, 125, 250, 500, 625, 750 and 1000µmol/l.

Trolox® (Standard): For the stock solution, 62,58mg of Trolox was dissolved in 100ml of PBS buffer for a concentration of 2500µmol/l and placed in an ultrasonic bath until completely dissolved. 1ml of the Trolox stock solution was filled in cups and frozen at -20°C for further use. To establish a standard curve, the solution was diluted each day before measurement to concentrations of 50, 100, 200, 300, 400 and 550µmol/l.

Sample Plasma: Before analyzing, the samples were defrosted and centrifuged.

Reference Plasma (Control): To ensure accurate performance, the same plasma from a healthy female subject was used for every measurement and prepared exactly the same way as the plasma sample.
3.3.3 Instrument settings

<table>
<thead>
<tr>
<th></th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test name</td>
<td>FRAP</td>
</tr>
<tr>
<td>Mode</td>
<td>Absorbance</td>
</tr>
<tr>
<td>Microplate</td>
<td>96-well</td>
</tr>
<tr>
<td>No of cycles</td>
<td>10</td>
</tr>
<tr>
<td>Start measurement</td>
<td>1s</td>
</tr>
<tr>
<td>Cycle time</td>
<td>36s</td>
</tr>
<tr>
<td>Filter and Integration</td>
<td>Excitation filter: 540nm</td>
</tr>
<tr>
<td>Incubation</td>
<td>On, 37°C</td>
</tr>
<tr>
<td>Shaking options</td>
<td>Orbital, 5s, before first cycle</td>
</tr>
</tbody>
</table>

Tab. 10: Basic Instrument settings FRAP

First, the FLUOstar Optima had to be activated, the appropriate settings adjusted and the incubation enabled (37°C). After finishing the preparation of the solutions, the reagents were transferred into the wells of the microplate with electronic Eppendorf pipettes.

The blank, standards, controls and samples were analyzed in duplicate. All steps summarized in table 11 were considered, beginning with 30µl of distilled water in each well, except for the blank where 40µl was applied. The procedure then continued as 10µl plasma, standard and/or control were pipetted and subsequently, 300µl of preheated (37°C) Frap-solution was added into every well. The time between adding the solution and starting the measurement was less than one minute.

<table>
<thead>
<tr>
<th>Reagents / well</th>
<th>Standard (µl)</th>
<th>Control (µl)</th>
<th>Sample (µl)</th>
<th>Blank (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron(II)-solution</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trolox®</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Aqua dest.</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>FRAP-solution</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
</tbody>
</table>

Tab. 11: Pipetting schemata FRAP
Prior to the start of the assay, the microplate reader was adjusted to shake for 5 seconds and then measure at intervals of 36 seconds for a total duration of 6 minutes. After shaking had been completed, the final value was obtained, which had to be applied against the blank value. An Iron(II)-standard series was used simultaneously with samples, blanks and Trolox (control) to quantify the concentrations of the antioxidative capacity.

3.3.4 Work plan for the FRAP assay

Following the pipette table below, 2 columns of a plate were filled and measured while 2 columns of a second microplate were simultaneously prepared. This procedure was alternately continued until all samples were analyzed. Each plate was completely filled, though only used once.

(a)

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Fe²⁺ 50 µmol</td>
<td>Trolox</td>
<td>50 µmol</td>
<td>S 1</td>
<td>S 5</td>
<td>S 9</td>
<td>S 13</td>
<td>S 17</td>
<td>S 21</td>
<td>S 26</td>
<td>S 30</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Fe²⁺ 125 µmol</td>
<td>Trolox</td>
<td>100 µmol</td>
<td>S 1</td>
<td>S 5</td>
<td>S 9</td>
<td>S 13</td>
<td>S 17</td>
<td>S 21</td>
<td>S 26</td>
<td>S 30</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Fe²⁺ 250 µmol</td>
<td>Trolox</td>
<td>200 µmol</td>
<td>S 2</td>
<td>S 6</td>
<td>S 10</td>
<td>S 14</td>
<td>S 18</td>
<td>S 22</td>
<td>S 27</td>
<td>S 31</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Fe²⁺ 500 µmol</td>
<td>Trolox</td>
<td>300 µmol</td>
<td>S 2</td>
<td>S 6</td>
<td>S 10</td>
<td>S 14</td>
<td>S 18</td>
<td>S 22</td>
<td>S 27</td>
<td>S 31</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Fe²⁺ 625 µmol</td>
<td>Trolox</td>
<td>400 µmol</td>
<td>S 3</td>
<td>S 7</td>
<td>S 11</td>
<td>S 15</td>
<td>S 19</td>
<td>S 23</td>
<td>S 28</td>
<td>S 32</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Fe²⁺ 750 µmol</td>
<td>Trolox</td>
<td>550 µmol</td>
<td>S 3</td>
<td>S 7</td>
<td>S 11</td>
<td>S 15</td>
<td>S 19</td>
<td>S 23</td>
<td>S 28</td>
<td>S 32</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>Fe²⁺ 1000 µmol</td>
<td></td>
<td></td>
<td>S 4</td>
<td>S 8</td>
<td>S 12</td>
<td>S 16</td>
<td>S 20</td>
<td>S 24</td>
<td>S 29</td>
<td>S 33</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td>S 4</td>
<td>S 8</td>
<td>S 12</td>
<td>S 16</td>
<td>S 20</td>
<td>S 25</td>
<td>S 29</td>
<td>S 33</td>
<td></td>
</tr>
</tbody>
</table>

B…Blank, Fe²⁺…Control, T…Trolox, S…Sample plasma

Tab. 12: Layout for the 96-well Microplate FRAP
3.3.5 Processing of data

Out of the different absorptions of both the blank and standard solvent Iron(II) (50, 125, 250, 500, 625, 750 and 1000µmol/l), a standard curve was established. The concentration of the plasma sample (µmol/l) was calculated using the linear regression formula (y = k*x + d).

