ANA PATRÍCIA REGO LIMA

PLASMA AMINOTHIOL PROFILE AND SOME OF ITS DETERMINANTS IN SUBJECTS FROM THE AZORES ARCHIPELAGO, PORTUGAL

Dissertação para obtenção do grau de Mestre em Ciências Biomédicas,
apresentada à Universidade dos Açores

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To my family

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PREFACE

Cardiovascular diseases, which major cause is atherosclerosis, still constitute the first cause of mortality on our country. Furthermore, the Azores population presents the highest standardized mortality rate caused by ischaemic heart disease when compared to mainland Portugal. This fact is a huge concern in terms of public health and one of the reasons of this thesis.

Many patients have precocious atherosclerosis without having any of the well establish risk factors. Identification of other markers that increase the risk of atherosclerosis may improve our understanding of the pathophysiologic mechanisms of this disorder and allow the development of new preventive or therapeutic measures. Homocysteine has been view as an independent risk factor for cardiovascular diseases. However, little attention has been paid to the other low molecular weight aminothiols (cysteine, cysteinylglycine and glutathione), which together with homocysteine define the redox thiol status of the organism. In addition, glutathione deficiency contributes to oxidative stress, which plays a key role in aging and in the pathogenesis of many diseases.

This work is part of a research project ("Dissecting environmental and genetic determinants of atherosclerosis: a study in isolated populations from Azores Islands"), and is mainly focused on plasma aminothiol profile and some of its determinants.

This Msc thesis is organized in three chapters. The first Chapter concerns a General Introduction to the study. It includes a brief review of the current knowledge on atherogenesis and its well established risk factors, namely those respecting the oxidative aspects of the disease. In particular, the metabolism of aminothiols is described. The objectives of the work are also defined.

Chapter II was prepared under the form of a scientific paper to be submitted to publication. This paper reports for the first time plasma aminothiol profile (PAP) and related vitamins (B_6, B_{12}, and folate), as well as serum γ-GT activity in a group of apparently healthy subjects born and living in the Azores archipelago. Analyses of parameters were carried out having into consideration two groups, one with a normal and another with an altered PAP. Both groups were then divided into different subgroups according to gender, age range, and lipid profile.

Finally, in Chapter III results are summarized along with an integrated discussion of the main conclusions. Suggestions for future research are also presented.

SUMMARY

As compared to mainland Portugal, the Azores Islands have the highest standardized mortality rate for cardiovascular diseases (CVD), which main cause is atherosclerosis. Since symptoms arise generally in advanced stages of the disease, there is a need to find precocious, non-invasive markers. Aminothiols, such as homocysteine (Hcy), cysteine (Cys), cysteinylglycine (Cys-Gly) and glutathione (GSH), are fundamental intra and extracellular components that serve numerous roles in metabolism and homeostasis, particularly as redox buffers and as components in antioxidant defense. Impairment of the redox thiol status might be linked to the occurrence of atherosclerosis and CVD.

This work presents, for the first time, the determination of the plasma aminothiol profile (PAP) and its major determinants (plasma folate, vitamin B_{12} and vitamin B_6 concentrations and serum γ-GT activity), as well as their relationship with gender, age, and serum lipid profile, in a group of apparently healthy volunteers, all born and living in the Azores. Albeit the mean concentrations of all thiols were within reference values, 76% of all participants had an altered PAP, mainly due to low plasma GSH levels. Ten percent of all subjects had moderate hyperhomocysteinemia, which is an independent risk factor for CVD, but only a small part had folate, B_{12} or B_6 deficiencies. An altered PAP, which we hypothesize to constitute an early marker of atherosclerosis, was particularly associated with age, high γ-GT activity, and hyperlipidemia; namely, serum triglyceride and plasma Cys levels were positively correlated. All these factors can lead to oxidative stress, which has a central role in atherogenesis and in CVD. Among subjects with low GSH levels, less than one-quarter had high γ-GT activity, whereas almost 70% had hyperlipidemia. Low plasma GSH levels could result from a high utilization of this tripeptide to fight a pro-oxidant status caused mainly by hyperlipidemia, which constitutes a prevalent risk factor for atherosclerosis in the study population. On the other hand, an impairment in aminothiol metabolism might also contribute to the observed pro-oxidant profile.

RESUMO

Comparados com Portugal Continental, os Açores possuem as mais altas taxas de mortalidade padronizadas por doenças cardiovasculares (CVD), cuja principal causa é a aterosclerose. Geralmente, os sintomas só surgem em fases avançadas da doença, havendo necessidade de encontrar marcadores precoces e não invasivos.

Os aminotióis, tais como a homocisteína (Hcy), a cisteína (Cys), a cisteinil-glicina (Cys-Gly) e o glutationo (GSH) são componentes intra e extracelulares fundamentais que servem numerosas funções no metabolismo e na homeostase, particularmente como tampões redox e como componentes da defesa antioxidante. Disfunções no estado redox tiólico podem estar relacionadas com a ocorrência da aterosclerose e CVD.

Este trabalho apresenta, pela primeira vez, a determinação do perfil aminotiólico plasmático (PAP) e dos seus principais determinantes (concentrações plasmáticas de folato, vitamina B_{12} e vitamina B_6 e actividade sérica do γ-glutamil transferase - γ-GT), bem como as suas relações com o género, a idade e o perfil lipídico sérico de um grupo de voluntários aparentemente saudáveis, naturais e residentes nos Açores. Apesar de as concentrações médias de todos os tióis estarem dentro dos intervalos de referência, 76% dos participantes tinham um PAP alterado, principalmente devido a baixos níveis de GSH. Dez por cento dos indivíduos tinham uma hiper-homocisteinémia moderada, a qual constitui um factor de risco independente para a aterosclerose e as CVD, sendo que apenas uma pequena parte daqueles era deficiente em folato, vitaminas B_{12} ou B_6.

O PAP alterado, que se crê poder constituir um marcador precoce de aterosclerose, estava particularmente associado à idade, a valores mais elevados de actividade do γ-GT e a hiperlipidémia; nomeadamente, as concentrações de triacilgliceróis e de Cys estavam positivamente correlacionadas. Todos estes factores podem gerar stresse oxidativo, o qual desempenha um papel central na aterogénese e no desenvolvimento das CVD. De entre os indivíduos com baixos níveis de GSH, menos de um quarto tinha elevada actividade de γ-GT, ao passo que quase 70% eram hiperlipidémicos. Os baixos níveis de GSH no plasma poderão resultar da sua maior utilização no combate a um estado pró-oxidante originado sobretudo pela hiperlipidémia, que constitui um factor de risco de aterosclerose prevalente nesta população. Por outro lado, uma disfunção no metabolismo dos tióis poderá também contribuir para o perfil pró-oxidante observado.

LIST OF ABBREVIATIONS AND SYMBOLS

AdoMet – *S*-adenosylmethionine

AdoHcy - *S*-adenosylhomocysteine

BHMT - betaine homocysteine methyltransferase

CBS – cystathionine β-synthase

Cys - cysteine

CySS – cystine

Cys-Gly - cysteinylglycine

CVD- cardiovascular diseases

HC - haptocorin

Hcy – homocysteine

HcySS – homocystine

HDL - high density lipoprotein

HDLc - high density lipoprotein cholesterol

HHcy - hyperhomocysteinemia

H_2O_2 – hydrogen peroxide

$HO^{\bullet}$ - hydroxyl radical

HPLC - high performance/pressure liquid chromatography

IF - intrinsic factor

γ-GT - γ-glutamyl transferase or γ-glutamyl transpeptidase

GCS - γ-glutamyl-cysteine synthetase

Glu – glutamate

Gly – glycine

GSH – glutathione

GSSG – glutathione disulfide

LDL - low density lipoprotein

LDLc - low density lipoprotein cholesterol

MCP-1 - Monocyte chemoattractant protein-1

Met - methionine

Mrp/Abcc – multidrug-resistance-related protein, a subfamily of the ATP-binding cassette (ABC) transporters

MS - methionine synthase

MTHFR - 5,10-methylene tetrahydrofolate reductase

NADPH – nicotinamide adenine dinucleotide phosphate

NF-kB – nuclear factor- kappa B

NO – nitric oxide

$O_2^{-\bullet}$ - superoxide anion radical

PAP – plasma aminothiol profile

PLP - pyridoxal 5'-phosphate

ROS - reactive oxygen species

RSD - relative standard deviation

SHMT – serine hydroxyl-methyltransferase

TC- total cholesterol

TG - triglycerides

THF – tetrahydrofolate

5-methylTHF - 5-methyltetrahydrofolate

CHAPTER I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Cardiovascular diseases (CVD) are the main cause of death in Portugal, and the most common are of atherosclerotic origin, notably cerebrovascular disease and ischaemic heart disease (World Health Organization, 2009). In Portugal, the Azores Islands have the highest standardized mortality rate for ischaemic heart disease (Direcção-Geral da Saúde, 2006).

The knowledge on atherosclerosis pathogenesis has been almost revolutionized over the past decades and more than 300 risk factors (including genetic and environmental) have been associated with the genesis, progression and complication of this disorder. Particularly, biochemical risk factors play a crucial role in all steps of this disease, and entail a great relevance in risk stratification and early prevention (Montagnana *et al*., 2008). As symptoms of atherosclerosis are usually deadly, there is a need to identify precocious markers for this condition.

1. Atherosclerosis

Atherosclerosis is actually understood as a progressive, multifactorial, chronic inflammatory disorder, which results from the interaction of personal genetic components and environmental risk factors (Roy *et al*., 2009). Therefore, its underlying mechanisms are complex and can diverge in different populations. Atherosclerosis involves primarily the elastic arteries (e.g. aorta) and large and medium-sized muscular arteries (e.g. coronary and carotid arteries). It is characterized by an intima medial thickening of these arteries where the two most important non-modifiable risk factors are male gender and advanced age (Schoen, 2005).

Atherosclerosis begins with endothelial dysfunction in the intima, which increases leukocyte, low density lipoprotein (LDL), and platelet adhesion to the endothelium. This process is hastened by high serum cholesterol, diabetes, obesity, high blood pressure, smoking, and other risk factors (Patrick & Uzick, 2001). As a result of this dysfunction, there is an accumulation of LDL in the subendothelial matrix, which is greater when levels of circulating LDLc are raised (Lusis, 2000).

According to the oxidation theory of atherosclerosis, the oxidative modification of LDL in the intima, by reactive oxygen species (ROS) secreted from endothelial cells, macrophages and smooth muscle cells, is a prerequisite for the formation of the lipid laden macrophage, known as “foam cells”. With the progression of the lesion, the number and the size of foam cells and the amount of extracellular lipid deposition increase significantly, leading to the formation of the lipid core (fatty streak) (Lusis, 2000; Libby *et al*., 2002).

The oxidized LDL stimulates the overlying endothelial cells to produce pro-inflammatory molecules, including adhesion molecules and growth factors. It can, as well inhibit the production of nitric oxide (NO), which has anti-inflammatory properties. The inflammatory response initiates migration of smooth muscle cells into the fatty streak and eventually the inflammatory cells produce a lesion of necrotic cellular debris inside the artery wall. The lesion then becomes covered with a “fibrous cap” that protects the area, forming the atherosclerotic plaque. The artery dilates to accommodate this lesion initially and then the lumen begins to narrow. If the fibrous cap is uneven or thinning rupture can occur, and a blood clot is formed, with occlusion of the arterial wall (Lusis, 2000; Zhou & Austin, 2009).

Symptoms associated with atherosclerosis depend on the stage of the disease. In the early ones, which may last for decades, they are rare. In the later stages, the symptoms are caused by the obstruction of blood flow, which is clinically manifested as myocardial infarction (heart attack), cerebral infarction (stroke), aortic aneurysms or peripheral vascular disease (Schoen, 2005). Consequently, it is necessary to identify expedite markers to predict earlier this condition.

1.1. Atherosclerosis and dyslipidemia

The main plasma lipids are cholesterol (both free and esterified) and triglycerides, which are carried by lipoproteins. Thus, lipoproteins are an association of lipids (cholesterol, triglycerides and phospholipids) and proteins (known as apolipoproteins). There are four major classes: chylomicrons and very low density lipoproteins, which carry the triglycerides (TG), and LDL and HDL, carriers of cholesterol (Hachem & Mooradian, 2006).

Dyslipidemia is an abnormal amount of lipids and consequently lipoproteins in the blood. This term includes abnormally high levels of total serum cholesterol (TC), LDLc or TG, as well as a low level of HDLc. It is generally accepted as a major risk factor for atherosclerosis, and

can be broadly classified as either primary (genetic) or secondary to a complicating event, such as organ failure, diabetes mellitus or use of some medications (Hachem & Mooradian, 2006).

The most frequent form of dyslipidemia is hypercholesterolemia. In fact, the majority of circulating TC is contained in LDL, which can penetrate the endothelial wall and contribute to the creation of lipid foam and fatty streaks (Libby *et al*., 2002; Schoen, 2005). According to recommendations of ATP III guidelines (NCEP, 2002) and its recent update (Grundy *et al*., 2004) serum LDLc concentrations above 100 mg/dL are considered as atherogenic.

Elevated serum TG and reduced HDLc contribute as well for atherosclerosis development. However, since lipoprotein metabolism is integrally linked, elevations of serum TG can be confounded by significant correlations with TC, LDLc, and HDLc levels. Also, non-lipid risk factors of obesity, hypertension, diabetes, and cigarette smoking are interrelated with TG content (NCEP, 2002).

Conversely, high levels of HDLc appear to protect against atherogenesis, as these lipoproteins promote the reverse cholesterol transport (from peripheral tissues to liver), and have antioxidant and anti-inflammatory properties (Lusis, 2000; Libby *et al*., 2002).

2. Redox state and oxidative stress

While the medium surrounding living organisms is usually characterized by oxidative conditions, extra and intracellular redox state is maintained in the range of reducing values. Maintaining normal cellular redox state is therefore a central question in the normal function of cells, such as DNA synthesis, gene expression, enzymatic activity, (Oktyabrsky & Smirnova, 2007), cell proliferation, and apoptosis (Aw, 2003). Thus, redox homeostasis is very important for vital cellular functions, and its disruption can be accompanied by an increase in the level of ROS, resulting in oxidative damage of lipids, DNA, and proteins. The sources of ROS, such as superoxide anion radical ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and others, can be either of endogenous or exogenous origin. Exogenous sources like ionizing radiation, diet, and smoking can be at least partly prevented. ROS, particularly H_2O_2, are now known to be important second messengers in intracellular

signaling, but due to its high reactivity, they can provoke oxidative damage when present in high concentrations (Oktyabrsky & Smirnova, 2007).

The imbalance between oxidants and antioxidants in favor of the former is characteristically at the basis of the oxidative stress, which is considered to be a potential contributor to the development/progression of many diseases, such atherosclerosis and consequent CVD (Ceconi *et al*., 2003). ROS initiate lipid peroxidation of polyunsaturated fatty acids of LDL, which are particularly prone to this reaction. This is one of the most important events in atherogenesis, as previously described. The apolipoprotein moieties of the LDL can also experience oxidation modifications (Libby *et al*., 2002). On the other hand, the presence of an antioxidant defense system under normal conditions maintains intracellular concentration of oxidants at a safe level (Oktyabrsky & Smirnova, 2007).

The blood antioxidant defense system comprises enzymes, such as glutathione peroxidases, superoxide dismutases, and catalases, which are reinforced by non-enzymatic antioxidants such as glutathione (GSH), protein thiol groups (-SH), vitamins A, C and E (provided by diet) and by other molecules present in extracellular fluids, including albumin, transferrin and uric acid (Ceconi *et al*., 2003).

3. Plasma aminothiols

The term “thiol” refers to an organic compound that contains a sulfhydryl group (-SH). Thiols can be classified as high molecular weight thiols and low molecular weight thiols. In addition to their central role in antioxidant biochemistry (due to the thiol group), they have other functions in protein synthesis and structure, receptor modifications, redox-sensitive signal transduction, cell growth and proliferation, regulation of programmed cell death, xenobiotic metabolism, and immune regulation (Sen & Packer, 2000).