![Iron(II) standard curve (FRAP)](image)

**Fig. 11:** Illustration of the Iron(II)-standard curve (FRAP)
Trolox solution was used as a control substance.

![Graph of Trolox curve (FRAP)](image)

Fig. 12: Illustration of the Trolox-curve (FRAP)

### 3.3.6 Quality assurance
FRAP was measured within a day, therefore control plasma was determined tenfold. The table below shows the standard deviation (SD) and the coefficient of variation (CV%) of the method.

<table>
<thead>
<tr>
<th>FRAP (µmol Fe²⁺ Eq/l)</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (n=10)</td>
<td>426.17</td>
<td>22.29</td>
</tr>
</tbody>
</table>

Tab. 14: Accuracy of FRAP

### 3.4 Malondialdehyde (MDA)

#### 3.4.1 The Principle of MDA
This method is based on the publications by BANNI and LUCCHI et al. [1996] and THOMSON and SMITH [1985]. Sampled plasma is hydrolyzed in the presence of phosphoric acid to accumulate a colored complex with thiobarbituric acid (TBA), during a one hour heating procedure at 100°C. Following the precipitation of plasma proteins in methanol dissolved samples,
the MDA-TBA-adducts were separated on the basis of HPLC (emission 563nm; excitation 532nm).

### 3.4.2 Reagents and Instruments

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Chemical Formula</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetraethoxypropan (TEP)</td>
<td>C₇H₁₆O₄</td>
<td>Sigma-Aldrich (D)</td>
</tr>
<tr>
<td>Thiobarbituric acid (TBA)</td>
<td>C₄H₄N₂O₄S</td>
<td>Sigma-Aldrich (D)</td>
</tr>
<tr>
<td>Ethanol 96% p.a.</td>
<td>C₂H₆O</td>
<td>Riedel de Haen (D)</td>
</tr>
<tr>
<td>Methanol/Sodium Hydroxide</td>
<td>CH₃NaO</td>
<td>Riedel de Haen (D)</td>
</tr>
<tr>
<td>Methanol chromasolv</td>
<td>CH₃OH</td>
<td>Riedel de Haen (D)</td>
</tr>
<tr>
<td>Phosphoric acid (density 1.71 g/ml; 85%)</td>
<td>H₃PO₄</td>
<td></td>
</tr>
<tr>
<td>1 N sodium hydroxide</td>
<td>NaOH</td>
<td></td>
</tr>
<tr>
<td>Distilled Water (H₂O)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Apparatus and Manufacturer**

| HPLC: LC-20AT Dual Reciprocating Plunger HPLC Pump | Shimadzu |
| Column: Merck LichroCart 125-4 Lichrosphere 100 RP-18 (5µm) |          |
| Detector: Hitachi Model F-1050 Fluorescence Spectrophotometer |        |
| Integrator: Hitachi model D-7500                  |          |

**Tab. 15: Reagents and Instruments for MDA**

- **For the eluent (mobile phase):**

  Phosphate buffer, pH 6.8: 6.8g of potassium dihydrogen phosphate was dissolved in 800ml of distilled water, the pH adjusted to 6.8 and consequently, the distilled water increased to 1l.

  Eluent's composition: Phosphate buffer and methanol chromasolv were mixed at a rate of 60:40. After filtering the eluent through filter paper, it was degassed in an ultrasonic bath for 15 minutes.
- **For the standards:**

  Thiobarbituric acid (TBA) solution: 0.6g of TBA was dissolved in 100ml of distilled water.

  Methanol/Sodium Hydroxide solution: 4.5ml of 1molar sodium hydroxide was dissolved in 50ml of methanol chromasolv.

  Phosphoric acid solution (0.44 mol/l): 3ml of concentrated ortho-phosphoric acid (density 1.71 g/ml; 85%) was mixed with 100ml with distilled water.

  1 N sodium hydroxide solution: 4g of caustic soda was dissolved in 100ml of distilled water.

  For the stock solution, 50µl of TEP (9% density = 0.92g/ml) was dissolved in 50ml of ethanol: water solution (40:60), of which 100µl was added to 50ml of ethanol: water solution (40:60) for the base material. To establish a standard line, the base material was further diluted to concentrations of 0 µM (distilled water), 0.195 µM up to 8.12 µM.

### 3.4.3 Instrument settings

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Autosampler</strong></td>
<td>2 trays; one for 8 standard samples and 70 for samples</td>
</tr>
<tr>
<td><strong>Filter</strong></td>
<td>Extinctions filter: 532 nm</td>
</tr>
<tr>
<td></td>
<td>Emissions filter: 563 nm</td>
</tr>
<tr>
<td><strong>Mobile Phase</strong></td>
<td>buffer : methanol (60:40)</td>
</tr>
<tr>
<td><strong>Flow Rate</strong></td>
<td>1 ml/min</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td>20</td>
</tr>
</tbody>
</table>

*Tab. 16: Basic Instrument settings MDA*
3.4.4 Implementation

<table>
<thead>
<tr>
<th>Reagents / pyrex glass</th>
<th>Blank (µl)</th>
<th>Control (µl)</th>
<th>Standard (µl)</th>
<th>Sample (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoric acid</td>
<td>700</td>
<td>700</td>
<td>700</td>
<td>700</td>
</tr>
<tr>
<td>TBA-solution</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Water</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference Plasma</td>
<td></td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Sample Plasma</td>
<td></td>
<td></td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

Tab. 17: Pipetting schemata for MDA

The solution was boiled in water for 60 minutes and afterwards cooled down in ice water for 10 minutes. Subsequently, 100µl of methanol/sodium hydrogen solution were added to the solution in the test tubes. The contained methanol denaturized the plasma proteins, and sodium hydroxide neutralized the phosphoric acid. Simultaneously there was 100µl of standards or sample pipette into the test tubes, followed by the centrifugation of the samples for 2 minutes at 3000 rpm. With pH indicator paper, the pH was adjusted and 7,2 µl of each sample was injected into the HPLC apparatus.