Aminothiols are low molecular weight thiol-containing amino acids, like homocysteine (Hcy), cysteine (Cys), glutathione (GSH), and cysteinylglycine (Cys-Gly), that occur in intra and extracellular milieu, being Cys and GSH the most abundant in plasma and cells, respectively. All these species are metabolically interrelated (Figure 1) and are important in determining the redox environment and free radical interactions, by acting as redox buffers (Giustarini *et al*., 2006). Their concentration in plasma is mostly a reflection of their intracellular

concentrations (Walmsley *et al*., 1997; Rossi *et al*., 2002) and of the integrity of the various pathways responsible for their metabolism.

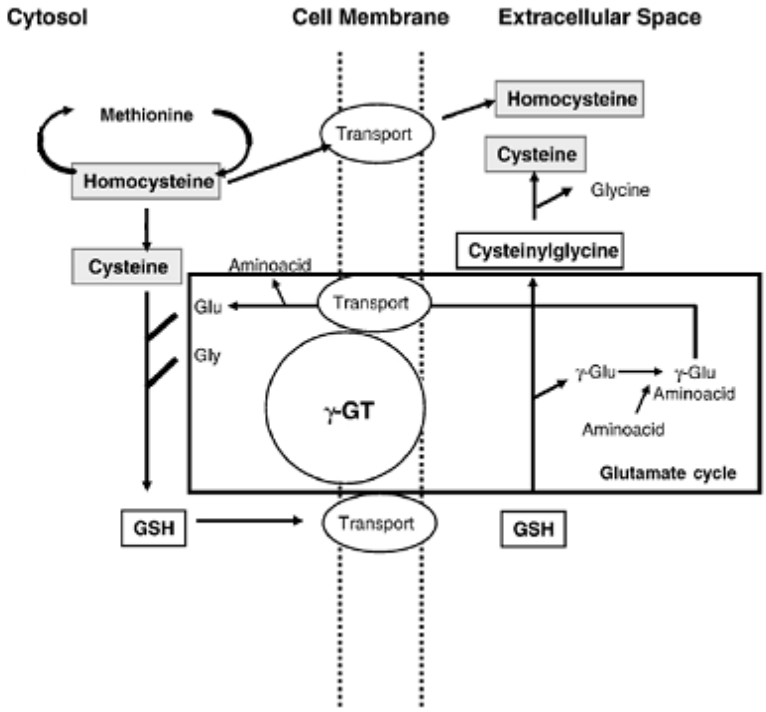

Figure 1. Aminothiol metabolic pathways in the intracellular and extracellular space (adapted from Campolo *et al*., 2007).

Thiols respond quickly to ROS, forming disulfides in plasma to remove the deleterious compound. Normally, in vivo the thiol/disulfide balance is highly controlled. Variations can overcome, typically reflected as an altered plasma aminothiol profile, in response to disease, to environmental factors and mutation.

Consequently, thiols act as good markers to quantify oxidative stress. In turn, the recognition of an oxidative stress status indicates the risk of suffering from diseases that are product of this oxidative damage. Furthermore, as conventional risk factors fail to account for part of the atherosclerosis cases, plasma aminothiols are actually being viewed with mounting interest (Özkan *et al*., 2002; McMenamin *et al*., 2009).

Total plasma "redox" thiol status can be assessed by measurements of the free forms (reduced and oxidized) and protein-bound forms of all aminothiols (Hcy, Cys, Cys-Gly and GSH). However, the free forms may be more likely to play a role in the pathogenesis of disease. Perturbations in the redox thiol status have been linked to several cardiovascular

diseases (Williams *et al*., 2001). Consequently, plasma aminothiol concentrations are being measured for investigating oxidative stress, and for routine clinical diagnosis and monitoring of vascular injury. HPLC with fluorescence detection has been the most commonly used method for determination of aminothiols (McMenamin *et al*., 2009).

3.1. Homocysteine

Hcy is a non-protein, sulphur-containing amino acid and is formed as an intermediate, during the essential amino acid methionine (Met) metabolism. It is at the intersection of two metabolic pathways, i.e. transsulfuration and remethylation (Figure 2). In the transsulfuration pathway, the condensation of Hcy with serine to form cystathionine is catalyzed by the enzyme cystathionine β-synthase (CBS). In a subsequent step, cystathionine is hydrolysed by the enzyme γ-cystathionase to yield Cys and α-ketobutyrate. Both these reactions require the physiologically active form of vitamin B_6, pyridoxal 5'-phosphate (PLP), as essential cofactor (Selhub, 2008). The catabolism of Hcy via transsulfuration is restricted to certain tissues (i.e. liver and kidneys).

On the other hand, the remethylation of Hcy (Figure 2) occurs in all cells and is catalyzed by methylcobalamin-containing methionine synthase (MS) in a vitamin B_{12}-dependent reaction which transfers the methyl group from 5-methyltetrahydrofolate (5-methylTHF) to Hcy resulting in the formation of Met. The methyl group of 5-methylTHF is in fact synthesized *de novo* when a carbon unit is transferred from a suitable source (e.g. serine) to THF, a reaction requiring PLP as cofactor. This reaction produces 5,10-methyleneTHF which is subsequently reduced to 5-methylTHF by the riboflavin-dependent enzyme 5,10-methylene tetrahydrofolate reductase (MTHFR) (Selhub, 2008).

Alternatively, Hcy may acquire a methyl group from betaine in a reaction catalyzed by betaine homocysteine methyltransferase (BHMT). The reaction with 5-methylTHF occurs in all tissues, while the reaction with betaine is confined mainly to the liver and is vitamin B_{12}-independent (Figure 2). Effective cellular Hcy metabolism is therefore dependent on an adequate status of the essential nutritional factors mentioned above. Yet, there is a third way of disposal of intracellular Hcy which is the export to the extracellular compartment, which has been suggested to occur when the intracellular production of Hcy exceeds its utilization (Selhub, 2008).

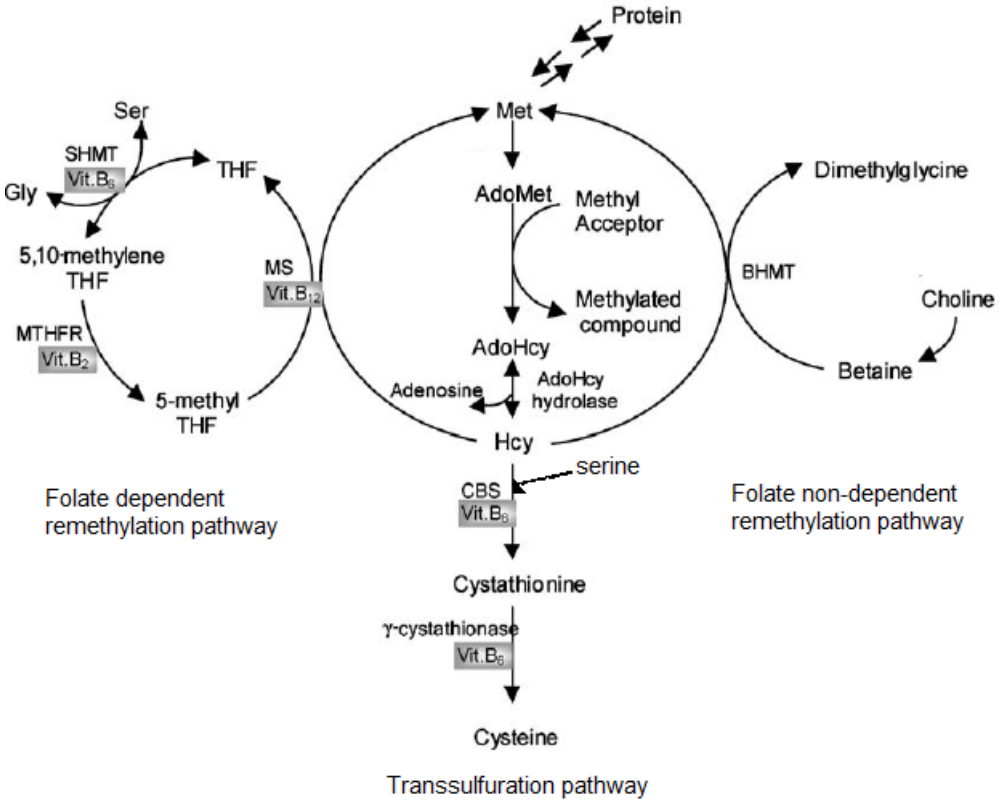

Figure 2. Hcy metabolism (adapted from Castro *et al*., 2006).

The normal level of total plasma Hcy, which includes free Hcy, homocystine (HcySS), and mixed disulfides, (protein-bound Hcy and Cys-Hcy) is between 5 and 15 µM (Kaul *et al*., 2006; Antoniades *et al*., 2009). About 98% of the total plasma Hcy pool consists of oxidized forms (disulfides) (Dröge, 2002), and it is determined by genetic and non-genetic factors. Modulation of Hcy includes sex, age, renal function, inadequate plasma concentrations of related B-vitamins, etc. In fact, B-vitamin deficiency is probably the most common non-genetic cause of moderate hyperhomocysteinemia (Zhou & Austin, 2009).

3.1.1. Hyperhomocysteinemia and atherogenesis

Much data have recently been reported which support the significance of hyperhomocysteinemia (HHcy) as an independent risk factor for cardiovascular diseases, including ischaemic heart disease, stroke, and peripheral vascular disease.

HHcy is classified according to fasting total plasma Hcy levels, as moderate (fasting total plasma Hcy levels 15–30 µM), intermediate (30–100 µM), or severe (>100 µM), and this classification is important to help clinicians to decide whether individuals should be treated

or not (Antoniades *et al*., 2009). Mutations in genes responsible for the metabolism of Hcy, including *CBS* or *MTHFR* can result in severe forms of HHcy, causing homocystinuria (Selhub, 2008).

Hcy may induce cell injury/dysfunction through a mechanism involving auto-oxidation and oxidative damage (Antoniades *et al*., 2009; Zhou & Austin, 2009), because its highly reactive thiol group (-SH) is readily oxidized to form ROS, like $O_2^{-\bullet}$and H_2O_2. Excess of $O_2^{-\bullet}$ leads to the formation of $OH^{\bullet}$, which highly reacts with NO to form peroxynitrite radicals, leading to low NO bioavailability and consequent endothelial dysfunction. All these ROS initiate lipid peroxidation and may induce the oxidation of LDL (Antoniades *et al*., 2009; Zhou & Austin, 2009).

An elevated Hcy level also causes protein homocysteinylation, thrombogenicity, endoplasmic reticulum stress, proinflammatory activity and proliferation of smooth muscle cells (Kaul *et al*., 2006; Zhou & Austin, 2009). Moreover, Hcy has the ability to inhibit the antioxidant enzymes superoxide dismutase and glutathione peroxidase (Kerkeni *et al*., 2008).

3.1.2. Hyperhomocysteinemia and B-vitamins

Vitamin deficiency (folate, B_{12} and B_6) is by far the leading cause of moderate HHcy, and it may be due to inadequate intake, reduced absorption from the gastrointestinal tract, alcohol abuse, and drug interactions. Individuals who do not eat a balanced diet (e.g., vegetarians), elderly people, pregnant women, patients with renal disease, malabsorption (inflammatory bowel disease) or malignant disease are at risk for clinically significant vitamin deficiency (Stanger *et al*., 2004).

At the present time, nutritional status for vitamins is assessed by measuring their concentrations in blood. However, despite B-vitamins can diminish plasma Hcy, there are currently not enough reliable data from randomized controlled trials that this will prevent “hard” vascular events (Kaul *et al*., 2006).

Folate

Folate (also known as vitamin B_9) is present in a wide range of foods: liver, green leafy vegetables, legumes, and citrus fruit are particularly rich in this vitamin. It is easily destroyed

by light, heat, and oxygen. In particular, the folate of fresh vegetables and fruit is destroyed by cooking and storage. Therefore fresh, uncooked vegetables and fruit are the best dietary sources of folate (FAO & WHO, 2004).

Dietary folates exist mainly in polyglutamate forms, which must be deconjugated, through hydrolysis in the intestine, to the monoglutamyl form before absorption. Subsequently, folate monoglutamates are absorbed and reach the bloodstream. 5-methylTHF monoglutamate is the principal circulating form of folate, and is transported across the cell membranes. Once in cells, 5-methylTHF can be demethylated to THF polyglutamate, trapping folate inside cells (see Figure 2). Vitamin B_{12} is required in this conversion, and in its absence, folate is "trapped" as 5-methylTHF. Subsequently, in normal conditions, intracellular remethylation of THF takes place, by a succession of reactions that yields in 5-methylTHF, which is available for the remethylation of Hcy (see Figure 2) and for maintaining an adequate supply of S-adenosylmethionine (AdoMet). This last one acts as a methyl donor to a wide range of methyltransferases, which methylate lipids, hormones, DNA, and proteins. Thus, folate cycle is dependent of Hcy metabolism and vice versa (FAO & WHO, 2004; Castro *et al*., 2006).

Vitamin B_{12}

Vitamin B_{12} or cobalamin consists of a corrin ring made up of four pyrroles with cobalt at the centre of the ring. In humans, there are just two vitamin B_{12}-dependent enzymes: one of these enzymes, MS, uses the chemical form of the vitamin which has a methyl group attached to the central cobalt, and is called methylcobalamin. As described, MS is crucial for the conversion of Hcy into Met and to maintain the availability of the methyl donor, AdoMet (see Figure 2). The other enzyme, methylmalonyl CoA mutase, which is not involved in Hcy metabolism, uses a 5'-adeoxyadenosyl moiety attached to the cobalt and is called 5'-deoxyadenosyl-cobalamin (FAO & WHO, 2004).

Absorption of dietary vitamin B_{12} (present only in animal products) involves binding to haptocorin (HC), after being released from food by stomach peptidases. In the duodenum, vitamin B_{12} is released from HC and bound to intrinsic factor (IF), produced by stomach parietal cells. IF-vitamin B_{12} complex enters the enterocyte, where the vitamin is released and bound to transcobalamin and reaches the bloodstream. Cells have receptors for

transcobalamin-vitamin B_{12} complex, where this vitamin is released in cytoplasm and converted into its active forms: adenosyl-cobalamin and methylcobalamin (FAO & WHO, 2004; Castro *et al*., 2006).

Without B_{12}, folate is trapped as 5-methylTHF (see Figure 2), so both vitamins are interconnected. Vitamin B_{12} is mainly stored in liver and bile is the main form of excretion. However, most of the vitamin B_{12} that is secreted in the bile is recycled via enterohepatic circulation; therefore, nutritional deficiency of this vitamin is rare. An elevated total plasma Hcy is recognized as a functional indicator of folate or vitamin B_{12} deficiency state (FAO & WHO, 2004).

Vitamin B_6

There are three natural vitamers of vitamin B_6, namely pyridoxine, pyridoxamine, and pyridoxal. All three come from the diet and must be phosphorylated to trap them inside enterocytes. The 5'-phosphates of the first two vitamers are oxidized to the functional PLP, which is the biologically active form of the vitamin. PLP is the major form of vitamin B_6 in all tissues and the plasma PLP concentration reflects liver PLP stores (FAO & WHO, 2004).

Vitamin B_6 functions as a coenzyme in the metabolism of amino acids, glycogen, and sphingoid bases. As described above, PLP is involved in the folate cycle by acting as a coenzyme in the conversion of THF to 5,10-methylTHF, and in the transsulfuration pathway of Hcy metabolism (see Figure 2). The products of vitamin B_6 metabolism are excreted in the urine, being 4-pyridoxic acid the major product (FAO & WHO, 2004; Castro *et al*., 2006).

3.2. Glutathione

GSH is a tripeptide (γ-glutamyl-cysteinyl-glycine) and the most important intracellular non-protein thiol antioxidant. GSH is crucial for maintaining redox homeostasis, protecting cells from oxidative stress by reacting directly with ROS or by participating as a substrate to several transferases and peroxidases, which catalyses the elimination of electrophilic compounds and peroxides, respectively. Moreover, GSH can help in the redox cycling of antioxidants such as vitamin C and E (Townsend *et al*., 2003; Forman *et al*., 2009; Lee & Jacobs JR, 2009).

GSH in cells is mainly in the reduced form (>98%), explaining why it is a great antioxidant, however some is also present in the disulfide form (GSSG), thioether, mercaptide or other thioester forms (glutathione S-conjugates and complexes) (Ballatori *et al*., 2009).