3.4.5 Calculation

A calibration curve with 8 standards was chosen, and the concentrations were calculated using the linear regression formula \( y = k \cdot x + d \), which was stated in µmol MDA/ml plasma.

3.4.6 Quality assurance

A control plasma was used daily as an internal standard to verify the accuracy of the method and the standard deviation in order to calculate the variation coefficient.
### 3.5 Statistical evaluation

The statistical analysis was performed with SPSS Statistics 17.0 for Windows. To test on normal distribution, the Kolmogorow-Smirnow-Test (KS-Test) was applied. The mean values of the two groups were then compared by utilizing both the t-Test and the Mann-Whitney-Test (U-Test). The t-Test was used for independent samples with normal distributed data, and the U-Test was used for data that did not follow a normal distribution. Three or more groups were compared by using Oneway Anova with the Tukey post hoc test for equal variances and the Tamhane’s T2 for non-homogenous data. To determine correlations between different groups, the “Pearson Correlation” and the “Spearman Correlation” were performed, dependent on the normal distribution. All data are shown as mean values ± standard deviation. The significant differences were expressed as significant (p<0,05)*, highly significant (p<0,01)**, and very highly significant (p<0,001)***. If a trend was observed, it was marked as (p=0,05 – 0,10)#.

### 4 Results and Discussion

#### 4.1 Study characteristics

100 subjects participated in the study, 76 of which successfully completed both the evaluation and met the inclusion criteria. The 76 individuals were then divided into two groups, the GS group, which was defined by UCB levels at or over 17 µmol/l, and the healthy control group with had UCB levels under 17 µmol/l. Each group included 28 male and 10 female individuals.

<table>
<thead>
<tr>
<th>control</th>
<th>MDA (µmol/l)</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 intraday</td>
<td>0,976</td>
<td>0,005</td>
<td>0,5</td>
</tr>
<tr>
<td>Day 2 intraday</td>
<td>0,941</td>
<td>0,041</td>
<td>4,4</td>
</tr>
<tr>
<td>Interday mean</td>
<td>0,958</td>
<td>0,025</td>
<td>2,6</td>
</tr>
<tr>
<td>Interday total</td>
<td>0,958</td>
<td>0,031</td>
<td>3,3</td>
</tr>
</tbody>
</table>

Tab. 18: Accuracy of MDA
The measured UCB levels in the total group ranged from $4,10 \, \mu mol/l$ to $73,53 \, \mu mol/l$, which is analog to the results of BOON et al. [2012] with UCB concentrations between $5,1$ and $85,3 \, \mu mol/l$. With $p<0,001$, the GS group showed significantly higher UCB levels ($32,0 \pm 13,64 \, \mu mol/l$) compared to the control group ($10,3 \pm 3,31 \, \mu mol/l$).

The recorded UCB concentration roughly corresponds to the results of VITEK et. al, who detected significantly higher serum bilirubin levels in GS subjects with $27,8 \pm 9,7 \mu mol/l$, compared to the control group with $9,9 \pm 3,0 \mu mol/l$. [2007]

### 4.2 Evaluation of the used methods ORAC, FRAP and MDA

In the following chapters, the results of ORAC, FRAP and MDA are discussed; hence, the study population was subdivided into different groups to determine potential distinctions based on gender, age, body mass index and UCB. ORAC values are expressed in $\mu$mol Trolox Equivalent (TE)/l, and FRAP levels are expressed in $\mu$mol Fe$^{2+}$ Equivalent/l. However, for convenience, the data are stated as $\mu$mol/l. To our knowledge, ORAC was never before used in context with GS.
4.2.1 Differences between the GS and control group

The study population was divided into a healthy control group and a GS group in order to determine the differences.

<table>
<thead>
<tr>
<th>mean ± SD (µmol/l)</th>
<th>GS</th>
<th>Control</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>38</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>ORAC</td>
<td>9144 ± 822</td>
<td>9420 ± 1130</td>
<td>0,23</td>
</tr>
<tr>
<td>FRAP</td>
<td>548 ± 59</td>
<td>440 ± 55</td>
<td>&lt; 0,001***</td>
</tr>
<tr>
<td>MDA</td>
<td>0,825 ± 0,339</td>
<td>0,785 ± 0,234</td>
<td>0,55</td>
</tr>
</tbody>
</table>

Tab. 21: ORAC, FRAP and MDA levels in GS and control group

ORAC and MDA showed no significant differences between the GS and control group. However, FRAP exhibited very highly significant elevated concentrations compared to the healthy control group (p<0,001). This is according to the results of BLUMER et al. who discovered significant increased FRAP levels (p<0,05), although there were no significant lower MDA plasma levels (p=0,500) in the GS group. [2008]
4.2.2 UCB (unconjugated bilirubin) in Tertiles

The entire study population was divided into different UCB-tertiles to detect the different effects. The UCB-ranges were separated into 3 groups:

- **Tertile 1** (<12µM): low bilirubin levels
- **Tertile 2** (12,5-21,4µM): moderate bilirubin levels
- **Tertile 3** (>21,4µM): high bilirubin levels

<table>
<thead>
<tr>
<th>mean ± SD (µmol/l)</th>
<th>Tertile 1</th>
<th>Tertile 2</th>
<th>Tertile 3</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>26</td>
<td>25</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>Age</td>
<td>31,5 ± 11,1</td>
<td>30,7 ± 11,6</td>
<td>34,1 ± 11,8</td>
<td>0,555</td>
</tr>
<tr>
<td>UCB range</td>
<td>4,10 - 12,48</td>
<td>12,65 - 21,38</td>
<td>22,06 - 73,53</td>
<td>-</td>
</tr>
<tr>
<td>ORAC</td>
<td>9490 ± 1118</td>
<td>9092 ± 1037</td>
<td>9257 ± 782</td>
<td>0,358</td>
</tr>
<tr>
<td>FRAP</td>
<td>427 ± 55</td>
<td>487 ± 44</td>
<td>571 ± 55</td>
<td>&lt; 0,001***</td>
</tr>
<tr>
<td>MDA</td>
<td>0,795 ± 0,242</td>
<td>0,798 ± 0,245</td>
<td>0,823 ± 0,376</td>
<td>0,933</td>
</tr>
</tbody>
</table>

Tab. 22: ORAC, FRAP and MDA levels divided into UCB tertiles

The ORAC and MDA levels showed no significant differences between the three UCB ranges. The FRAP data present a very high significant difference between all bilirubin tertile groups (p<0,001). Thus, leading to the conclusion that the FRAP levels and consequently, the antioxidant capacity increases in correlation to elevated UCB levels.
4.2.3 UCB in Quartiles

The study population was further divided into UCB-Quartiles:

- Quartile 1 (<10 µmol/l): 4.10 – 8.21 µmol/l
- Quartile 2 (10 – 17.1 µmol/l): 10.26 – 17.10 µmol/l
- Quartile 3 (17.2 – 36 µmol/l): 17.78 – 35.40 µmol/l
- Quartile 4 (≥ 36 µmol/l): 36.77 – 73.53 µmol/l

<table>
<thead>
<tr>
<th>mean ± SD (µmol/l)</th>
<th>Quartile 1</th>
<th>Quartile 2</th>
<th>Quartile 3</th>
<th>Quartile 4</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>16</td>
<td>23</td>
<td>23</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>Age</td>
<td>33.0 ± 12.3</td>
<td>30.8 ± 10.5</td>
<td>31.4 ± 10.9</td>
<td>34.4 ± 13.6</td>
<td>0.800</td>
</tr>
<tr>
<td>UCB range</td>
<td>4.10 - 8.21</td>
<td>10.26 - 17.10</td>
<td>17.78 - 35.40</td>
<td>36.77 - 73.53</td>
<td>-</td>
</tr>
<tr>
<td>ORAC</td>
<td>9115 ± 1039</td>
<td>9612 ± 1147</td>
<td>9054 ± 856</td>
<td>9305 ± 802</td>
<td>0.240</td>
</tr>
<tr>
<td>FRAP</td>
<td>421 ± 60</td>
<td>453 ± 47</td>
<td>529 ± 56.8</td>
<td>585 ± 39</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>MDA</td>
<td>0.826 ± 0.274</td>
<td>0.766 ± 0.204</td>
<td>0.830 ± 0.366</td>
<td>0.805 ± 0.311</td>
<td>0.885</td>
</tr>
</tbody>
</table>

Tab. 23: ORAC, FRAP and MDA levels divided into UCB-quartiles

There were no significant differences between the four UCB groups in ORAC and MDA. The FRAP values were very highly significant different within all
quartiles (p<0.001). The levels showed a considerable increase from the first to the forth quartile (Quartile 1 < Quartile 2 < Quartile 3 < Quartile 4).

4.2.4 Differences between gender

Due to an existing gender bias in GS (~25%) the study population was subdivided into men and women to establish occurring distinctions. [VITEK et al. 2002]

<table>
<thead>
<tr>
<th>mean ± SD (µmol/l)</th>
<th>Male</th>
<th>Female</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>56</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Age</td>
<td>31,5 ± 11,4</td>
<td>33,9 ± 11,7</td>
<td>0,426</td>
</tr>
<tr>
<td>UCB</td>
<td>22,4 ± 15,5</td>
<td>17,5 ± 11,6</td>
<td>0,184</td>
</tr>
<tr>
<td>ORAC</td>
<td>9368 ± 953</td>
<td>9043 ± 1082</td>
<td>0,212</td>
</tr>
<tr>
<td>FRAP</td>
<td>505 ± 64</td>
<td>462 ± 104</td>
<td>0,033*</td>
</tr>
<tr>
<td>MDA</td>
<td>0,797 ± 0,299</td>
<td>0,828 ± 0,268</td>
<td>0,681</td>
</tr>
</tbody>
</table>

Tab. 24: ORAC, FRAP and MDA levels considering gender

The UCB and ORAC levels were slightly higher, and the MDA levels were marginally lower in male subjects, everything not significant. Only FRAP showed significantly (p < 0,05) higher levels in men (505 ± 64), compared to female subjects (462 ± 104). This might indicate that men, due to an increased bilirubin level, have a higher antioxidative capacity and correspondingly, less oxidative damage.

Male subjects generally show higher bilirubin concentrations (12,4%), and are more often affected by GS when compared to females (4,8%). An explanation might be the increased bilirubin load per kilogram body weight in men, as well as an inhibition of the enzymatic glucuronidation of bilirubin, due to androgen steroids. [RADU and ATSMON 2001]

A Slovakian study that investigated the effect of mineral wool exposure on oxidative DNA damage and lipid peroxidation, found no differences between the exposed and the control group, however, general male individuals showed significantly higher concentrations of FRAP than female subjects, which is similar to our outcome. [STARUCHOVA et al., 2008]
4.2.5 Differences between 2 groups BMI

<table>
<thead>
<tr>
<th>mean ± SD (µmol/l)</th>
<th>BMI &lt; 25 kg/m²</th>
<th>BMI ≥ 25 kg/m²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>53</td>
<td>23</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>29.7 ± 9.9</td>
<td>37.7 ± 13.0</td>
<td>0.005**</td>
</tr>
<tr>
<td>UCB</td>
<td>29.3 ± 12.3</td>
<td>10.1 ± 3.2</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>ORAC</td>
<td>9127 ± 888</td>
<td>9640 ± 1139</td>
<td>0.038*</td>
</tr>
<tr>
<td>FRAP</td>
<td>502 ± 81</td>
<td>474 ± 70</td>
<td>0.156</td>
</tr>
<tr>
<td>MDA</td>
<td>0.821 ± 0.291</td>
<td>0.768 ± 0.291</td>
<td>0.466</td>
</tr>
</tbody>
</table>

Tab. 25: ORAC, FRAP and MDA levels divided into 2 BMI groups

In the total study population, more subjects had a normal body mass index < 25 kg/m² than an increased BMI ≥ 25 kg/m², (approximate ratio= 2:1). The group with elevated BMI levels (37.7 ± 13.0) was significantly older (p=0.005), compared to the group < 25 kg/m² (29.7 ± 9.9), which is evidence that the BMI is increasing with the advancement of age.