Oxidation-reduction and thiol-disulfide exchange reactions during oxidative perturbations cause a redistribution of GSH and GSSG; the resultant quantitative shift in the ratio of GSH to GSSG in favor of GSSG directly reflects an oxidized redox status and is a convenient expression of oxidative stress within a cell (Aw, 2003; Wu *et al*., 2004). This shift can affect signaling pathways by influencing a number of target proteins (Townsend *et al*., 2003).

The concentration of GSH to support redox cycle activity in cells depends on two factors: GSH neosynthesis (principally) and GSH regeneration from GSSG, by NADPH-dependent glutathione reductase (Sen & Packer, 2000).

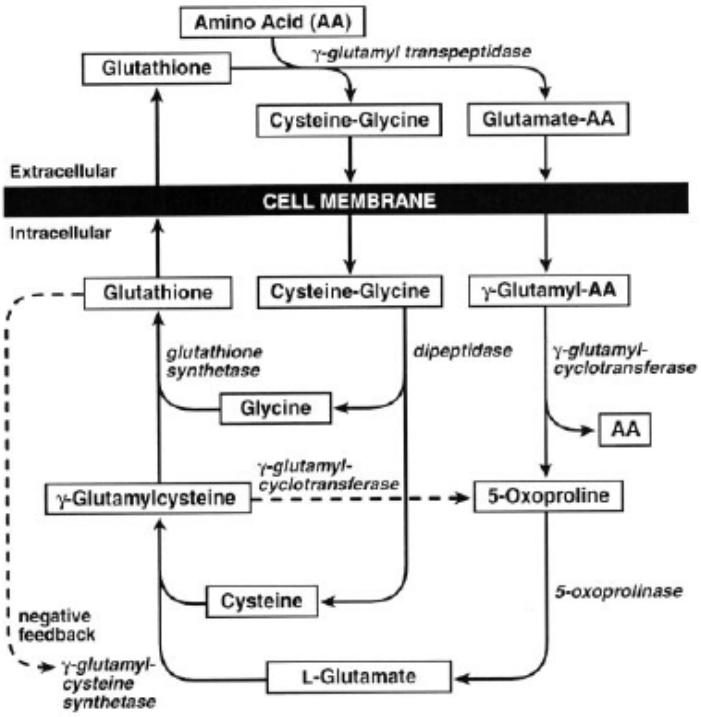

Figure 3. Glutathione metabolism (from Fenves *et al*., 2006).

GSH is synthesized via the γ-glutamyl cycle (in cytosol) from its constituent amino acids (Figure 3). Its synthesis is catalyzed sequentially by two cytosolic ATP-dependent enzymes, γ-glutamyl-cysteine synthetase (GCS) and GSH synthetase (Figure 3). This pathway occurs in virtually all cell types, with the liver being the major producer and exporter of GSH (Wu *et*

al., 2004). GCS is the rate-controlling enzyme in *de novo* synthesis of GSH. Oxidants, inflammatory cytokines, GSH conjugation, prostaglandin A_2, insulin, among others, increase GCS transcription. Also, GSH itself regulates the activity of GCS via a negative feedback mechanism (Figure 3). Polymorphisms in the genes governing GSH levels and post-translational modifications of GCS may contribute to the etiology of GSH-related disorders (Townsend *et al*., 2003).

An increase in urinary excretion of 5-oxoproline, an intermediate on the γ-glutamyl cycle (Figure 3), is a useful indicator of reduced availability of glycine (Gly) for GSH synthesis (Wu *et al*., 2004).

After its synthesis, some of the GSH is delivered into specific intracellular compartments, including mitochondria and endoplasmic reticulum, but much of the tripeptide is delivered to extracellular spaces. Its degradation occurs exclusively in the extracellular space, and only on the surface of cells that express the enzyme γ-glutamyl transferase (γ-GT), also known as γ-glutamyl transpeptidase. Thus, export from the cell is required for normal GSH turnover from all mammalian cells. In the liver, GSH is released at high rates into both blood plasma and bile. GSH transport into bile plays an important role in the transport and hepatic detoxification of reactive compounds of both endogenous and exogenous origin. GSH is also released into blood plasma, for delivery of Cys to other tissues. Despite GSH transporters are still elusive, recent studies have implicated a major role for some Mrp/Abcc proteins in this process (Ballatori *et al*., 2009).

Almost all blood GSH is contained in the cellular compartment, primarily in the erythrocytes, while human blood plasma contains only trace amounts of GSH, owing to the rapid catabolism of the tripeptide in the circulation (Sen & Packer, 2000; Ballatori *et al*., 2009).

Cellular reuptake of GSH is accomplished by two enzymes commonly found on the surfaces of the majority cells. The γ-GT (see Figure 3) transfers a glutamate (Glu) to other amino acids releasing Cys-Gly, which in turn can be broken down by a dipeptidase to produce Cys and Gly. Cys and Gly as well as γ-glutamyl amino acids are moved into cells by specific amino acid transporters and are used for GSH biosynthesis (Forman *et al*., 2009). So, augment of γ-GT activity is an attempt to increase intracellular GSH synthesis. However, as γ-GT initiates the breakdown of extracellular GSH, Cys-Gly, one of the products of γ-GT action, has a strong ability to reduce Fe^{3+} a Fe^{2+}, which promotes generation of ROS with high reactivity. This pro-oxidant effect of γ-GT is more prominent in the elderly than in the young, since serum γ-GT

tends to increase with age and body iron stores are great in the elderly (Lee & Jacobs JR, 2009). Persistent increase of serum γ-GT and γ-GT-dependent oxidation of LDL may represent a potential mechanism in atherosclerosis (Turgut *et al*., 2006).

3.3. Cysteinylglycine

As stated above Cys-Gly is one of the products of extracellular GSH hydrolysis produced by γ-GT activity, and has important pro-oxidant activities (Drozdz *et al*., 1998; Enoiu *et al*., 2000; Lin *et al*., 2007), which can cause LDL oxidation. There is just a small case-control study that reported a significantly positive association between serum Cys-Gly levels and risk for ischaemic heart disease (Mendis *et al*., 1997) and a prospective study that showed an association between plasma Cys-Gly and myocardial infarction (Drogan *et al*., 2010).

3.4. Cysteine

Cys, whether formed from Met and serine via transsulfuration pathway (see Figure 2) or supplied preformed in the diet, serves as a precursor for synthesis of proteins and several other essential molecules. These metabolites include GSH, coenzyme A, taurine, and inorganic sulfur (Stipanuk, 2004).

Synthesis of GSH in the cell is rate-limited by the availability of Cys (since Glu and Gly are present in comparatively higher concentrations in the cell), which, in its reduced form, is highly unstable. Thus, more than 90% of total Cys in the human circulation is present in oxidized (disulfide) forms, including cystine (CySS). Consequently, one strategy to enhance intracellular GSH is to improve Cys availability within the cell (Sen & Packer., 2000; Wu *et al*., 2004).

Neither Cys nor Met are stored in the body. Any dietary excess of Cys is readily oxidized to sulfate and taurine to be excreted in the urine, or used to protein or GSH synthesis (Stipanuk, 2004). In situations where Cys supply is low, extracellular GSH concentration declines in order to maintain adequate levels of this amino acid. This loss of GSH impairs antioxidant defenses. As stated above, plasma GSH is mostly derived from hepatic efflux, but when hepatic stores are decreased skeletal muscle is a major source of extracellular GSH and consequent Cys pool (Moriarty-Craige & Jones, 2004).

Cys is also present in the active site of many proteins, enzymes, receptors, ion channels, transporters and transcription factors, which are common targets of oxidation, and extracellular thiol-disulfide redox state of Cys residues can affect function of diverse plasma proteins (Moriarty-Craige & Jones, 2004).

There are a huge number of cells that cannot synthesize Cys from Met; moreover, most have little uptake for CySS. Therefore Cys uptake, in its reduced form, is very important to support cellular protein synthesis and GSH needs (Dröge, 2002; Wu *et al*., 2004).

Cys and CySS, are transported into the cell via sodium dependent and independent transporters, respectively. CySS and Glu share the system Xc^- amino acid antiport, in which CySS enters in cells (Wu *et al*., 2004). An excess of extracellular Glu inhibits CySS uptake. Within cells, CySS is rapidly reduced to Cys (Sen & Packer, 2000).

The association between total plasma Cys and CVD has not been investigated to the same extent as Hcy, but some studies (Jacob *et al*., 1999; Özkan *et al*., 2002; Wronska-Nofer *et al*., 2007) have shown raised total Cys in patients with vascular diseases.

OBJECTIVES OF THE STUDY

Redox homeostasis is critical for normal function of cell and extracellular proteins (Moriarty-Craige & Jones, 2004). Aminothiols are important intra and extracellular redox buffers (Campolo *et al*., 2007), and their concentration in plasma reflects mostly their intracellular level and the integrity of the various pathways responsible for their metabolism. An overproduction of oxidants or an impairment of antioxidant systems can disrupt the normal redox state, leading to changes in thiol content and metabolism (Dickinson & Forman, 2002). Consequently, thiols can act as good markers of oxidative stress, which is deeply involved in atherogenesis and consequent CVD.

The objectives of this dissertation were:

1. To evaluate total plasma aminothiols (Hcy, Cys, Cys-Gly and GSH) concentrations in an apparently healthy population born and living in the Azores archipelago, in order to define its plasma aminothiol profile.
2. To assess serum γ-GT activity and plasma B-vitamins (folate, B_6, B_{12}) as fundamental determinants of aminothiol metabolism.
3. To study eventual relationships between aminothiol profile and lipid profile.
4. To identify in the study population endogenous and/or exogenous factors affecting the normal thiol profile, that can be taken as risk factors for atherosclerosis.

REFERENCES

Antoniades C, Antonopoulos AS, Tousoulis D, *et al*. Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. *Eur Heart J.* 2009; **30**: 6-15.

Aw TY. Cellular Redox: A Modulator of Intestinal Epithelial Cell Proliferation. *News Physiol Sci.* 2003; **18**: 201-204.

Ballatori N, Krance SM, Marchan R. Plasma membrane glutathione transporters and their roles in cell physiology and pathophysiology. *Mol Aspects Med* 2009; **30**: 13–28.

Campolo J, De Maria R, Caruso R, *et al*. Blood glutathione as independent marker of lipid peroxidation in heart failure. *Int J Cardiol* 2007; **117**: 45–50.

Castro R, Rivera I, Blom HJ, *et al*. Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: An overview. *J Inherit Metab Dis* 2006; **29**: 3–20.

Ceconi C, Boraso A, Cargnoni A. Oxidative stress in cardiovascular disease: myth or fact? *Arch Biochem Biophys* 2003; **420**: 217–221.

Dickinson DA, Forman HJ. Cellular glutathione and thiols metabolism. *Biochem Pharmacol* 2002; **64**: 1019-1026.

Direcção-Geral da Saúde. Risco de morrer em Portugal 2006 (vol I). Retrieved from http://www.dgs.pt/

Drogan D, Weikert C, Dierkes J. Plasma γ-Glutamyltransferase, Cysteinyl-Glycine, and Oxidized Low-Density Lipoprotein: A Pathway Associated With Myocardial Infarction Risk? *Arterioscler Thromb Vasc Biol* 2010; **30**: 2053-2058.

Dröge W. Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants. *Exp Gerontol* 2002; **37**: 1331-1343.

Drozdz R, Parmentier C, Hachad H, *et al*. γ-Glutamyltransferase dependent generation of reactive oxygen species from a glutathione/transferrin system. *Free Radic Biol Med* 1998; **25**: 786–792.

Enoiu M, Aberkane H, Salazar J-F. Evidence for the pro-oxidant effect of γ-glutamyltranspeptidase–related enzyme. *Free Radic Biol Med* 2000; **29**: 825–833.

FAO & WHO. Vitamin and mineral requirements in human nutrition. Report of a joint FAO/WHO expert consultation. 2nd ed. Bangkok, Thailand, 2004.

Fenves AZ, Kirkpatrick HM 3rd, Patel VV, *et al*. Increased Anion Gap Metabolic Acidosis as a Result of 5-Oxoproline (Pyroglutamic Acid): A Role for Acetaminophen. *Clin J Am Soc Nephrol* 2006; **1**: 441-447.

Forman HJ, Zhang H, Rinna A. Glutathione: Overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med* 2009; **30**: 1-12.

Giustarini D, Dalle-Donne I, Lorenzini S, *et al*. Age-Related Influence on Thiol, Disulfide, and Protein-Mixed Disulfide Levels in Human Plasma. *J Gerontol A Biol Sci Med Sci* 2006; **61**: 1030-1038.

Grundy SM, Cleeman JI, Merz CN, Brewer HB Jr, Clark LT, Hunninghake DB, Pasternak RC, Smith SC Jr, Stone NJ; National Heart, Lung, and Blood Institute; American College of Cardiology Foundation; American Heart Association. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. *Circulation* 2004; **110**: 227–239.

Hachem SB, Mooradian AD. Familial Dyslipidaemias An Overview of Genetics, Pathophysiology and Management. *Drugs* 2006; **66**: 1949-1969.

Jacob N, Bruckert E, Giral P, *et al*. Cysteine is a cardiovascular risk factor in hyperlipidemic patients. *Atherosclerosis* 1999; **146**: 53-59.

Kaul S, Zadeh AA, Shah PK. Homocysteine Hypothesis for Atherothrombotic Cardiovascular Disease: not validated. *J Am Coll Cardiol* 2006; **48**: 914-923.

Kerkeni M, Added F, Ben Farhat M, *et al*. Hyperhomocysteinaemia and parameters of antioxidative defence in Tunisian patients with coronary heart disease. *Ann Clin Biochem* 2008; **45**: 193-198.

Lee D, Jacobs JR, DR. Is serum gamma-glutamyltransferase a marker of exposure to various environmental pollutants? *Free Radic Res* 2009; **43**: 533-537.

Libby P, Ridker PM, Maseri A. Inflammation and Atherosclerosis. *Circulation* 2002; **105**: 1135-1143.

Lin J, Manson JE, Selhub J. Plasma Cysteinylglycine Levels and Breast Cancer Risk in Women. *Cancer Res* 2007; **67**: 11123-11127.

Lusis AJ. Atherosclerosis. *Nature* 2000; **407**: 233-241.

McMenamin ME, Himmelfarb J, Nolin TD. Simultaneous analysis of multiple aminothiols in human plasma by high performance liquid chromatography with fluorescence detection. *J Chromatogr B* 2009; **877**: 3274-3281.

Mendis S, Athauda SB, Takashi K. Association between hyperhomocysteinemia and ischemic heart disease in Sri Lankans. *Int J Cardiol* 1997; **62**: 221-225.

Montagnana M, Lippi G, Salvagno GL *et al*. Role of biochemical risk factors and markers for the risk of atherosclerosis. *Recenti Prog Med* 2008; **99**: 215-222.

Moriarty-Craige SE, Jones DP. Extracellular thiols and thiol/disulfide redox in metabolism. *Annu Rev Nutr* 2004; **24**: 481–509.

National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation* 2002; **106**: 3143–3421.

Oktyabrsky ON, Smirnova GV. Redox Regulation of Cellular Functions. *Biochemistry (Mosc)* 2007; **72**: 132-145.

Özkan Y, Özkan E, Şimşek B. Plasma total homocysteine and cysteine levels as cardiovascular risk factors in coronary heart disease. *Int J Cardiol* 2002; **82**: 269–277.

Patrick L, Uzick M. Cardiovascular Disease: C-Reactive Protein and the Inflammatory Disease Paradigm: HMG-CoA Reductase Inhibitors, alpha-Tocopherol, Red Yeast Rice, and Olive Oil Polyphenols. A Review of the Literature. *Altern Med Rev* 2001; **6**: 248-271.

Rossi R, Milzani A, Dalle-Donne I, *et al*. Blood Glutathione Disulfide: In Vivo Factor or in Vitro Artifact? *Clin Chem* 2002; **48**: 742-753.

Roy H, Bhardwaj S, Yla-Herttuala S. Molecular genetics of atherosclerosis. *Hum Genet* 2009; **125**: 467-491.

Schoen FJ. Blood Vessels. In: Kumar V, Abbas AK, Fausto N, eds. Robbins and Cotran: Pathologic Basis of Disease. 7th ed. Philadelphia, Pennsylvania: Elsevier Saunders; 2005.

Selhub J. Public health significance of elevated homocysteine. *Food Nutr Bull* 2008; **29**: S116-S125.

Sen CK, Packer L. Thiol homeostasis and supplements in physical exercise. *Am J Clin Nutr* 2000; **72**: 653S–69S.

Stanger O, Herrmann W, Pietrzik, *et al*. Clinical use and rational management of homocysteine, folic acid, and B vitamins in cardiovascular and thrombotic diseases. *Z Kardiol* 2004; **93**: 439-453.

Stipanuk, MH. Sulfur amino acid metabolism: Pathways for Production and Removal of Homocysteine and Cysteine. *Annu Rev Nutr* 2004; **24**: 539-577

Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. *Biomed Pharmacother* 2003; **57**: 145–155.

Turgut O, Yilmaz A, Yalta K, *et al*. γ-Glutamyltransferase is a promising biomarker for cardiovascular risk. *Med Hypotheses* 2006; **67**: 1060.1064.

Walmsley SL, Winn LM, Harrison ML. Oxidative stress and thiol depletion in plasma and peripheral blood lymphocytes from HIV-infected patients: toxicological and pathological implications. *AIDS* 1997; **11**: 1689–1697.

Williams RH, Maggiore JA, Reynolds RD, *et al*. Novel Approach for the Determination of the Redox Status of Homocysteine and Other Aminothiols in Plasma from Healthy Subjects and Patients with Ischemic Stroke. *Clin Chem* 2001; **47**: 1031–1039.

World Health Organization. World Health Statistics 2009. Retrieved from http://www.who.int/whosis/whostat/2009/en/index.html

Wronska-Nofer T, Nofer J-R, Stetkiewicz J, *et al*. Evidence for oxidative stress at elevated plasma thiol levels in chronic exposure to carbon disulfide (CS_2) and coronary heart disease. *Nutr Metab Cardiovasc Dis* 2007; **17**: 546-53.

Wu G, Fang Y-Z, Yang S, *et al*. Glutathione metabolism and its implications for health. *J Nutr* 2004; **134**: 489-92.

Zhou J, Austin RC. Contributions of hyperhomocysteinemia to atherosclerosis: Causal relationship and potential mechanisms. *Biofactors* 2009; **35**: 120-129.

CHAPTER II

PLASMA AMINOTHIOL PROFILE AND SOME OF ITS DETERMINANTS IN SUBJECTS FROM THE AZORES ARCHIPELAGO, PORTUGAL

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(to submit)

PLASMA AMINOTHIOL PROFILE AND SOME OF ITS DETERMINANTS IN SUBJECTS FROM THE AZORES ARCHIPELAGO, PORTUGAL

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ABSTRACT

Background: The Azores Islands have the highest standardized mortality rate for cardiovascular diseases (CVD) as compared to mainland Portugal, and the most common are of atherosclerotic origin, where oxidative stress plays a central role. Plasma aminothiols, such as homocysteine (Hcy), cysteine (Cys), cysteinylglycine (Cys-Gly) and glutathione (GSH), have recently received greater attention than have conventional risk factors. The aim of this study was to evaluate, for the first time, plasma aminothiol profile (PAP), some of its major determinants (plasma folate, vitamin B_{12} and vitamin B_6 concentrations and serum γ-GT activity), as well as its relationship with serum lipid profile, in apparently healthy subjects from the Azores archipelago.

Methods: This was a cross-sectional study, where participants were split in two groups: one with a normal PAP (where all aminothiol levels were within the reference ranges) and another with an altered PAP. Plasma aminothiols and vitamin B_6 concentrations were measured by HPLC. The other parameters were determined by commercial kits.

Results: Seventy-six percent of the participants had an altered PAP, mainly due to low GSH levels. That profile was more frequent in male gender, in older or hyperlipidemic subjects or in those with high γ-GT activity. Older subjects or hyperlipidemics showed decreased GSH and increased Cys levels and serum γ-GT activity, as compared to the respective counterparts. Hyperhomocysteinemia was present in 10% of participants, where only a small part had B-vitamin deficiencies.

Conclusions: An altered PAP reflects a pro-oxidant status, thus favoring atherogenesis and consequent CVD. Since subjects were apparently healthy, an altered PAP, namely originated

by low GSH levels, can constitute an early marker of atherosclerosis. Decreased plasma GSH concentrations could result from a high utilization of the tripeptide in the antioxidant defenses of subjects where hyperlipidemia is also a prevalent risk factor for atherosclerosis. It might also be caused by an impairment in aminothiol metabolism and therefore contribute to a condition of oxidative stress.

Key words: Azores, atherosclerosis, plasma aminothiol profile, glutathione, B-vitamins, γ-glutamyl transferase activity.

INTRODUCTION

Atherosclerosis, the major cause of cardiovascular diseases (CVD), is actually considered as a chronic inflammatory disease, involving interactions of multiple genetic and environmental factors (Roy *et al*., 2009). It usually begins in early life but its clinical symptoms generally arise as a consequence of advanced stages of the disease. Consequently, there is a need to identify precocious, non-evasive and expedite markers for this condition. Oxidative processes play a central role in atherogenesis and consequent CVD (Ceconi *et al*., 2003), and therefore redox homeostasis is very important for maintaining normal cellular functions.

In recent years, there has been an increasing interest in the measurement of aminothiols in human plasma, more than other conventional risk factors, since disturbances in thiol homeostasis have been linked to several human health disorders, including CVD (Sen & Packer, 2000). The major plasma aminothiols are homocysteine (Hcy), cysteine (Cys), cysteinylglycine (Cys-Gly), and glutathione (GSH), that serve numerous vital functions in metabolism, including detoxification and regulation of cellular metabolism, enzymatic activity, and protein trafficking and degradation (McMenamin *et al*., 2009). All these aminothiols interact via redox, namely disulfide exchange reactions, and reduced, oxidized and protein bound forms of these species comprise a dynamic system referred to as the redox thiol status (Atmaca, 2004). Their metabolic pathways are strongly linked. Hcy is produced from the essential amino acid methionine and can be remethylated again to methionine. This last reaction is catalyzed by methionine synthase, which requires vitamin B_{12} as a cofactor and 5-methyltetrahydrofolate, the circulating form of folate, as a substrate.

An alternative route of Hcy disposal is degradation to Cys through the transsulfuration pathway by two sequential vitamin B_6-dependent reactions (Selhub, 2008). Besides being an Hcy byproduct, Cys is a GSH precursor inside cells, which has important antioxidant functions. The action of γ-glutamyl transferase (γ-GT) on extracellular GSH results in its cleavage to produce glutamate and Cys-Gly, the latter can be broken down by a dipeptidase to produce Cys and glycine (Forman *et al*., 2009). This enzyme activity has been shown to correlate with CVD (Drogan *et al*., 2010; Mason *et al*., 2010). Oxidative processes leads to depletion of GSH, induces the expression of γ-GT, and subsequently elevates serum γ-GT activity (Turgut *et al*., 2006). So, γ-GT may predict various diseases, such as CVD, as a marker of oxidative stress.

Perturbations in the redox thiol status have been reported in patients with CVD, through mainly an oxidative mechanism. High total plasma Hcy concentration (hyperhomocysteinemia) is an independent risk factor for CVD (Stanger *et al*., 2004; Zhou & Austin, 2009). However, the association between total plasma Cys and CVD has not received much attention to the same extent as Hcy. Some studies have shown elevated total Cys in patients with vascular diseases (Jacob *et al*., 1999; Özkan *et al*., 2002; Wronska-Nofer *et al*., 2007). Only two papers reported the association of total Cys-Gly concentrations with CVD risk (Mendis *et al*., 1997; Drogan *et al*., 2010). Disturbances in GSH homeostasis, namely low circulating levels of total GSH, have also been implicated in the etiology and/or progression of a number of human diseases, including CVD (Morrison *et al*., 1999; Shimizu *et al*., 2004; De Chiara *et al*., 2007).

The Azores Islands (Portugal) have the highest standardized mortality rate for ischaemic heart disease in the country (Direcção-Geral da Saúde, 2006), which is a major public health concern. Therefore, the main objective of this study was to evaluate the plasma aminothiol profile (PAP) and its major determinants (plasma folate, vitamin B_{12} and vitamin B_6 concentrations and serum γ-GT activity) in a group of apparently healthy subjects, all born and living in the Azores archipelago, as well as their relationship with gender, age, and serum lipid profile. In addition, subjects were divided in two groups, one with a normal and another with an altered PAP.

MATERIALS AND METHODS

Subjects

The Azores are an Atlantic Portuguese archipelago with 244,006 habitants (SREA, 2007) formed by nine islands. These are distributed by three geographical groups: the Eastern (São Miguel and Santa Maria), the Central (Terceira, Pico, Faial, São Jorge and Graciosa) and the Western group (Flores and Corvo).

The study group consisted of 333 volunteers (191 women and 142 men) aged 18-63 years, all born and residing in one of four islands of this archipelago: São Miguel (Nordeste (71) and Povoação (86) villages), São Jorge (71), Graciosa (86) and Flores (19).

The participants were requested to complete a questionnaire on their medical history and medicine intake. Subjects with a history of CVD, with diabetes mellitus or other chronic diseases, as well as those who were taking vitamin supplementation were excluded from the study. Other exclusion criteria was serum triglycerides >400 mg/dL. All subjects provided written informed consent to participate in this study.

Study design

This study had a cross-sectional design where subjects were tested for plasma aminothiol content, B-vitamins (folate, vitamin B_{12} and B_6), serum γ-GT activity and lipid profile. Subjects were assigned into 2 groups based on their PAP: one with a normal PAP, where all aminothiols were within the respective concentration reference range, and one with an altered PAP [i.e. Hcy ≥15 µM (Kaul *et al*., 2006) and/or Cys >250 µM (Moat *et al*., 2001) and/or Cys-Gly >36 µM (Turell *et al*., 2009) and/or GSH < 1.5 µM]. Hyperhomocysteinemia (HHcy) was defined as fasting total plasma Hcy ≥15 µM, and high serum γ-GT activity was defined as >36 U/L for women and >61 U/L for men, according to manufacturer's instruction. For B-vitamin deficiencies, folate deficiency was defined as plasma folate concentration ≤4 ng/mL (in agreement with manufacturer's instruction kit); vitamin B_6 deficiency was defined as plasma pyridoxal-5'-phosphate (PLP) concentration <20 nmol/L (Morris *et al*., 2008). Vitamin B_{12} deficiency was defined as plasma vitamin B_{12} concentration <250 pg/mL (Food and Nutrition Board & Institute of Medicine, 1998).

Individuals were deemed hyperlipidemic when their total cholesterol concentration was ≥200 mg/dL, and/or their triglyceride level was ≥150 mg/dL, and/or when receiving lipid-

lowering drugs; normolipidemics had both parameters below these references values, in accordance with recommendations of ATP III guidelines (NCEP, 2002) and its recent update (Grundy *et al*., 2004).

Sample collection

A single fasting venous blood sample was obtained from all subjects. The blood was drawn into 4.9 ml vacutainer tubes with heparin and into 10 ml tubes without anticoagulant. After centrifugation at 1,500 x *g* for 15 min at 4°C for serum and 2,500 x *g* for 15 min at 4°C for plasma, both fractions were separated and divided into 200 µl aliquots and stored at -80°C until analysis. One aliquot of serum was immediately used to evaluate the lipid parameters. Blood samples were collected from 2007 to 2009 at the respective local health center.

Chromatographic equipment and Chemicals

HPLC analysis was performed using an Agilent Technologies system (Avondale, PA, USA) model 1200 Series equipped with quaternary pump, manual injection valve, thermostatted column compartment, fluorescence detector, and controlled by HP ChemStation software. Pyridoxal-5'-phosphate monohydrate, sodium bisulfite, perchloric acid and HPLC-grade acetonitrile were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4) was obtained from Merck (Darmstadt, Germany). DL-Homocysteine, oxidized L-glutathione, L-cysteine, cysteinylglycine, cysteamine hydrochloride, tri-n-butylphosphine (TBP), ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), potassium borate, ethylenediaminetetraacetic acid (EDTA), dimethylformamide, sodium perchlorate monohydrate, ortho-phosphoric and trichloroacetic (TCA) acids were obtained from Fluka (Sigma-Aldrich chemie, Steinheim, Germany). Deionized water was obtained with in-house Milli-Q water purification system (Millipore, Bradford, MA, USA).

Biochemical analyses

Determination of serum lipids, γ-GT activity and plasma folate and vitamin B_{12}

Serum total cholesterol (TC), HDL-cholesterol (HDLc), triglycerides (TG), and γ-GT were measured enzymatically using Roche diagnostic kits on a Cobas Integra® 400 plus (Roche Diagnostics). LDL-cholesterol (LDLc) was determined by Friedewald formula. Plasma folate and vitamin B_{12} levels were determined by electrochemiluminescence competitive immunoassay kits on Cobas® 6000 analyzer (Roche Diagnostics). The intra-assay coefficients of variation for all these biochemical parameters were ≤ 7%.

Quantification of plasma aminothiols

The plasma concentrations of Hcy, Cys, Cys-Gly, and GSH were measured as a total, i.e., the sum of reduced, oxidized, and protein-bound species. For that, 100 µL of plasma was reduced with 15 µl of 100 ml/L of TBP in dimethylformamide. Fifty µl of 5 µM cysteamine hydrochloride was introduced as internal standard. The mixture was incubated at 4°C for 30 min; subsequently 150 µl of TCA (10%) cointaining 1 mM EDTA was added. After centrifugation at 15,500 x *g* at room temperature for 10 min, a 60 µl of the clear supernatant was added to a mixture of 120 µl of 125 mmol/L of potassium borate (pH 10.5), containing 4 mmol/L of EDTA and 60 µl of SBD-F (1 g/L dissolved in 125 mmol/L of potassium borate, pH 9.5). This mixture was incubated at 60°C for 60 min and then, stored at 4°C until further analysis. The reaction mixture was filtered through a 0.45 µm Alltech filter and an aliquot of 20 µl was used for HPLC analysis.

Separation was achieved using a Platinum EPS C18 (53 x 7 mm I.D., 3 µm) column (Phenomenex) at 35°C coupled with a C18 guard-column (30 x 2 mm), and a fluorescence detector with an excitation at 385 nm and emission at 515 nm. The mobile phase used was an isocratic mixture of 0.1 mol/L KH_2PO_4 buffer (pH 2), containing 40 ml/L of acetonitrile, at a flow rate of 1 ml/min. This is a new method with a recovery ranging between 92% and 100%.

The RSD for the intraday and interday repeatability of the retention times were 0.54% and 0.32% for Cys, 0.67% and 0.91% for Cys-Gly, 1.28% and 0.52% for Hcy, and 1.84% and 1.71% for GSH, respectively.

Determination of plasma vitamin B_6

Thawed plasma samples were prepared for analysis of pyridoxal-5'-phosphate (PLP) according to the method of Kimura *et al*. (1996), and following the advices of Deitrick *et al*. (2001). In detail, sample preparation consisted of plasma protein precipitation by the addition of 0.1 ml 0.8 mol/L perchloric acid to 0.1 ml of plasma. This mixture was then vigorously vortex-mixed for 1 minute. The sample was then centrifuged at 16,000 x *g* for 15 min, and 20 µl of the clear supernatant was used as sample for chromatographic HPLC analysis.

PLP was separated on an Onyx monolithic C18 (100 x 4.6 mm) column (Phenomenex) kept at 35°C coupled with a guard-column Onyx monolithic C18 (10 x 4.6 mm). The fluorescence detector was operating at an excitation wavelength of 300 nm and an emission wavelength of 400 nm. For mobile phase A, 0.1 mol/L KH_2PO_4 containing 0.1 mol/L sodium perchlorate and 0.5 g/L sodium bisulfite, was adjusted to pH 3.0 with ortho-phosphoric acid. Sodium bisulfite was used for on-line derivatization. A clean up mobile phase (B) consisted of acetonitrile/water (3:7, v/v). Phase A was run for 5 minutes followed by a partial clean up with phase B for 10 min, and finally an equilibration time of phase A for 5 minutes. Samples were run at a flow rate of 0.75 ml/min.

The RSD for the intraday and interday repeatability of the PLP retention times were 0.58% and 0.87%, respectively.

The quantitative analysis was achieved by external standard method, which all parameters quantified by HPLC were calculated from the average of triplicate measurements of the peak area under the chromatographic curve and using data derived from calibration curve.