MDA and FRAP showed no significant differences between the two categories, although FRAP hints at slightly higher values in the group < 25 kg/m² (502.1 ± 81.1µmol/l), compared to the group ≥ 25 kg/m² (474.2 ± 69.7µmol/l).

The UCB levels are highly significantly increased in subjects with a BMI < 25 kg/m² (p<0.001***), which leads to the conclusion that patients with GS generally have normal bodyweight and, are therefore not overweight or obese.

Interestingly, opposite to former results, the ORAC values were significantly lower (p=0.038) in the group < 25 kg/m² (9127 ± 888 µmol/l), compared to the overweight group (9640 ± 1139 µmol/l), which subsequently showed a higher antioxidant capacity. A possible reason for the elevated data in overweight subjects might be the increased serum lipid level, which is able to bind and neutralize circulating endotoxins, and therefore, inhibit the subsequent stimulation of inflammatory cytokines. Furthermore, a higher production of soluble TNF-α receptor neutralizes the inflammatory effect of TNF-α. Another explanation might be the ability of adipose tissue to neutralize toxic metabolites
and the antioxidative effect of the musculature. Thus, an increased BMI in subjects with higher age does not necessarily lead to a higher mortality rate. For individuals over 65 years of age, a BMI of 27 – 30 kg/m² was established to be in correlation to the mortality risk. Therefore, a BMI ≥ 25 kg/m² proved to be advantageous, especially in elderly people with diseases, such as chronic inflammation, chronic heart failure, renal failure and rheumatoid arthritis. [DORNER and RIEDER, 2010]

4.2.5.1 Differences between GS and C group divided in 2 groups BMI

<table>
<thead>
<tr>
<th></th>
<th>GS &lt; 25 kg/m²</th>
<th>C &lt; 25 kg/m²</th>
<th>GS ≥ 25 kg/m²</th>
<th>C ≥ 25 kg/m²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>30</td>
<td>23</td>
<td>8</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>UCB</td>
<td>29.3 ± 12.3</td>
<td>10.1 ± 3.2</td>
<td>35.6 ± 10.5</td>
<td>21.1 ± 14.7</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>ORAC</td>
<td>9015 ± 847</td>
<td>9273 ± 937</td>
<td>9630 ± 516</td>
<td>9645 ± 1381</td>
<td>0.160</td>
</tr>
<tr>
<td>FRAP</td>
<td>550 ± 62</td>
<td>440 ± 58</td>
<td>540 ± 47</td>
<td>439 ± 53</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>MDA</td>
<td>0.818 ± 0.322</td>
<td>0.825 ± 0.251</td>
<td>0.853 ± 0.418</td>
<td>0.722 ± 0.197</td>
<td>0.665</td>
</tr>
</tbody>
</table>

Tab. 26: ORAC, FRAP and MDA levels divided into GS, control and 2 BMI groups

MDA showed no significant differences within the groups. ORAC indicated a slight but not significant difference between the GS group < 25 kg/m² (9015 ± 847) and the control group ≥ 25 kg/m² (9645 ± 1381). The UCB and FRAP results significantly differed between the GS and control groups.

4.2.6 Differences between age in 2 groups

Age can possibly influence the antioxidant capacity and oxidative damage. Therefore, the study population, which ranged from 20-67 years, was separated into a younger group with individuals < 30 years and an older group ≥ 30 years.
4.2.6.1 Differences between GS and C group divided in 2 age groups

For further evaluation the two age groups were stratified for GS and control group.

<table>
<thead>
<tr>
<th>(µmol/l)</th>
<th>GS &lt; 30 years</th>
<th>C &lt; 30 years</th>
<th>GS ≥ 30 years</th>
<th>C ≥ 30 years</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>22</td>
<td>16</td>
<td>21</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td>UCB</td>
<td>29.3 ± 12.3</td>
<td>10.1 ± 3.2</td>
<td>35.6 ± 10.5</td>
<td>21.1 ± 14.7</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>ORAC</td>
<td>8881 ± 803</td>
<td>9187 ± 927</td>
<td>9507 ± 725</td>
<td>9708 ± 1312</td>
<td>0.048*</td>
</tr>
<tr>
<td>FRAP</td>
<td>542 ± 578</td>
<td>440 ± 59</td>
<td>556 ± 61.5</td>
<td>440 ± 52</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>MDA</td>
<td>0.786 ± 0.277</td>
<td>0.821 ± 0.199</td>
<td>0.879 ± 0.412</td>
<td>0.739 ± 0.270</td>
<td>0.566</td>
</tr>
</tbody>
</table>

Tab. 28: ORAC, FRAP and MDA levels divided into GS, control and 2 age groups

There were no significant differences observed in MDA concerning the GS, the control group and age. FRAP and UCB data was highly significantly increased (p<0.001) in GS under and over 30 years, compared to both the younger and the older control group. ORAC was significantly higher in GS < 30 years than in the control group ≥ 30 years (p=0.045).

4.3 Correlations

Correlations between the methods ORAC, FRAP, MDA and various clinical and biochemical parameters were calculated. In the following chapters the most significant correlations are discussed.
4.3.1 Correlations with ORAC

4.3.1.1 ORAC and UCB
There was no significant correlation between ORAC and UCB in the total study population ($r=0.068$, $p=0.746$), as well as in the GS and control group.