Statistical analysis

The SPSS 17.0 software (Statistical Packages for Social Sciences, for Windows) was used for evaluation of data. Results were expressed as mean±SD and P <0.05 value was considered

statistically significant. Because the outcome variables were not normally distributed, the following non-parametric methods were used: chi-square test or Fisher's exact test for categorical variables, Mann–Whitney U-test in the case of two independent samples, and Spearman's rank correlation coefficients were calculated to test relationship between two variables. All tests were two-tailed.

RESULTS

Baseline characteristics of the study population

The main characteristics of the whole study population (57% of women and of 43% of men) are shown in Table 1. No differences in age were observed between genders. The mean plasma concentrations of all aminothiols were within the reference ranges, but those of GSH were at the low limit of the reference range. In fact, 57% of all subjects (especially women) had low GSH levels, whilst only 10% had moderate HHcy, mainly male gender. None subject had intermediate or severe HHcy (>30 μM). Furthermore, among subjects with low GSH concentration 29% had also some other aminothiol content above normal values, namely Cys-Gly (data not shown). One-quarter of all individuals had a high Cys-Gly content, whereas hypercysteinemia was unfrequent.

The mean values of plasma Hcy, Cys-Gly, GSH concentrations, as well as of serum γ-GT activity were higher in men than in women, while those of Cys did not differ between genders. The average serum γ-GT activity was within the normal range, but a relevant amount of subjects had high enzymatic activity. However, among subjects with low GSH levels, only 23% exhibited high serum γ-GT activity (data not shown).

The mean concentrations of all vitamins in plasma were within the normal range for both genders. No significant differences in folate and vitamin B_{12} were found between men and women, whilst men had higher values of vitamin B_6. A small number of subjects had deficient plasma folate, vitamin B_{12} or vitamin B_6 levels. However, only 33% of the hyperhomocysteinemics had some B-vitamin deficiencies (data not shown), being folate deficiency the most frequent.

Concerning serum lipids, either TC or LDLc mean levels were above the reference values, whereas both HDLc and TG levels were within the normal range. No significant gender-

related differences were observed in these parameters, except for HDLc, which was higher in women than in men, as expected. Hyperlipidemia, which did not differ between genders, was present in the majority of subjects, expressed mainly as hypercholesterolemia. Among normolipidemic subjects, 55% had LDLc concentration above 100 mg/dL (data not shown). Furthermore, ten percent of all participants were under treatment with lipid-lowering drugs and 22% of all women were at the post-menopausal stage.

Table 1. Baseline characteristics of the study population, according to gender.

	All (333)	Women (191)	Men (142)
Age (years)	41±10	42±10	39±10
Plasma thiols (µM)			
Hcy	10±3	9±3	11±4***
Cys	200±39	197±39	204±39
Cys-Gly	32±6	29±5	35±6***
GSH	1.5±0.6	1.4±0.5	1.6±0.6**
Hyperhomocysteinemia (%)	10	6	16**
Hypercysteinemia (%)	9	7	13
High Cys-Gly (%)	25	13	41***
Low GSH (%)	57	63	48**
Serum γ-GT activity (U/L)	33±35	24±27	45±42***
High γ-GT activity (%)	15	13	17
Plasma vitamins			
Folate (ng/mL)	8±3	8±3	8±3
Deficiency (%)	8	10	5
Vitamin B_{12} (pg/mL)	568±311	568±357	568±231
Deficiency (%)	5	9	1**
Vitamin B_6 (nmol/L)	53±28	48±26	61±28***
Deficiency (%)	4	6	1*
Serum lipids (mg/dL)			
Triglycerides	120±63	115±58	128±70
Total cholesterol	207±37	209±35	204±40
HDL cholesterol	59±15	63±15	53±13***
LDL cholesterol	128±35	126±35	129±36
Hyperlipidemia (%)	65	66	64

Values are presented as mean±SD, except otherwise indicated. Figures in parenthesis are the number of subjects. Asterisks denote significant sex-differences ($^{***}P$ <0.001; $^{**}P$ <0.01; $^{*}P$ <0.05).

Analysis of parameters, according to plasma aminothiol profile

When grouping subjects according to their plasma aminothiol profile (PAP), more than three-quarters exhibited an altered one (see Table 2), mainly due to the existence of very low GSH concentrations. Also, the average ages of either men or women were significantly higher in the altered than in the normal PAP group. Nevertheless, both PAP groups were sex-matched (P >0.05).

As expected, mean Hcy, Cys and Cys-Gly concentrations were significantly higher (namely in men) and GSH was lower (in both genders) in the altered PAP group as compared to their normo counterparts. In the altered PAP group all plasma aminothiols correlated positively with each other, but in the normal group just Cys correlated with Hcy.

The gender-related difference in plasma GSH observed in the whole population (Table 1) seems to arise mainly from the altered PAP group. However, the small number of men in the normal PAP group may have conditioned the analysis. Conversely, Cys, whose levels did not appear to differ with gender (Table 1) showed to be significantly higher in men than in women of the altered PAP group.

Albeit still within the reference range, serum γ-GT activity in the altered PAP group was about twice than in the normal one, in both genders. Moreover, in the altered PAP group, serum γ-GT activity was positively associated with Hcy, Cys, and Cys-Gly, and was negatively associated with GSH (r= 0.28, P <0.001; r= 0.23, P <0.001; r= 0.22, P <0.001; r= -0.29, P <0.001, respectively), whilst in the normal PAP group, just Cys-Gly was significantly correlated with that enzyme activity.

Concerning vitamins, their concentration in plasma did not change with PAP. Though, not significant, folate concentration was lower in men than in women with an altered PAP. The number of hyperlipidemics (mostly men) was significantly increased in the altered PAP group as compared to the normal one. In fact, serum TG, as well as TC and LDLc concentrations were higher in the former than in the latter group. In addition, the levels of serum HDLc in women with an altered PAP were lower than in their normo counterparts. Like Cys, TG concentration showed to be significantly higher in men than in women with an altered PAP, which was not apparent in the whole population (Table 1).

In the normal PAP group, CT was higher in women than in men. Again, the small size of the male normal group may have conditioned the analysis.

Table 2. Concentrations of plasma aminothiols and vitamins, serum γ-GT activity and lipids, according to plasma aminothiol profile.

	Normal			**Altered**		
	All (81)	Women (47)	Men (34)	All (252)	Women (144)	Men (108)
Age (years)	36±10[a]	37±11[b]	35±9[c]	42±9[a]	43±9[b]	41±10[c]
Plasma thiols (μM)						
Hcy	9±2[d]	8±2	9±2*[e]	10±4[d]	9±3	12±4***[e]
Cys	190±33[f]	193±29	185±37[g]	203±40[f]	198±41	210±38*[g]
Cys-Gly	30±3[h]	29±3	31±3**[i]	32±7[h]	30±6	36±6***[i]
GSH	2.0±0.5[j]	1.9±0.5[k]	2.0±0.5[l]	1.3±0.5[j]	1.2±0.4[k]	1.4±0.5***[l]
Serum γ-GT activity (U/L)	20±13[m]	14±6[n]	28±16***[o]	37±39[m]	28±30[n]	50±46***[o]
Plasma vitamins						
Folate (ng/mL)	8±3	8±3	8±3	8±3	8±3	7±3
Vitamin B_{12} (pg/mL)	571±300	587±358	547±188	567±314	562±358	574±243
Vitamin B_6 (nmol/L)	54±24	47±19	66±28**	53±29	49±28	59±28**
Serum lipids (mg/dL)						
Triglycerides	101±47[p]	108±47	92±47[q]	127±67[p]	117±61	139±72*[q]
Total cholesterol	200±39[r]	205±33	194±45*[s]	209±37[r]	210±36	207±38[s]
HDL cholesterol	60±13	66±12[t]	52±11***	58±15	63±15[t]	53±13***
LDL cholesterol	123±35[u]	121±33	125±38	129±35[u]	128±35	131±35
Hyperlipidemia (%)	56[v]	66	41*[w]	68[v]	66	71[w]

Values are presented as mean±SD, except otherwise indicated. Figures in parenthesis are the number of subjects. Asterisks denote significant sex-differences within the same PAP group ($^{***}P$ <0.001; $^{**}P$ <0.01; $^{*}P$ <0.05). Values in each row sharing a common superscript letter are significantly different ($^{a,b,e,i,j,k,l,m,n,o,q}P$ <0.001; $^{c,d,g,p,w}P$ <0.01; $^{f,h,r,s,t,u,v}P$ <0.05).

Plasma aminothiol profile, its determinants and serum lipids, according to age range

Table 3 shows plasma aminothiols and vitamin levels, as well as serum γ-GT activity and lipid concentration in subjects with normal or altered PAP, according to age range.

Hcy seemed not to be correlated with age. However, it decreased with age in men of the normal group, nevertheless, the small number of men ≥40 in this group did not allow to do a reliable comparative analysis. When considering the whole population (data not shown) the older subjects showed higher Cys values than the younger ones (P <0.05). A similar tendency (although without statistical significance) was observed in both men and women in the altered PAP group, but not in the normal one (Table 3). This could be the result of the smaller size of the latter group. Cys-Gly levels were unchanged in both genders across the age range.

Only men with ≥40 years seemed to justify the increased of both Hcy and Cys levels observed in the altered PAP group, as previously reported (Table 2). The opposite was found with respect to Cys-Gly concentrations, which were increased only in the younger male subjects. Once more, the small number of men ≥40 in the normal PAP group might be responsible for these results.

On the other hand, plasma GSH levels decreased and serum γ-GT activity increased significantly with age, particularly in women in the altered PAP group. However, the two age groups within the altered PAP group were not sex-matched (P <0.01). Moreover, in both PAP groups GSH was strongly negatively associated with age (r= -0.32, P <0.001) while serum γ-GT activity was positively related to age (r= 0.25, P <0.001).

Concerning plasma vitamins, no influence of age was observed in subjects with a normal PAP. However, in the altered PAP group, both folate and vitamin B_{12} levels increased with age in women, whereas vitamin B_6 decreased in men. Albeit not significant, a similar trend was found in men with respect to folate levels.

As expected, the number of hyperlipidemic subjects increased with age, mainly in the altered PAP group. Both genders in the altered PAP group, independently of age range, exhibited significantly higher TG than the normal group, except for women <40 years (Table 3). In fact, both TG and Cys were higher in younger women with normal PAP than in their counterparts in the altered one.

Table 3. Concentrations of plasma aminothiols and vitamins, serum γ-GT activity and lipids, in subjects with normal or altered plasma aminothiol profile, according to age range and gender.

	Normal				Altered			
	< 40 years (50)		≥ 40 years (31)		< 40 years (100)		≥ 40 years (152)	
	Women (28)	Men (22)	Women (19)	Men (12)	Women (47)	Men (53)	Women (97)	Men (55)
Plasma thiols (µM)								
Hcy	8±1	10±2^{**g}	9±2	8±1^{Eg}	9±4	12±4***	9±3	11±4^{***E}
Cys	194±29	185±37	191±29	187±39^{G}	187±38	206±40	203±42	215±36^{*G}
Cys-Gly	29±3	32±4^{**A}	29±3	31±3	30±6	37±5^{***A}	29±6	35±7***
GSH	2.0±0.6^{Ag}	2.0±0.5^{D}	1.7±0.3^{Bg}	2.0±0.5^{C}	1.3±0.5^{Ai}	1.5±0.6^{*D}	1.1±0.4^{Bi}	1.3±0.5^{*C}
Serum γ-GT activity (U/L)	14±6^{E}	27±16^{***F}	15±6^{D}	29±17^{***G}	20±11^{aE}	50±60^{***F}	31±35^{aD}	50±29^{***G}
Plasma vitamins								
Folate (ng/mL)	8±3	8±3	8±4	8±2	7±3^{g}	7±2	8±4^{g}	8±3
Vitamin B_{12} (pg/mL)	560±326	567±205	629±409	511±155	470±261^{g}	577±237*	606±389^{g}	572±251
Vitamin B_6 (nmol/L)	45±15	70±29**	49±23	59±27	44±20	66±28^{***e}	51±31	53±27^{e}
Serum lipids (mg/dL)								
Triglycerides	116±47^{I}	79±40^{**Fg}	95±45^{H}	116±51^{g}	100±62^{eI}	133±80^{*F}	126±59^{eH}	144±63
Total cholesterol	205±26	184±33*	205±42	213±59	199±33^{g}	198±34	215±36^{g}	216±40
HDL cholesterol	67±11	54±12***	65±13	49±10**	67±19^{g}	53±16***	60±13^{g}	52±11***
LDL cholesterol	119±24	116±28	123±43	143±49	115±31^{e}	123±29^{g}	135±35^{e}	139±39^{g}
Hyperlipidemia (%)	64	36^{I}	68	50^{G}	49^{e}	64^{I}	74^{e}	78^{G}

Values are presented as mean±SD, except otherwise indicated. Figures in parenthesis are the number of subjects. Asterisks denote significant sex-differences in subjects within the same age range and PAP group ($^{***}P$ <0.001; $^{**}P$ <0.01; $^{*}P$ <0.05). Values in each row sharing a common superscript letter are significantly different ($^{a,A,B,C,D}P$ <0.001; $^{e,E,F}P$ <0.01; $^{g,G,H,I,i}P$ <0.05).

Plasma aminothiol profile and its determinants, according to lipid profile

When taking into consideration the lipid profile of subjects with an altered or normal PAP (Table 4), no significant differences were observed in what concerns the aminothiol levels. However, a non-significant slight tendency to an elevation of Cys concentration in hyperlipidemics was observed mainly in men. Moreover, in the whole population (data not shown) especially hyperlipidemic men showed higher Cys values than the normo ones (P <0.05). In fact, in the altered PAP group, Cys was significantly positively associated with TG (r= 0.20, P <0.01) and LDLc (r= 0.19, P <0.01), and was negatively correlated with HDLc (r= -0.23, P <0.001). In the normal PAP group, Cys was only significantly positively associated with TG (r= 0.23, P <0.05). Even though GSH did not correlate with lipid profile, 68% of subjects with low GSH levels exhibited hyperlipidemia (data not shown).

In both PAP groups, serum γ-GT activity was higher in hyper- than in normolipidemic subjects, namely in men. These findings were further supported by the positive relationships found between γ-GT activity and TC or TG concentration (r= 0.16, P <0.01; r= 0.34, P <0.001, respectively) in the whole population.

In the altered PAP group, but not in the normal one, all the three vitamins revealed to be in significantly higher concentrations in hyper- than in normolipidemic subjects (namely in men for folate, and in women for the other two B-vitamins). Similar differences were found in the whole population, regardless PAP.

Normolipidemic men with an altered PAP had significantly lower folate and higher Hcy concentration than their counterparts with a normal one. Furthermore, negative associations between Hcy and folate or vitamin B_{12} (r= -0.45, P <0.001; r= -0.18, P <0.01, respectively) were found in the whole population. Vitamin B_6 did not correlate with Hcy in both groups, however it correlated with Cys in the normal one (r= 0.30, P <0.05).

Table 4. Concentrations of plasma aminothiols and vitamins and serum γ-GT activity in subjects with normal or altered plasma aminothiol profile, according to serum lipid profile and gender.

	Normal				**Altered**			
	NL (36)		HL (45)		NL (80)		HL (172)	
	Women (16)	Men (20)	Women (31)	Men (14)	Women (49)	Men (31)	Women (95)	Men (77)
Age (years)	36±10	33±10	38±12^{E}	38±7	39±9^{a}	38±11	45±9^{aE}	42±9*
Thiols (μM)								
Hcy	9±2	10±2^{G}	8±2	9±2^{J}	9±3	13±5**^{G}	9±3	11±3***^{J}
Cys	194±27	179±40^{G}	193±30	195±31	191±41	204±37^{G}	201±41	213±38
Cys-Gly	29±3	32±3*^{E}	29±3	31±4^{F}	29±6	35±6***^{E}	30±5	36±6***^{F}
GSH	2.0±0.6^{A}	2.0±0.4^{D}	1.8±0.4^{B}	2.0±0.6^{C}	1.1±0.4^{A}	1.5±0.6***^{D}	1.2±0.4^{B}	1.4±0.5*^{C}
Serum γ-GT activity (U/L)	12±6^{Ag}	22±11***^{Ij}	15±6^{Dg}	35±19***^{j}	25±18^{A}	37±25**^{hI}	29±35^{D}	55±52***^{h}
Plasma vitamins								
Folate (ng/mL)	8±5	8±3^{J}	8±3	8±3	7±2	7±2^{gJ}	8±4	8±3^{g}
Vitamin B_{12} (pg/mL)	575±434	504±171	594±318	600±200	457±197^{g}	526±252	616±407^{g}	593±238
Vitamin B_6 (nmol/L)	45±18	60±29	48±19	71±26**	41±19^{g}	54±26*	52±31^{g}	61±29*

Values are presented as mean±SD. Figures in parenthesis are the number of subjects. Asterisks denote significant sex-differences between normolipidemic (NL) or hyperlipidemic (HL) subjects within the same PAP group ($^{***}P$ <0.001; $^{**}P$ <0.01; $^{*}P$ <0.05). Values in each row sharing a common superscript letter are significantly different ($^{a,A,B,C,D}P$ <0.001; $^{E,F}P$ <0.01; $^{g,G,h,I,j,J}P$ <0.05).

DISCUSSION

This work reports, for the first time, the plasma aminothiol profile and some of its major determinants in apparently healthy Azorean subjects. The relationship with serum lipid profile was also considered. As far as we know this is the first study where main plasma aminothiols are taken together to define a normal or an altered plasma aminothiol profile.

Plasma aminothiol profile of the study population

When splitting our sample according to PAP, the majority of subjects had an altered one. In these subjects, all thiols were significantly associated with each other. This is consistent with their close metabolic connection and in agreement with other studies (Jacob *et al*., 1999; Özkan *et al*., 2002; Edgar *et al*., 2008).

GSH was the major parameter responsible for the high prevalence of an altered PAP in this population. In fact, the tripeptide was present at very low concentrations, with almost 60% of all subjects, mainly in women, having plasma GSH levels < 1.5 μM. Yet, some authors (Moriarty-Craige & Jones, 2004) take 2 μM as the low limit of the reference range, which if adopted would enhance that number for 82%. Low plasma GSH levels affect more women than men. A similar gender-related difference, which is not fully understood, was also reported by other authors (Sen & Packer, 2000; Pastore *et al*., 2003). To our knowledge, no similar data on plasma GSH concentrations have been obtained in other Portuguese populations.

Mean Cys-Gly levels were within the normal range in this population. However, a relevant number of subjects with high Cys-Gly concentration have also contributed to form the altered PAP group. Such a condition has already been associated to the development of CVD (Mendis *et al*., 1997; Drogan *et al*., 2010). In agreement with others, men exhibited higher plasma Cys-Gly levels than women. These gender-related differences could be due to discrepancies in life-style, genetic and hormonal factors (Jacobsen *et al*., 1994; Jacob *et al*., 1999; Bates *et al*., 2002; Drogan *et al*., 2010).

Elevated plasma Hcy and Cys concentrations also contributed, though in a smaller extent than the other aminothiols, to the size of the altered PAP group. Yet, prevalence of HHcy was twice that the value recently found for the general population (Brustolin *et al*., 2010). Men had as well higher plasma Hcy and Cys levels than women, as usually observed

(Jacobsen *et al*., 1994; Jacob *et al*., 1999; El-Khairy *et al*., 2003; Real *et al*., 2009). This can be explained by the effect of estrogens in women because this difference disappears rapidly after menopause (Stanger *et al*., 2004). In fact, it is recognized that in pre-menopausal women, the incidence and severity of vascular diseases is lower than in men at similar ages or than in post-menopausal women (Tan *et al*., 2009).

The Azorean subjects had higher plasma Hcy concentrations than those recently reported for healthy subjects with similar age from Lisbon (Castro *et al*., 2010), albeit different analytical methods were used for Hcy determination.

Plasma aminothiol profile and its determinants

Aminothiols and γ-GT activity

It is generally considered that γ-GT activity is higher in male gender (Song *et al*., 2007) and that it is the main regulator of GSH circulating concentrations (Giral *et al*., 2008). Therefore a negative correlation between the two parameters was expected, as observed by other authors (Sedda *et al*., 2008). However, in this study, only less than one-quarter of individuals with low GSH levels exhibited a serum γ-GT activity above normal values. Other reasons could explain the occurrence of low plasma GSH levels: a decreased GSH synthesis inside cells (Cys availability does not seem to be a limiting factor, but its influx could be one); a deficiency on GSH efflux by GSH transporters; and/or a large utilization of GSH by cells, namely in antioxidant defense. Further research is needed to clarify this point.

Besides correlating with GSH, γ-GT activity was associated as well with the other aminothiols in the altered PAP group. Unsurprisingly, it was more elevated in this group, because the enzyme activity is higher in perturbed metabolism of thiol compounds (Giral *et al*., 2008) and in older subjects (Lee & Jacobs JR, 2009), as was the case. In fact, the action of γ-GT on GSH results in its cleavage to Cys-Gly and then to Cys; therefore γ-GT also regulates their circulating concentrations (Giral *et al*., 2008). In the normal PAP group, only Cys-Gly levels correlated with γ-GT. However, in the altered PAP group, even Hcy correlated well with γ-GT activity, suggesting that an altered PAP, whatever its cause, is indicative of oxidative stress. Similar results have already been reported in other populations (Lippi *et al*., 2008; Sakuta *et al*., 2007). Since γ-GT is regarded as a marker of oxidative stress, it is conceivable that oxidative damage generated by Hcy may elevate this parameter (Sakuta *et al*., 2007).

Hyperhomocysteinemia and B-vitamins

As expected, an inverse relationship was observed between Hcy and folate or vitamin B_{12} concentration, as these vitamins are essential co-factors in Hcy metabolism. This is why they are the first therapeutic targets in the treatment of HHcy (Antoniades *et al*., 2009). However, only 33% of subjects with HHcy had B-vitamin deficiencies. These results seem to confirm that Hcy metabolic pathway is very complex and under the control of many factors, both genetic and non-genetic (Castro *et al*., 2010).

A low prevalence of B-vitamin deficiencies was observed in this population, maybe due to the relatively young age of subjects, as these deficiencies usually enhance with increasing age (Clarke *et al*., 2004). As with Hcy, Azorean subjects had also higher folate and vitamin B_{12} concentrations than those reported from a similar group of individuals from Lisbon (Castro *et al*., 2010). Still concerning folate and vitamin B_{12} concentrations, no gender-related differences were found in this study, which is in accordance with data reported by Castro *et al*. (2003) and Cascalheira *et al*. (2008) for other Portuguese populations. On the contrary, men had higher vitamin B_6 levels than women, strongly suggesting that estrogens play a role on this vitamin concentration in plasma (Morris *et al*., 2008). In the normal PAP group, vitamin B_6 was positively associated with Cys, as expected, since higher concentrations of this vitamin lead to an increased transsulfuration of Hcy to Cys. In the altered PAP group, vitamin B_6 no longer correlated with Cys, evidencing that there are other routes for the production of Cys, namely from a higher degradation rate of extracellular GSH by γ-GT (which contribute for reactive oxygen species production) or/and from protein catabolism. Furthermore, in this situation plasma Hcy concentrations can increase and contribute for oxidative stress.

Plasma aminothiol profile, its determinants and age

Among the four aminothiols only GSH was negatively correlated with age. This is a well-known relationship (Camera & Picardo, 2002; Dröge *et al*., 2002) and might support the claimed “free radical theory”, which proposes an increase of oxidative stress with aging (Pastore *et al*., 2003).

In this study, as in others (Lussier-Cacan *et al*., 1996; Edgar *et al*., 2008; Real *et al*., 2009) there was no association of Hcy with age, possibly because of the fairly young age of this population. In a study conducted by Cascalheira *et al*. (2008) Hcy correlated with age for

individuals aged > 50 years, where all women were in post-menopausal stage. Furthermore, in our case, the majority of the participants were women in the premenopausal stage.

On the other hand, since γ-GT increases with age (Lee & Jacobs JR, 2009), it would be expected that the older subjects would have poor levels of plasma GSH and high contents of Cys and Cys-Gly. In fact, this was observed for Cys in the whole population (mainly due to the altered PAP group). These observations are in agreement with data from other authors (Jacob *et al*., 1999; El-Khairy *et al*., 2003; Giustarini *et al*., 2006; Edgar *et al*., 2008), confirming that Cys contributes for the oxidative stress observed in older subjects (Giustarini *et al*., 2006). However, in our study, Cys-Gly concentrations remained unchanged with age, whereas serum γ-GT activity increased with it, namely in women.

In the altered PAP group, folate and vitamin B_{12} levels increased with age, which could also justify why Hcy did not. A diet rich in vitamins might contribute for that, since subjects were not taking any supplements. However, no information was available on their previous consumptions. Vitamin B_6 decreased with age in men, as reported by Morris *et al*. (2008). Possible explanations for B_6 age-related decrease include deficient absorption, increased catabolism, and defective phosphorylation of B_6 vitamers.

Plasma aminothiol profile, its determinants and lipid profile

Although being apparently healthy, this study population revealed to be mostly affected by hyperlipidemia, namely hypercholesterolemia, which is a well-known risk factor for the development of atherosclerosis and vascular diseases (Yang *et al*., 2008). In addition, more than a half of normolipidemics had LDLc levels above the borderline. Furthermore, albeit within normal range, TG concentration in the Azorean population was about 23% higher than in subjects from Lisbon (Lopes *et al*., 2009). As expected, women had higher levels of HDLc and lower levels of TG than men, which confer them a lower risk of atherosclerosis as compared to men (NCEP, 2002).

Curiously, subjects in the normal PAP group had a better lipid profile and were younger than those in the altered one. In fact, older subjects had higher TC, LDLc and TG concentrations than the younger ones, as observed in other Azorean populations (Pavão *et al*., 2003; Pavão *et al*., 2006). Moreover, our data suggest that an altered PAP was associated with high TG concentrations, especially in men, independently of the age range. Unsurprisingly, Hcy did

not correlate with lipid profile (Lussier-Cacan *et al*., 1996; Jacob *et al*., 1999), thus confirming that HHcy is an independent risk factor for vascular diseases.

As stated by others, Cys tended to be higher in hyperlipidemics (Jacob *et al*., 1999; El-Khairy *et al*., 2003; Giral *et al*., 2008), namely in men. The correlation between Cys and lipid profile could reflect a prooxidant state, where Cys is readily oxidizable, giving rise to the production of free radical species (Drozdz *et al*., 1998; Giral *et al*., 2008), thereby promoting oxidative damage of LDL and facilitating foam cell formation. On the other hand, Page *et al*. (2010) believe that elevated plasma Cys might be a marker of the body's attempt to increase intracellular GSH in response to augmented oxidative stress. Our results seem to be in accordance with van den Brandhof *et al*. (2001), who hypothesized that Cys levels may predict a high-risk profile for CVD, namely in hyperlipidemics, as a marker of oxidative stress. Nevertheless, further studies should be carried out to better characterize the relationship between Cys and lipid profile.

Despite PAP, hyperlipidemic subjects (mainly men) had higher γ-GT activity than the normo ones, which sustains the role of this enzyme as a marker of oxidative stress (Turgut *et al*., 2006; Lippi *et al*., 2007). Also, a recent cross-sectional study in metabolic syndrome has shown that patients with individual components of metabolic syndrome, including dyslipidemia, have higher γ-GT activity, higher plasma levels of Cys-Gly and Cys, and lower plasma GSH levels (Giral *et al*., 2008).

In the study population, GSH concentration was not directly correlated with serum lipid profile. Yet, almost 70% of subjects with low GSH levels were also hyperlipidemic. This suggests a high cell consumption of the tripeptide to fight oxidative stress created by hyperlipidemia.

Regarding B-vitamins, all were higher in hyperlipidemics in comparison with the normo ones, which is against others (Semmler *et al*., 2010). Again, a healthy diet could have contributed for these results.

FINAL REMARKS

Our findings showed that although this population was apparently healthy, mostly subjects had an altered PAP, mainly due to low plasma GSH levels, which were more frequent in older, hyperlipidemics, and in subjects with high γ-GT activity. These are all markers of oxidative stress, which is crucial in atherogenesis and CVD. However, not all subjects with an altered PAP fulfill those conditions, which puts forwards that other reasons may exist for that alteration. As subjects were apparently healthy, an altered PAP, namely provoked by low GSH levels, can constitute an early marker of atherosclerosis.

With respect to limitations of this investigation, no dietary data were still available, neither smoking nor alcohol habits. Also, our variables were not normally distributed neither could be transformed, so we had to use less powerful, non-parametric tests. Another restriction of our study was the relatively small number of subjects in the normal PAP group, especially men.

Finally, thiol profile does seem to be affected, though with diverse extent, by gender, age, γ-GT activity, B-vitamin concentrations, and lipid profile. All these factors should be considered when evaluating the plasma aminothiol profile.

REFERENCES

Antoniades C, Antonopoulos AS, Tousoulis D, *et al*. Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. *Eur Heart J* 2009; **30**: 6-15.

Atmaca G. Antioxidant effects of sulfur-containing amino acids. *Yonsei Med J* 2004; **45**: 776-788.

Bates CJ, Mansoor MA, Gregory J, *et al*. Correlates of plasma homocysteine, cysteine and cysteinyl-glycine in respondents in the British National Diet and Nutrition Survey of young people aged 4-18 years, and a comparison with the survey of people aged 65 years and over. *Br J Nutr* 2002; **87**: 71-79.

Brustolin S, Giugliani R, Félix TM. Genetics of homocysteine metabolism and associated disorders. *Braz J Med Biol Res* 2010; **43**: 1-7.

Camera E, & Picardo M. Analytical methods to investigate glutathione and related compounds in biological and pathological processes. *J Chromatogr B Analyt Technol Biomed Life Sc* 2002; **781**: 181–206.

Cascalheira JF, Parreira MC, Viegas AN, *et al*. Serum Homocysteine: Relationship with Circulating Levels of Cortisol and Ascorbate. *Ann Nutr Metab* 2008; **53**: 67–74.

Castro R, Barroso M, Rocha M, *et al*. The *TCN2* 776C>G polymorphism correlates with vitamin B_{12} cellular delivery in healthy adult populations. *Clin Biochem* 2010; **43**: 645–649.

Castro R, Rivera I, Ravasco P, *et al*. 5,10-Methylenetetrahydrofolate reductase 677C→T and 1298A→C mutations are genetic determinants of elevated homocysteine. *Q J Med* 2003; **96**: 297–303.

Ceconi C, Boraso A, Cargnoni A. Oxidative stress in cardiovascular disease: myth or fact? *Arch Biochem Biophys* 2003; **420**: 217–221.

Clarke R, Evans JG, Schneede J. Vitamin B_{12} and folate deficiency in later life. *Age Ageing* 2004; **33**: 34-41.

De Chiara B, Mafrici A, Campolo J, *et al*. Low plasma glutathione levels after reperfused acute myocardial infarction are associated with late cardiac events. *Coron Artery Dis* 2007; **18**: 77-82.

Deitrick CL, Richard E. Katholi RE, David J. Huddleston DJ, *et al*. Clinical adaptation of a high-performance liquid chromatographic method for the assay of pyridoxal 5′ phosphate in human plasma. *J Chromatogr B Biomed Sci Appl* 2001; **751**: 383-387.

Direcção-Geral da Saúde. Risco de morrer em Portugal 2006 (vol I). Retrieved from http://www.dgs.pt/

Drogan D, Weikert C, Dierkes J. Plasma γ-Glutamyltransferase, Cysteinyl-Glycine, and Oxidized Low-Density Lipoprotein: A Pathway Associated With Myocardial Infarction Risk? *Arterioscler Thromb Vasc Biol* 2010; **30**: 2053-2058.

Dröge W. Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants. *Exp Gerontol* 2002; **37**: 1331-1343.

Drozdz R, Parmentier C, Hachad H, *et al*. γ-Glutamyltransferase dependent generation of reactive oxygen species from a glutathione/transferrin system. *Free Radic Biol Med* 1998; **25**: 786–792.

Edgar KS, Woodside JV, Skidmore P, *et al*. Thiol and Cardiovascular Risk Factor Status in a Male Northern Irish Population. *Int J Vitam Nutr Res* 2008; **78**: 208-216.

El-Khairy L, Vollset S, Refsum H. Predictors of Change in Plasma Total Cysteine:

Longitudinal Findings from the Hordaland Homocysteine Study. *Clin Chem* 2003; **49**: 113-120.