4.3.1.2 ORAC and uric acid

<table>
<thead>
<tr>
<th></th>
<th>r-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>total population</td>
<td>0.256</td>
<td>0.025*</td>
</tr>
<tr>
<td>GS</td>
<td>0.205</td>
<td>0.218</td>
</tr>
<tr>
<td>control</td>
<td>0.338</td>
<td>0.038*</td>
</tr>
</tbody>
</table>

Tab. 29: Correlation between ORAC and uric acid

A minor significant positive relationship between ORAC and uric acid was observed in the whole study cohort. ($r=0.256$, $p=0.025^*$) ORAC exhibited no significant correlation with uric acid in the GS group ($r=0.205$, $p=0.218$), however in the control group a more potent significant correlation was found. ($r=0.338$, $p=0.038^*$)

Fig. 15: Correlation between ORAC and uric acid in (a) the whole cohort and (b) control
Uric acid is a potent natural antioxidant and is responsible for up to 60% of the free radical scavenging activity in human blood. [AMES et al., 1981] Elevated plasma levels of uric acid are further linked with markers for CVD. However, it is not clear if the increased concentrations are based on a protective antioxidant response to augmented oxidative stress or constitute a cause of CVD. Therefore, uric acid might provide antiatherosclerotic activity on the one hand, and exhibit proatherogenic effects at increased concentrations on the other hand. [GAGLIARDI et al. 2009, KAYA et al. 2010] A recent study suggested that uric acid serves as a substrate for myeloperoxidase, and the subsequent formed uric acid radical could influence atherogenic and inflammatory processes due to reactions with O2* and NO*. [MEOTTI et al., 2011]

4.3.1.3 ORAC and BMI
ORAC showed a slight but significant positive correlation with the body mass index in the total study population (r=0,269, p=0,019), with a middle strong correlation in the 3. UCB-tertile (r=0,419, p=0,037). A positive correlation of ORAC and BMI in male subjects proved to be significant (r=0,324, p=0,015), opposite to the ORAC levels of females, which indicated no significant correlation (r=0,120, p=0,614). In the GS group, the correlation was somewhat stronger (r=0,350, p=0,038), whereas the control group showed no significant relationship (r=0,177, p=0,289).

Fig. 16: Correlation of ORAC and BMI in (a) the whole cohort and (b) 3. UCB-tertile
4.3.2 Correlations with FRAP

4.3.2.1 FRAP and UCB

<table>
<thead>
<tr>
<th></th>
<th>r-value</th>
<th>p-value</th>
<th>r-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>total population</td>
<td>0.812</td>
<td>&lt;0.001***</td>
<td>BMI &lt; 25</td>
<td>0.832</td>
</tr>
<tr>
<td>GS</td>
<td>0.696</td>
<td>&lt;0.001***</td>
<td>BMI ≥ 25</td>
<td>0.672</td>
</tr>
<tr>
<td>control</td>
<td>0.451</td>
<td>&lt;0.001***</td>
<td>1. Tertile</td>
<td>0.351</td>
</tr>
<tr>
<td>male</td>
<td>0.777</td>
<td>&lt;0.001***</td>
<td>2. Tertile</td>
<td>0.547</td>
</tr>
<tr>
<td>female</td>
<td>0.877</td>
<td>&lt;0.001***</td>
<td>3. Tertile</td>
<td>0.431</td>
</tr>
<tr>
<td>&lt; 30 years</td>
<td>0.837</td>
<td>&lt;0.001***</td>
<td>≥ 30 years</td>
<td>0.767</td>
</tr>
</tbody>
</table>

Tab. 30: Correlation between FRAP and UCB in various groups

FRAP and UCB showed a highly significant positive correlation in all groups, except for the lowest UCB-tertile, where there was no significant correlation, but a tendency (r=0.351, p=0.079#). This indicates a higher antioxidant capacity in GS subjects due to an elevated UCB status.
4.3.2.2 FRAP and Albumin

<table>
<thead>
<tr>
<th></th>
<th>r-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>total population</td>
<td>0.302</td>
<td>0.008**</td>
</tr>
<tr>
<td>control</td>
<td>0.375</td>
<td>0.020*</td>
</tr>
<tr>
<td>female</td>
<td>0.501</td>
<td>0.024*</td>
</tr>
<tr>
<td>&lt; 30 years</td>
<td>0.498</td>
<td>0.001**</td>
</tr>
<tr>
<td>BMI &lt; 25</td>
<td>0.336</td>
<td>0.014*</td>
</tr>
<tr>
<td>1. UCB-Tertile</td>
<td>0.394</td>
<td>0.046*</td>
</tr>
</tbody>
</table>

Tab. 31: Correlations between FRAP and Albumin in various groups

Amongst the entire cohort, a positive highly significant relationship was detected between FRAP and albumin (r=0.302, p=0.008). The correlation proved to be strongest in females (r=0.501, p=0.024) and in subjects < 30 years (r=0.498, p=0.001).

![Correlation between FRAP and Albumin](image1)

![Correlation between FRAP and Albumin](image2)

Fig. 18: Correlation between FRAP and albumin in (a) females and (b) <30 years

Like uric acid, albumin acts as an important contributor to the total antioxidant status in humans. [CAO and PRIOR, 1998] As previously mentioned, albumin serves as a transporter for the hydrophobic unconjugated bilirubin in plasma and therefore, increased albumin concentrations are associated with elevated levels of bilirubin. STOCKER et al. [1987] proved that one mole of albumin-bound bilirubin scavenges two moles peroxyl radicals in competition with uric
acid. Albumin-bound bilirubin protects linoleic acid as well as the albumin molecule itself from peroxidation. In absence of antioxidants, the amino acids of albumin are attacked by OH* and O2* radicals. However, if UCB is present, the attacked amino acids decrease by half, which indicates a more potent effect in preventing protein damage in comparison to vitamin E and trolox. [NEUZIL et al., 1993]

4.3.3 Correlations with MDA

4.3.3.1 MDA and UCB

There were no significant correlations between UCB and plasma MDA in the whole cohort (r=0.032, p=0.782). The outcome is similar to Blumer et al. [2008], who found no significant differences in the oxidative stress marker MDA in the GS and control group (p=0.500).