Food and Nutrition Board, Institute of Medicine. Dietary reference intakes: thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin B_{12}, pantothenic acid, biotin, and choline. Washington, DC: National Academy Press; 1998.

Forman HJ, Zhang H, Rinna A. Glutathione: Overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med* 2009; **30**: 1-12.

Giral P, Jacob N, Dourmap C, *et al*. Elevated gamma-glutamyltransferase activity and perturbed thiol profile are associated with features of metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2008; **28**: 587-593.

Giustarini D, Dalle-Donne I, Lorenzini S, *et al*. Age-Related Influence on Thiol, Disulfide, and Protein-Mixed Disulfide Levels in Human Plasma. *J Gerontol A Biol Sci Med Sci* 2006; **61**: 1030-1038.

Grundy SM, Cleeman JI, Merz CN, Brewer HB Jr, Clark LT, Hunninghake DB, Pasternak RC, Smith SC Jr, Stone NJ; National Heart, Lung, and Blood Institute; American College of Cardiology Foundation; American Heart Association. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. *Circulation* 2004; **110**: 227–239.

Jacob N, Bruckert E, Giral P, *et al*. Cysteine is a cardiovascular risk factor in hyperlipidemic patients. *Atherosclerosis* 1999; **146**: 53-59.

Jacobsen DW, Gatautis VJ, Green R, *et al*. Rapid HPLC Determination of Total Homocysteine and Other Thiols in Serum and Plasma: Sex Differences and Correlation with Cobalamin and Folate Concentrations in Healthy Subjects. *Clin Chem* 1994; **40**: 873-881.

Kaul S, Zadeh AA, Shah PK. Homocysteine Hypothesis for Atherothrombotic Cardiovascular Disease: not validated. *J Am Coll Cardiol* 2006; **48**: 914-923.

Kimura M, Kanehira K, Yokoi K. Highly sensitive and simple liquid chromatographic determination in plasma of B_6 vitamers, especially pyridoxal 5'-phosphate. *J Chromatogr A* 1996; **722**: 295-301.

Lee D, Jacobs JR, DR. Is serum gamma-glutamyltransferase a marker of exposure to various environmental pollutants? *Free Radic Res* 2009; **43**: 533-537.

Lippi G, Salvagno GL, Targher G, *et al*. Plasma γ-glutamyl Transferase Activity Predicts Homocysteine Concentration in a Large Cohort of Unselected Outpatients. *Intern Med* 2008; **47**: 705-707.

Lippi G, Targher G, Montagnana M, *et al*. Relationship between γ-glutamyltransferase, lipids and lipoprotein(a) in the general population. *Clin Chim Acta* 2007; **384**: 163–166.

Lopes PA, Rodrigues OR, Napoleão P, *et al*. Influence of Dyslipidemia and Smoking on Redox Markers in Humans – a Critical Study. *Rev Port Cardiol* 2009; **28**: 37-47.

Lussier-Cacan S, Xhignesse M, Piolot A, *et al*. Plasma total homocysteine in healthy subjects: sex-specific relation with biological traits. *Am J Clin Nutr* 1996; **64**: 587-593.

Mason JE, Starke RD, Van Kirk JE. Gamma-glutamyl transferase: a novel cardiovascular risk biomarker. *Prev Cardiol* 2010; **13**: 36-41.

McMenamin ME, Himmelfarb J, Nolin TD. Simultaneous analysis of multiple aminothiols in human plasma by high performance liquid chromatography with fluorescence detection. *J Chromatogr B* 2009; **877**: 3274-3281.

Mendis S, Athauda SB, Takashi K. Association between hyperhomocysteinemia and ischemic heart disease in Sri Lankans. *Int J Cardiol* 1997; **62**: 221-225.

Moat SJ, Bonham JR, Powers HJ. Role of aminothiols as a component of the plasma antioxidant system and relevance to homocysteine-mediated vascular disease. *Clin Sci (Lond)* 2001; **100**: 73-79.

Moriarty-Craige SE, Jones DP. Extracellular thiols and thiol/disulfide redox in metabolism. *Annu Rev Nutr* 2004; **24**: 481–509.

Morris MS, Picciano MF, Jacques PF, *et al*. Plasma pyridoxal 5'-phosphate in the US population: the National Health and Nutrition Examination Survey, 2003-2004. *Am J Clin Nutr* 2008; **87**: 1446-1454.

Morrison JA, Jacobsen DW, Sprecher DL, *et al*. Serum Glutathione in Adolescent Males Predicts Parental Coronary Heart Disease. *Circulation* 1999; **100**: 2244-2247.

National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation* 2002; **106**: 3143–3421.

Özkan Y, Özkan E, Şimşek B. Plasma total homocysteine and cysteine levels as cardiovascular risk factors in coronary heart disease. *Int J Cardiol* 2002; **82**: 269–277.

Page JH, Ma J, Chiuve SE, *et al*. Plasma total cysteine and total homocysteine and risk of myocardial infarction in women: A prospective study. *Am Heart J* 2010; **159**: 599-604.

Pastore A, Federici G, Bertini E, *et al*. Analysis of glutathione: implication in redox and detoxification. *Clin Chim Acta* 2003; **333**: 19–39.

Pavão ML, Cordeiro C, Costa A, *et al*. Comparison of Whole-Blood Glutathione Peroxidase Activity, Levels of Serum Selenium, and Lipid Peroxidation in Subjects from the Fishing and

Rural Communities of "Rabo de Peixe" Village, San Miguel Island, The Azores' Archipelago, Portugal. *Biol Trace Elem Res* 2003; **92**: 27-40.

Pavão ML, Figueiredo T, Santos V, *et al*. Whole blood glutathione peroxidase and erythrocyte superoxide dismutase activities, serum trace elements (Se, Cu, Zn) and cardiovascular risk factors in subjects from the city of Ponta Delgada, Island of San Miguel, The Azores Archipelago, Portugal. *Biomarkers* 2006; **11**: 460-471.

Real JT, Martinez-Hervas S, Garcia-Garcia AB, *et al*. Association of C677T Polymorphism in MTHFR Gene, High Homocysteine and Low HDL Cholesterol Plasma Values in Heterozygous Familial Hypercholesterolemia. *J Atheroscler Thromb* 2009; **16**: 815-820.

Roy H, Bhardwaj S, Yla-Herttuala S. Molecular genetics of atherosclerosis. *Hum Genet* 2009; **125**: 467-491.

Sakuta H, Suzuki T, Ito T. Serum γ-glutamyl transferase is associated with plasma total homocysteine in Japanese patients with type 2 diabetes. *Acta Diabetol* 2007; **44**: 177-180.

Sedda V, De Chiara B, Parolini M, *et al*. Plasma glutathione levels are independently associated with gamma-glutamyltransferase activity in subjects with cardiovascular risk factors. *Free Radic Res* 2008; **42**: 135–141.

Selhub J. Public health significance of elevated homocysteine. *Food Nutr Bull* 2008; **29**: S116-S125.

Semmler A, Moskau S, Grigull A, *et al*. Plasma folate levels are associated with the lipoprotein profile: a retrospective database analysis. *Nutr J* 2010; **9**(31).

Sen CK, Packer L. Thiol homeostasis and supplements in physical exercise. *Am J Clin Nutr* 2000; **72**: 653S–69S.

Shimizu H, Kiyohara Y, Kato I. Relationship Between Plasma Glutathione Levels and Cardiovascular Disease in a Defined Population: The Hisayama Study. *Stroke* 2004; **35**: 2072-2077.

Song SH, Kwak IS, Kim YJ, *et al*. Can gamma-glutamyltransferase be an additional marker of arterial stiffness? *Circ J* 2007; **71**: 1715-1720.

SREA: Regional Service of Statistics from the Azores. Demografia 2007. Retrieved from http://estatistica.azores.gov.pt/

Stanger O, Herrmann W, Pietrzik, *et al*. Clinical use and rational management of homocysteine, folic acid, and B-vitamins in cardiovascular and thrombotic diseases. *Z Kardiol* 2004; **93**: 439-453.

Tan T-Y, Lu C-H, Lin T-K, *et al*. Factors associated with gender difference in the intima-media thickness of the common carotid artery. *Clin Radiol* 2009; **64**: 1097-1103.

Turell L, Carballal S, Botti H, *et al*. Oxidation of the albumin thiol to sulfenic acid and its implications in the intravascular compartment. *Braz J Med Biol Res* 2009; **42**: 305-311.

Turgut O, Yilmaz A, Yalta K, *et al*. γ-Glutamyltransferase is a promising biomarker for cardiovascular risk. *Med Hypotheses* 2006; **67**: 1060.1064.

van den Brandhof WE, Haks K, Schouten EG, *et al*. The relation between plasma cysteine, plasma homocysteine and coronary atherosclerosis. *Atherosclerosis* 2001; **157**: 403–409.

Wronska-Nofer T, Nofer J-R, Stetkiewicz J, *et al*. Evidence for oxidative stress at elevated plasma thiol levels in chronic exposure to carbon disulfide (CS_2) and coronary heart disease. *Nutr Metab Cardiovasc Dis* 2007; **17**: 546-53.

Yang R-L, Shi Y-H, Hao G. Increasing Oxidative Stress with Progressive Hyperlipidemia in Human: Relation between Malondialdehyde and Atherogenic Index. *J Clin Biochem Nutr* 2008; **43**: 154-158.

Zhou J, Austin RC. Contributions of hyperhomocysteinemia to atherosclerosis: Causal relationship and potential mechanisms. *Biofactors* 2009; **35**: 120-129.

CHAPTER III

GENERAL DISCUSSION, CONCLUDING REMARKS AND FUTURE PERSPECTIVES

GENERAL DISCUSSION, CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The conventional risk factors for atherosclerosis and related CVD include hyperlipidemia, hypertension, diabetes, positive family history and smoking. However, many patients have precocious atherosclerosis without having any of these standard risk factors (Mayer *et al*., 1996). Identification of other markers that increase the risk of atherosclerosis may improve our understanding of the pathophysiologic mechanisms of this disorder and allow the development of new preventive or therapeutic measures.

This thesis intended to understand the behavior of the main aminothiols in plasma of apparently healthy Azorean populations, according to variables as gender, age range, related plasma B-vitamins concentrations, serum γ-GT activity and lipid profile. The main findings will be summarized and further discussed in the following pages.

1. Oxidative stress and aminothiol levels

Accumulated evidences suggest a contributing factor of oxidative stress in the genesis and development of atherosclerosis (Ceconi *et al*., 2003; Antoniades *et al*., 2009). Thus, redox homeostasis is very important for vital cellular functions, and its disruption can be accompanied by an increase in the level of ROS. However, organism has antioxidant defense systems, which under normal conditions maintain intracellular concentration of oxidants at a safe level (Oktyabrsky & Smirnova, 2007). Thiols belong to this antioxidant defense system, being GSH the most important in cells and Cys in plasma (Moriarty-Craige & Jones, 2004). The activity of GSH as an antioxidant can be expressed in two ways: as a function of GSH concentration and as a function of the redox state of the GSH/GSSG pool (Samiec *et al*., 1998). Several pathological conditions, namely CVD, are being characterized by GSH deficiency (Morrison *et al*., 1999; Shimizu *et al*., 2004) or by an imbalance in the GSSG/GSH ratio (Samiec *et al*., 1998; Ashfaq *et al*., 2006).

In almost 60% of all study participants, total plasma GSH concentration was very low and among those subjects only less than one-quarter had high γ-GT activity (the main regulator of the tripeptide values). The condition situation in the remaining individuals could result from an impairment in aminothiol metabolism, such as scarce influx of Cys; decreased GSH

synthesis; deficiency in GSH efflux by protein transporters; and/or increased utilization in redox and conjugation reactions. More research in this field is needed.

Decreased GSH synthesis could arise from GCS or GSH synthetase deficiencies, both conditions are very rare autosomal recessive disorders (Ristoff & Larsson, 2007). Also, GSH synthesis is rate-limited by the availability of Cys and a decrease in the Hcy transsulfuration pathway can lead to GSH deficiency (Mosharov *et al*., 2000). This pathway seems to occur in a less extent in the altered PAP. Measurement of cell Cys and GSH levels would clarify this point.

Another interesting point is the fact that oxidative stress induces CySS uptake by cells, while elevated extracellular glutamate and vitamin C levels inhibit it competitively, which decrease GSH synthesis and utilization (Siow *et al*., 1998; Ruiz *et al*., 2003). Also, Lenton et al. (2003) showed that vitamin C spares GSH consumption by competing with it for free radicals and by converting thiol radicals back to GSH. Therefore, vitamin C deficiency (defined by some authors as plasma ascorbate <33 μM) can result in decreased plasma GSH content (Henning *et al*., 1991) and vitamin C supplementation can restore it (Lenton *et al*., 2003).

Vitamin C plays an important role in regulating the redox state of cells and has anti-atherogenic proprieties (Ekuni *et al*., 2009), namely inhibition of LDL oxidation and leukocyte adhesion to the endothelium (Carr *et al*., 2000; Ginter 2007), and CVD have been associated with vitamin C deficiency (Frikke-Schmidt & Lykkesfeldt, 2009). This vitamin has been shown as well to ameliorate the lipid profile (McRae, 2008).

Albeit not object of this work, preliminary results in this study population point out to a mean plasma ascorbate concentration below the reference values. Moreover, this vitamin was reported to be in much higher concentration in subjects from mainland Portugal (Cascalheira *et al*., 2008). These results may express a significant effect of vitamin C in prevention of vascular damage.

The premise that oxidative stress, among several other factors, plays an important role in atherogenesis implies that the development and progression of atherosclerosis can be inhibited by antioxidants, such as vitamins C, E, and A. Thus, antioxidant therapies could be a preventive measure in this population. However, interventional trials in other populations have been controversial (Zhou & Austin, 2009).

Low plasma GSH levels also can result from alcohol abuse, which leads to hypocysteinemia (Moriarty-Craige & Jones, 2004). However the average plasma Cys levels and serum γ-GT activity were within the normal ranges.

In cell metabolism a same substrate is often used in many different reactions. Therefore, the normal mean plasma Cys and Cys-Gly levels found in this study could also result from the contribution of other processes, such as protein degradation, and not exclusively from degradation of GSH.

Currently available information indicates that dietary components contribute to plasma thiol concentrations and/or redox state. These include the intake of sulfur amino acids (Cys and Met), the availability of glutamine (a precursor for Glu and GSH), the presence of dietary antioxidants (such vitamins C and E) to inhibit oxidative processes and thereby contribute to a more reduced redox state, and the adequacy of other redox active micronutrients such as selenium, niacin and riboflavin (Moriarty-Craige & Jones, 2004).

2. HHcy and B-vitamins

In agreement with other studies, we also confirmed an association of plasma Hcy with folate and vitamin B_{12} concentrations. Furthermore, in Azorean subjects they were in higher amounts than in subjects from Lisbon (Castro *et al*., 2010). Namely, the prevalence of HHcy (personal communication) was more elevated in the Azores (10%) than in Lisbon (4%). Nutritional analyses in this population, which are in course, will contribute to clarify this point.

On the contrary to GSH levels, this study population was not very affected by HHcy. Moreover, among subjects with HHcy only 33% had B-vitamins deficiencies. In fact, prevalence of HHcy results from a complex interplay of genetic and environmental factors. Unfortunately, we did not investigate the frequency of *MTHFR* polymorphism to compare it with data from Lisbon. Yet, Branco *et al*. (2009) found for São Miguel population an allelic frequency on *MTHFR* C677T of 41.7%, which was more elevated than that in mainland Portugal (Castro *et al*., 2003). The pro-oxidative state in HHcy favors the activation of several inflammatory mediators, such as the nuclear factor-kappa B (NF-kB), responsible for the transcriptional regulation of many proinflammatory genes. This leads to the activation of endothelial cells and induces the expression of factors such as vascular cell adhesion

molecules and results on increased circulating proinflammatory cytokines (Antoniades *et al*., 2009).

Folate may be considered as an effective antioxidant in subjects with HHcy, since it will reduce Hcy concentration and therefore ROS production (Racek *et al*., 2005).

3. Aminothiols and relations to gender and age

Both male gender and advanced age are well recognized risk factors for atherosclerosis. As reported in Chapter II, men exhibited higher plasma concentrations of Hcy, Cys-Gly and GSH than women. In addition, when analyzing by PAP, also Cys revealed to be increased in men, which can be partly due to the hyperlipidemia condition present in the altered PAP.

GSH declines with age as a result of an augment in oxidative stress or a decrease in GSH effux with aging and/or a decrease in intracellular synthesis (Samiec *et al*., 1998, Wang *et al*., 2003). This is consistent with a decline of antioxidant defenses with aging and age-related disease processes (Samiec *et al*., 1998). In this study, age-dependent differences were only found for GSH and Cys. Usually, disulfide forms (with the exception of GSH) are more concentrated than the respective thiol forms (Giustarini *et al*., 2006). The determination of GSSG/GSH ratio could have highlighted some of the results.

With aging thiol/disulfide redox state becomes more oxidized, due at least in part, from oxidative damage inflicted by ROS, which leads to an increase in CySS (Hildebrandt *et al*., 2002). Thus, it seems that CySS and other disulfides are the main forms responsible for the increase of total Cys with age in our study. This augment could result from a decreased amino acid clearance capacity in the older subjects (which leads to an increased exposure of dietary Cys to the oxidative environment of the blood) or from an impaired aminothiol metabolism (Dröge, 2002).

The recognized age-related increase in total plasma Hcy can be as well partly explained, by a decline in renal function and/or by a decrease on enzyme activity involved in Hcy metabolism (Edgar *et al*., 2008). As stated in Chapter II, Hcy did not correlate with age, probably because our study population was relatively young.

4. Aminothiols and γ-GT

Although best known as a reliable index of hepatobiliary dysfunction and alcohol abuse, prospective cohort studies have revealed that serum γ-GT activity also exhibits a positive

association with CVD brought by atherosclerosis (Lee & Jacobs, 2009; Drogan *et al*., 2010; Mason *et al*., 2010). Indeed, it regulates plasma aminothiols concentrations and act as a pro-oxidant in the extracellular space, producing ROS (Drozdz *et al*., 1998). Furthermore, most serum γ-GT is bound to carriers, such lipoproteins and albumin, so LDL can carry γ-GT into the atherosclerotic plaque, where free iron is also present, which leads to LDL oxidation and atherogenesis (Emdin *et al*., 2005). As a result, the enzyme activity is considered a good marker of oxidative stress.

An elevation in γ-GT activity was closely associated with a potentially pro-oxidant profile of circulating thiol compounds, as decreased plasma levels of GSH, and increased concentrations of Cys-Gly and Cys. Such a profile is indicative of oxidative stress (Giral *et al*., 2008). As see in Chapter II, this enzyme activity was higher in men than in women, and increased with aging and with hyperlipidemia. Consequently, γ-GT activity was more elevated in the altered PAP group. Therefore, such a profile is predictive of an oxidant environment.

5. Aminothiols and hyperlipidemia

Hyperlipidemia, namely hypercholesterolemia, is a major risk factor for atherosclerosis and CVD, and was very present in this population. Moreover, TG concentration in this Azorean population was higher than what was recently report for mainland Portugal (Lopes *et al*., 2009). This could result from overweigh and obesity, physical inactivity, cigarette smoking, excess alcohol intake, very high-carbohydrate diets, among other factors (Grundy *et al*., 2004). Furthermore, an altered PAP seems to be predictive of a worse lipid profile, namely on TG concentration, mainly due to correlation with Cys levels. Also, Hildebrandt *et al*. (2002) pointed that hyperlipidemia induces a change on thiol/disulfide redox state.

Although Cys has many protective functions, including support of protein and GSH synthesis, and maintenance of cellular redox state, high plasma Cys concentration seems to be toxic and contributes for ROS production in hyperlipidemics. In plasma Cys is rapidly auto-oxidized to CySS, resulting in the generation of ROS, such H_2O_2, that in turn promotes the activation of the cellular immune system by enhanced induction of NF-kB and MCP-1 (Moriarty-Craige & Jones, 2004). Thus, Cys, mainly due to its oxidation, can modulate inflammatory events of early atherosclerosis (Go & Jones, 2005; Giustarini *et al*., 2006; Ashfaq *et al*., 2008). Consequently, GSH acts as a major transport non-toxic form of Cys in plasma (Wu *et al*.,

2004). Albeit in our work GSH did not correlate with lipid profile, a large proportion of subjects with low plasma GSH were hyperlipidemic. Another study (Mendoza-Núñez *et al*., 2010) found a decrease in intracellular GSH and GSH/GSSG ratio in subjects with familial combined hyperlipidemia. However, it remains to elucidate if low GSH concentration is just a reflex of a condition of oxidative stress (resulting namely from hyperlipidemia) or if it is at least partly the cause of that stress, due an impairment in its metabolism.

Also, in this study, serum γ-GT activity correlated with lipidemia, being especially higher in hyperlipidemic men. An increase in γ-GT activity can basically be a response to oxidative stress in these individuals, as γ-GT increases intracellular GSH synthesis (Lee & Jacobs JR, 2009). However about 80% of subjects with low GSH had a normal γ-GT activity.

FINAL REMARKS

1. The present thesis reports, for the first time, the determination of plasma aminothiol profile in apparently healthy subjects born and living in Azores archipelago, and its association with important variables, namely with gender, age, serum γ-GT activity and plasma B-vitamins concentrations.
2. This work provides reference values for these thiols and some of its determinants, thus allowing future comparisons with data from other populations.
3. The relationships among aminothiols (namely Cys), serum γ-GT activity and lipid profile were also studied.
4. The Azorean population seems to be very affected by an altered PAP, which is influenced by age, high γ-GT activity and hyperlipidemia, therefore demonstrating a general tendency to an oxidative profile. Consequently, having an altered PAP could be predictive of oxidative stress-related diseases, namely CVD.
5. Furthermore, male gender, namely after 40 years, is more affected by an altered PAP (specifically with higher Hcy, Cys, and Cys-Gly levels), high γ-GT activity and a worse lipid profile, all associated with an elevated risk for atherosclerosis.
6. Vitamin B_{12} and folate supplements after 40 years should be considered as a preventive measure for HHcy in this population.

7. Albeit apparently healthy, hyperlipidemia (and consequent oxidative stress) was a prevalent characteristic of this population. So, a healthy diet, as well as physical activity, and weight-loss should be considered.
8. There is a need of standardized procedures to quantify aminothiols or other parameters in order to carry out comparative studies among populations. Also, care must be taken in interpreting blood measurements, and ideally conduction of both intra- and extracellular parameters concentration should be done.
9. Due to the nature of this cross-sectional study, it remains to establish if the improvement of PAP would contribute to a decrease standardized mortality rate for CVD in the Azores. The data generated from this study will be of importance context of ongoing studies concerning the factors that influence the high mortality rates for CVD in the Azorean population.

FUTURE DIRECTIONS

1. Future research will include better characterization of the study population, including dietary patterns, smoking and alcohol habits.
2. Since oxidative mechanisms are involved in atherosclerosis, the interest in antioxidant vitamins is increasing. Among them, vitamin A, C and E are the most potent antioxidants preventing the oxidation of LDL. Consequently, some studies, which are already in course, will allow the evaluation of plasma antioxidant vitamins (A, C and E) and other markers of oxidative stress (GSSG/GSH ratio) in this population.
3. Further work should clarify why plasma concentrations of GSH were very low: by expression of *MRP* (GSH transporter), mutations on genes involved in its synthesis, or/and measurement of intracellular Cys and GSH levels. Also, measurement of plasma GSH levels in mainland Portugal should be realized.
4. As depletion of GSH results in increased susceptibility of the cell to oxidative stress, new knowledge of the nutritional regulation of GSH metabolism is critical for the development of effective strategies to improve health and to treat disease.

REFERENCES

Antoniades C, Antonopoulos AS, Tousoulis D, *et al*. Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. *Eur Heart J* 2009; **30**: 6-15.

Ashfaq S, Abramson JL, Jones DP, *et al*. The relationship between plasma levels of oxidized and reduced thiols and early atherosclerosis in healthy adults. *J Am Coll Cardiol* 2006; **47**: 1005-1011.

Ashfaq S, Abramson JL, Jones DP, *et al*. Endothelial function and aminothiol biomarkers of oxidative stress in healthy adults. *Hypertension* 2008; **52**: 80-85.

Branco CC, Pereirinha T, Cabral R, *et al*. Thrombotic genetic risk factors and warfarin pharmacogenetic variants in São Miguel's healthy population (Azores). *Thromb J* 2009; **7**(9).

Carr AC, Zhu B-Z, Frei B. Potential antiatherogenic mechanisms of ascorbate (Vitamin C) and alpha-tocopherol (Vitamin E). *Circ Res* 2000; **87**: 349-354.

Cascalheira JF, Parreira MC, Viegas AN, *et al*. Serum Homocysteine: Relationship with Circulating Levels of Cortisol and Ascorbate. *Ann Nutr Metab* 2008; **53**: 67–74.

Castro R, Barroso M, Rocha M, *et al*. The *TCN2* 776C>G polymorphism correlates with vitamin B_{12} cellular delivery in healthy adult populations. *Clin Biochem* 2010; **43**: 645–649.

Castro R, Rivera I, Ravasco P, *et al*. 5,10-Methylenetetrahydrofolate reductase 677C→T and 1298A→C mutations are genetic determinants of elevated homocysteine. *Q J Med* 2003; **96**: 297–303.

Ceconi C, Boraso A, Cargnoni A. Oxidative stress in cardiovascular disease: myth or fact? *Arch Biochem Biophys* 2003; **420**: 217–221.

Drogan D, Weikert C, Dierkes J. Plasma γ-Glutamyltransferase, Cysteinyl-Glycine, and Oxidized Low-Density Lipoprotein: A Pathway Associated With Myocardial Infarction Risk? *Arterioscler Thromb Vasc Biol* 2010; **30**: 2053-2058.

Dröge W. Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants. *Exp Gerontol* 2002; **37**: 1331-1343.

Drozdz R, Parmentier C, Hachad H, *et al*. γ-Glutamyltransferase dependent generation of reactive oxygen species from a glutathione/transferrin system. *Free Radic Biol Med* 1998; **25**: 786–792.

Edgar KS, Woodside JV, Skidmore P, *et al*. Thiol and Cardiovascular Risk Factor Status in a Male Northern Irish Population. *Int J Vitam Nutr Res* 2008; **78**: 208-216.

Ekuni D, Tomofuji T, Sanbe T, *et al*. Vitamin C intake attenuates the degree of experimental atherosclerosis induced by periodontitis in the rat by decreasing oxidative stress. *Arch Oral Biol* 2009; **54**: 495-502.

Emdin M, Pompella A, Paolicchi A. Gamma-glutamyltransferase, atherosclerosis, and cardiovascular disease: triggering oxidative stress within the plaque. *Circulation* 2005; **112**: 2078-2080.

Frikke-Schmidt H, Lykkesfeldt J. Role of Marginal Vitamin C Deficiency in Atherogenesis: In Vivo Models and Clinical Studies. *Basic Clin Pharmacol Toxicol* 2009; **104**: 419-433.

Ginter E. Chronic vitamin C deficiency increases the risk of cardiovascular diseases. *Bratisl Lek Listy* 2007; **108**: 417-421.

Giral P, Jacob N, Dourmap C, *et al*. Elevated gamma-glutamyltransferase activity and perturbed thiol profile are associated with features of metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2008; **28**: 587-593.

Giustarini D, Dalle-Donne I, Lorenzini S, *et al*. Age-Related Influence on Thiol, Disulfide, and Protein-Mixed Disulfide Levels in Human Plasma. *J Gerontol A Biol Sci Med Sci* 2006; **61**: 1030-1038.

Go Y-M, Jones DP. Intracellular Proatherogenic Events and Cell Adhesion Modulated by Extracellular Thiol/Disulfide Redox State. *Circulation* 2005; **111**: 2973-2980.

Grundy SM, Cleeman JI, Merz CN, Brewer HB Jr, Clark LT, Hunninghake DB, Pasternak RC, Smith SC Jr, Stone NJ; National Heart, Lung, and Blood Institute; American College of Cardiology Foundation; American Heart Association. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. *Circulation* 2004; **110**: 227–239.

Henning SM, Zhang JZ, McKee RW, *et al*. Glutathione blood levels and other oxidant defense indices in men fed diets low in vitamin C. *J Nutr* 1991; **121**: 1969-1975.

Hildebrandt W, Kinscherf R, Hauer K, *et al*. Plasma cystine concentration and redox state in aging and physical exercise. *Mech Ageing Dev* 2002; **123**: 1269-1281.

Lee D, Jacobs JR, DR. Is serum gamma-glutamyltransferase a marker of exposure to various environmental pollutants? *Free Radic Res* 2009; **43**: 533-537.

Lenton KJ, Sané AT, Therriault H, *et al*. Vitamin C augments lymphocyte glutathione in subjects with ascorbate deficiency. *Am J Clin Nutr* 2003; **77**: 189–195.

Lopes PA, Rodrigues OR, Napoleão P, *et al*. Influence of Dyslipidemia and Smoking on Redox Markers in Humans – a Critical Study. *Rev Port Cardiol* 2009; **28**: 37-47.

Mason JE, Starke RD, Van Kirk JE. Gamma-glutamyl transferase: a novel cardiovascular risk biomarker. *Prev Cardiol* 2010; **13**: 36-41.

Mayer EL, Jacobsen DW, Robinson K. Homocysteine and coronary atherosclerosis. *J Am Coll Cardiol* 1996; **27**: 517-527.

McRae MP. Vitamin C supplementation lowers serum low-density lipoprotein cholesterol and triglycerides: a meta-analysis of 13 randomized controlled trials. *J Chiropr Med* 2008; **7**: 48-58.

Mendoza-Núñez VM, Beristain-Pérez A, Pérez-Vera SP. Age-Related Sex Differences in Glutathione Peroxidase and Oxidative DNA Damage in a Healthy Mexican Population. *J Womens Health* 2010; **19**: 919- 926.

Moriarty-Craige SE, Jones DP. Extracellular thiols and thiol/disulfide redox in metabolism. *Annu Rev Nutr* 2004; **24**: 481–509.

Morrison JA, Jacobsen DW, Sprecher DL, *et al*. Serum Glutathione in Adolescent Males Predicts Parental Coronary Heart Disease. *Circulation* 1999; **100**: 2244-2247.

Mosharov E, Cranford MR, Banerjee R. The quantitatively important relationship between homocysteine metabolism and glutathione synthesis by the transsulfuration pathway and its regulation by redox changes. *Biochemistry* 2000; **39**: 13005-13011.

Oktyabrsky ON, Smirnova GV. Redox Regulation of Cellular Functions. *Biochemistry (Mosc)* 2007; **72**: 132-145.

Racek J, Rusňáková H, Trefil L, *et al*. The Influence of Folate and Antioxidants on Homocysteine Levels and Oxidative Stress in Patients with Hyperlipidemia and Hyperhomocysteinemia. *Physiol Res* 2005; **54**: 87-95.

Ristoff E, Larsson A. Inborn errors in the metabolism of glutathione. *Orphanet J Rare Dis* 2007; **2**(16).

Ruiz E, Siow RCM, Bartlett SR, et al. Vitamin C inhibits diethylmaleate-induced L-cystine transport in human vascular smooth muscle cells. *Free Radic Biol Med* 2003; **34**: 103-110.

Samiec, PS, Drews-Botsch C, Flagg EW, *et al*. Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes. *Free Radic Biol Med* 1998; **24**: 699-704.

Shimizu H, Kiyohara Y, Kato I. Relationship Between Plasma Glutathione Levels and Cardiovascular Disease in a Defined Population: The Hisayama Study. *Stroke* 2004; **35**: 2072-2077.

Siow RCM, Sato H, Leake DS, et al. Vitamin C protects human arterial smooth muscle cells against atherogenic lipoproteins: effects of antioxidant vitamins C and E on oxidized LDL-induced adaptive increases in cystine transport and glutathione. *Arterioscler Thromb Vasc Biol* 1998; **18**: 1662-1670.

Wang H, Liu H, Liu R-M. Gender difference in glutathione metabolism during aging in mice. *Exp Gerontol* 2003; **38**: 507-517.

Wu G, Fang Y-Z, Yang S, *et al*. Glutathione metabolism and its implications for health. *J Nutr* 2004; **134**: 489-92.

Zhou J, Austin RC. Contributions of hyperhomocysteinemia to atherosclerosis: Causal relationship and potential mechanisms. *Biofactors* 2009; **35**: 120-129.