MDA is a commonly used method to measure the antioxidant status in infants with hyperbilirubinemia. Compared to adults, infants with increased bilirubin levels show a prolonged lag phase and a reduced slope of serum oxidation. [WIEDERMANN et al., 2003]

KUMAR et al. investigated the influence of bilirubin as an antioxidant in newborns with increased bilirubin levels by assessing MDA as a marker of oxidative stress. Jaundiced neonatal subjects showed significantly lower MDA levels. Moreover, plasma bilirubin exhibited a significant negative correlation with antioxidant enzyme activities, which led to the conclusion that neonatal hyperbilirubinemia is associated with decreased oxidative stress. [2007] However, another study discovered elevated MDA concentrations and a positive correlation between MDA and bilirubin levels in infants with manifest hyperbilirubinemia, due to hemolysis compared to a healthy control group. [YIGIT et al., 1999]

Therefore, a recent study investigated the reason of the different outcomes and came to the following conclusion: the antioxidant effect of bilirubin is linked to the concentration until a certain threshold, after which kernicterus is developed.
with a toxic impact on cells. Hence, further research is necessary to investigate the point at which bilirubin shows a toxic effect when it passes the blood-brain-barrier. [DOGAN et al., 2011]

4.3.3.2 MDA and high density lipoprotein (HDL)

<table>
<thead>
<tr>
<th></th>
<th>r-value</th>
<th>p-value</th>
<th></th>
<th>r-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>total population</td>
<td>0.313</td>
<td>0.007**</td>
<td>BMI &lt; 25</td>
<td>0.300</td>
<td>0.032*</td>
</tr>
<tr>
<td>GS control</td>
<td>0.301</td>
<td>0.070*</td>
<td>BMI ≥ 25</td>
<td>0.341</td>
<td>0.112</td>
</tr>
<tr>
<td>male</td>
<td>0.260</td>
<td>0.058*</td>
<td>1. Tertile</td>
<td>0.459</td>
<td>0.018*</td>
</tr>
<tr>
<td>female</td>
<td>0.563</td>
<td>0.010*</td>
<td>2. Tertile</td>
<td>0.429</td>
<td>0.036*</td>
</tr>
<tr>
<td>female</td>
<td>0.563</td>
<td>0.010*</td>
<td>3. Tertile</td>
<td>0.208</td>
<td>0.330</td>
</tr>
<tr>
<td>&lt; 30 years</td>
<td>0.196</td>
<td>0.213</td>
<td>≥ 30 years</td>
<td>0.439</td>
<td>0.012*</td>
</tr>
</tbody>
</table>

Tab. 32: Correlation between MDA and HDL in various groups

There was a weak but highly significant positive relationship between MDA and HDL in the whole cohort observed (r=0.313, p=0.007). Higher levels of HDL are associated with elevated values of MDA and, therefore lead to an increased lipid peroxidation. In male subjects, there was no significant correlation, although a strong trend (r=0.260, p=0.058*) was found. In the GS group, no significant relationship was exhibited either (r=0.301, p=0.070*), which might be due to the fact that more men than women have GS (28 males : 10 females). The female population indicated the strongest correlation between MDA and HDL (r=0.563, p=0.010), which leads to the conclusion that women manifest a more potent lipid peroxidation compared to men, which is explainable by the higher HDL concentration in women.
High-density lipoprotein exhibits a protective role in the development of coronary heart disease and therefore, low plasma concentrations of HDL indicate a risk factor. The protective effect has been attributed in part in the reverse cholesterol transport, a multi-level process in which cholesterol is carried by plasma compartment from peripheral tissues back to the liver. [OLLSON, 2009] Moreover, HDL particles contain various elements that might account to cardioprotection through antioxidant, antithrombotic, anti-inflammatory and endothelial stabilizing effects. [BARTER et al., 2004] However, doubts about the beneficial attributes of HDL have arisen. ANSELL et al. [2007] suggested that HDL have either anti-inflammatory or pro-inflammatory properties, depending on whether proteins, enzymes or lipids are transported. Due to specific pathologic chemical and structural changes, HDL particles become dysfunctional, pro-inflammatory and lose the ability to decrease the oxidation of LDL. HDL particles also control the reverse cholesterol transport and suppress vascular inflammation. Patients with coronary heart disease exhibit HDL particles with pro-inflammatory properties independent of the HDL concentration. However, a high HDL is still considered as protective against CVD by several associations (e.g. American Heart Association (AHA)).
4.3.3.3 MDA and total cholesterol (TCH)

<table>
<thead>
<tr>
<th></th>
<th>r-value</th>
<th>p-value</th>
<th></th>
<th>r-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>total population</strong></td>
<td>0.322</td>
<td>0.005**</td>
<td><strong>BMI &lt; 25</strong></td>
<td>0.369</td>
<td>0.008**</td>
</tr>
<tr>
<td><strong>BMI ≥ 25</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.341</td>
<td>0.112</td>
</tr>
<tr>
<td><strong>GS</strong></td>
<td>0.326</td>
<td>0.049*</td>
<td>1. Tertile</td>
<td>0.478</td>
<td>0.018*</td>
</tr>
<tr>
<td><strong>BMI ≥ 25</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.429</td>
<td>0.034*</td>
</tr>
<tr>
<td><strong>control</strong></td>
<td>0.349</td>
<td>0.034*</td>
<td>2. Tertile</td>
<td>0.429</td>
<td>0.034*</td>
</tr>
<tr>
<td><strong>male</strong></td>
<td>0.228</td>
<td>0.097#</td>
<td>3. Tertile</td>
<td>0.197</td>
<td>0.355</td>
</tr>
<tr>
<td><strong>female</strong></td>
<td>0.584</td>
<td>0.007**</td>
<td></td>
<td>0.203</td>
<td>0.264</td>
</tr>
<tr>
<td><strong>&lt; 30 years</strong></td>
<td>0.478</td>
<td>0.001**</td>
<td>≥ 30 years</td>
<td>0.203</td>
<td>0.264</td>
</tr>
<tr>
<td><strong>≥ 30 years</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tab. 33: Correlation between MDA and HDL in various groups

A highly significant positive correlation between MDA and TCH in the whole study population was detected ($r=0.322$, $p=0.005$). The strongest relationship was found in female subjects ($r=0.584$, $p=0.007$), opposite to male individuals, who showed only a weak trend ($r=0.228$, $p=0.097$). In the GS group, there was more or less the same trend observed ($r=0.326$, $p=0.049$) as in the control group ($r=0.349$, $p=0.034$).

Cholesterol, a waxy fat steroid component of mammalian membranes, is an important factor of human health. However, increased concentrations in the blood have been strongly associated with damage to arteries and cardiovascular diseases. The majority of total serum cholesterol is contained in
low-density lipoprotein (LDL). Therefore, in various epidemiological studies a strong relationship between total cholesterol and coronary heart disease was found, which indicates that elevated LDL exhibits a powerful risk factor. Further it is revealed as the most frequent evident atherogenic lipoprotein, which is affirmed by significant elevation of LDL cholesterol in homozygous and heterozygous genetic disorders of familial hypercholesterolemia with an accelerated atherogenesis. [NCEP, 2002]
5 Conclusion
The present work aimed to analyze whether or not GS subjects exhibit an advanced protection against oxidative damage and subsequently, possess a superior antioxidant capacity. Thus, different assays with diverse mechanisms to determine the antioxidative capacity and oxidative stress caused by lipid peroxidation were used.

Whilst ORAC and MDA showed no significant differences between GS and control group, FRAP was significantly higher in GS. Furthermore, FRAP levels proved to be significantly linked to UCB through a simultaneous rise in concentration. Male subjects are more often affected by GS and showed elevated FRAP levels due to generally increased UCB concentrations and hence, manifest an elevated antioxidant capacity. A significantly positive relationship between FRAP and albumin was detected, which was strongest in women and individuals < 30 years of age and most presumably resulted from the fact that albumin acts as a transporter for UCB in plasma.

Interestingly, significantly higher ORAC levels in individuals ≥ 30 years of age and a significant correlation between ORAC and subjects with a BMI ≥ 25kg/m² was found. Moreover, ORAC was significantly positive correlated with uric acid in the control group compared to the GS population. This suggests that in healthy people, uric acid is the predominant antioxidant in human serum while in GS subjects, the competitive UCB is more dominant.

MDA was positively correlated with HDL and total cholesterol, especially in the female cohort, which proved a major susceptibility to oxidative damage in women in comparison to males.

The results of this study showed that females possess a higher potential for oxidative stress than do males. The GS subjects had significantly higher FRAP values than the age and sex matched healthy control group, which positively correlates with the elevation of UCB concentrations and therefore demonstrates that individuals with GS have a higher antioxidant capacity due to increased UCB.
6 Summary
In various studies, bilirubin has been proven to be an effective antioxidant with the potential to decrease the prevalence of oxidative stress-induced diseases. Therefore, it is assumed that individuals with GS exhibit a minor susceptibility to oxidative damage and subsequent diseases due to an elevated bilirubin status compared to healthy subjects.

The present case-control-study, which was funded by the FWF-Austrian Science Fund (project number P21162), aimed to investigate the antioxidant status of individuals with GS, a benign hyperbilirubinemia, compared to a group of healthy age and gender matched control subjects. 104 subjects were recruited and 76 of them divided according to their unconjugated serum bilirubin concentrations into an affected GS group and the control group.

To determine the antioxidant capacity the ORAC and FRAP assays were applied, and to further measure oxidative stress due to lipid peroxidation, the biomarker MDA was investigated. The outcome was not consistent, which might be a result of the different mechanisms of the used methods and moreover, a diverse affinity to the endogenous antioxidant bilirubin. Whilst ORAC and MDA showed no differences in the GS and control group, FRAP levels were significantly higher in GS subjects and exhibited a significantly positive correlation with UCB, which indicated a higher antioxidant capacity in GS based on increased UCB concentrations. Our findings therefore support the hypothesis that individuals with elevated UCB levels possess an improved protection against oxidative stress and subsequent diseases.

A significant relationship between ORAC and uric acid was discovered in the control group which suggests a predominance of uric acid in plasma of healthy individuals, contrary to GS subjects with bilirubin acting as predominating antioxidant.
7 Zusammenfassung

Unterschiedliche Studien konnten die antioxidative Wirkung von Bilirubin und dessen Potential für die Risikominderung von oxidativen Stress bedingten Erkrankungen belegen. Daher wird angenommen, dass Personen mit erhöhten Plasmabilirubinwerten (Gilbert Syndrome, GS) im Vergleich zu gesunden Personen eine geringere Empfindlichkeit gegenüber oxidativer Schädigung und einhergehenden Krankheiten besitzen.


Um die antioxidative Kapazität zu messen, wurden die Methoden ORAC und FRAP angewandt und um den oxidativen Stress zu beurteilen, wurde der Biomarker MDA untersucht. Die Ergebnisse waren unterschiedlich, was möglicherweise auf die verschiedenen Mechanismen der Methoden und deren unterschiedliche Affinität zu Bilirubin zurückzuführen ist. Während ORAC und MDA keine Unterschiede zwischen der GS- und Kontroll-Gruppe aufwiesen, waren die FRAP Werte bei den GS Personen signifikant erhöht und zeigten eine positive Korrelation mit unkonjugiertem Bilirubin (UCB). Dies weist auf eine gesteigerte antioxidative Kapazität bei Personen mit GS hin, die auf eine erhöhte UCB Konzentration zurückzuführen ist. Die vorliegenden Ergebnisse stützen daher die Hypothese, dass Menschen mit erhöhten UCB Werten einen besseren Schutz gegenüber oxidativem Stress und dessen Folgeerkrankungen besitzen.

Eine signifikant positive Korrelation zwischen ORAC und Harnsäure in der Kontroll-Gruppe zeigt das Potential von Harnsäure im Plasma, was sich in der Gruppe der GS zu Gunsten von erhöhtem UCB verschiebt.
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WIEDERMANN M, KONTUSH A, FINCKH B et al. Neonatal blood plasma is less susceptible to oxidation than adult plasma owing to its higher content of


9 Curriculum Vitae

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