Impact of Coffee-consuming Habits on Plasma Biomarkers in a Healthy Adult Population

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Before you begin a thing, remind yourself that difficulties and delays quite impossible to foresee are ahead. If you could see them clearly, naturally you could do a great deal to get rid of them, but you can't. You can only see one thing clearly and that is your goal. Form a mental vision of that and cling to it through thick and thin.

Kathleen Norris, from "Hands full of living"

The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Publications

Abstract and poster

Encarnação, Joana; Poquet, Laure and Williamson, Gary, Free Hydroxycinnamic Acids in Instant Coffee, abstract and poster *V International Conference on Polyphenols and Health*, Sitges, Spain, 2011

Paper

da Encarnação, Joana Amarante; Farrell, Tracy L.; Ryder, Alexandra; Kraut, Nicolai U. and Williamson, Gary, 2014. *In vitro* enzymic hydrolysis of chlorogenic acids in coffee. *Molecular Nutrition & Food Research*, 10-1002/mnfr201400498

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Abstract

Increasing epidemiological evidence for the beneficial health effects of (poly)phenol-rich products has led to a growing interest in the role of (poly)phenols in reducing the incidence of chronic diseases. Coffee is a major contributor to dietary chlorogenic acids. However, the majority of these, unlike free phenolic acids, first need to be cleaved in order to be absorbed and about 70 % of them reach the colon intact where they are processed by the microbiota population.

First, the analysis of major free phenolic acids in five commercially available soluble coffees is described. The influence of roasting and decaffeination and the contribution of free phenolic acids to the appearance of derived metabolites in plasma resulting from a pre-colonic absorption are then assessed. The hypothesis was that, as reported for chlorogenic acids, both roasting and decaffeination would have a negative impact on the quantified compounds. The contribution of free phenolic acids to the appearance of derived pre-colonic metabolites in plasma was hypothesized to be significant, as these are easily absorbed in the gastrointestinal tract. Results indicated that roasting and decaffeination reduce the amount of hydroxycinnamic acids, which in the amounts consumed with a regular coffee beverage do not significantly contribute to the early appearance of derived metabolites. The hydrolysis of 5-*O*-caffeoylquinic acid and 3-*O*-caffeoylquinic acid is a likely major contributing mechanism to the early appearance of derived metabolites in plasma.

In a second stage, the impact of habitual consumption of popular (poly)phenol-rich products on human health and the impact of habitual consumption of coffee on the absorption and metabolism of chlorogenic acids were assessed in an observational human study with 62 healthy adult participants. The health status of the study population was assessed by the quantification of selected biomarkers of health using optimized methods, which were based on published protocols. The major hypotheses in this second section were that the metabolism of the biomarkers was stable over the study period (i.e. max 16 weeks); that subjects with a higher regular consumption of (poly)phenol-rich products have a healthier profile of biomarkers and that subjects with a higher absorption of coffee (poly)phenols have a healthier

profile of biomarkers. A healthy and overall stable metabolism of the quantified inflammation and cardiovascular biomarkers (i.e. total aminothiols, glucose, insulin and uric acid) over a period of at least 8 - 16 weeks was confirmed. Subjects with a higher consumption were more likely to also be higher consumers of other (poly)phenol-rich products. A higher consumption of (poly)phenol-rich products was not associated with a better inflammatory and cardiovascular health profile, but the a higher presence of two colonic metabolites (i.e. vanilloylglycine and feruloylglycine) was associated with higher levels of glutathione. Overall, the results suggested a reduced bioavailability of chlorogenic acids with a higher habitual consumption of coffee including associated (poly)phenols, and encourage future investigations targeting the colonic microbiota populations.

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3, 4-dimethoxycinnamic acid	3, 4-DMCIN
6-phosphogluconate	6-PG
acetic acid	AcOH
N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinone-monoimine	ACSBM
adenosine diphosphate	ADP
anova with repeated measures	Anova RM
4-aminophenazone	APh
apolipoprotein E3	APOE3
adenosine triphosphate	ATP
area under the curve	AUC
buffer/Enzymes/4-aminophenazone reagent	BEA
blank	Bl
total blank	$Blank_{tot}$
body mass index	BMI
blood pressure	BP
caffeic acid	CA
collision energy for the first transition	CE1
collision energy for the second transition	CE2
chlorogenic acid(s)	CGA(s)
confidence interval	CI
carbon dioxide	CO2
coefficient of variation	CV
cysteinylglycine	CysGly
cystamine dihydrochloride	Cysm 2 HCl
D-glucose	D-Glc
5-dichloro-2-hydroxybenzene-sulfonic acid	DHBS
dihydroferulic acid	DHFA
diastolic blood pressure	Diast.

D,L-homocysteine	DL-HCys
ethylenediaminetetraacetic acid	EDTA
enzyme linked immunosorbent assays	ELISA
endothelial nitric oxide synthase	eNOS
electrospray ionisation	ESI
extracellular signal regulated kinase	ERK
ethanol	EtOH
ferulic acid	FA
food frequency questionnaire	FFQ
School of Food Science and Nutrition, University of Leeds	FS&N
glucose-6-phosphate	G-6-P
glucose-6-phosphate dehydrogenase	G-6-PDH
glyceraldehyde	GlyCHO
caffeinated golden roast coffee	GoC
decaffeinated golden roast coffee	GoD
coffee made from 35 % unroasted and 65 % roasted green coffee beans	GrC
hydrogen peroxide	H_2O_2
sulfuric acid	H_2SO_4
health assessment questionnaire	HAQ
hydroxycinnamic acid	HCA
hydrochloric acid	HCl
high density lipoprotein	HDL
hexokinase	HK
high pressure liquid chromatography	HPLC
high pressure liquid chromatography coupled to diode array detection, eectrospray ionisation and mass spectrometry	HPLC-DAD-ESI-QQQ
high pressure liquid chromatography coupled to mass spectrometry	HPLC-MS
hour(s)	h
isoferulic acid	iFA

Interleukin-18	IL-18
Janus kinase 2	JAK2
potassium hydrogen phosphate	K_2HPO_4
potassium dihydrogen phosphate	KH_2PO_4
liquid chromatography-mass spectrometry	LCMS
L-cysteine	L-Cys
low density lipoprotein	LDL
L-glutathione	L-GSH
limit of detection	LOD
limit of quantification	LOQ
caffeinated medium-dark roast coffee	MC
decaffeinated medium-dark roast coffee	MD
Maths, Engineering and Physical sciences committee	MEEC
methanol	MetOH
mass-to-charge ratio of negatively charged molecular ion	$M\text{-}H^-$
deionized millipore water	milliQ
not applicable	n.a.
not determined	n.d.
oxidized nicotinamide adenine dinucleotide	NAD^+
reduced nicotinamide adenine dinucleotide	NADH
nicotinamide adenine dinucleotide phosphate-oxidase	NADPH
sodium fluoride	NaF
sodium acetate	NaOAc
sodium hydroxide	NaOH
nuclear factor-kappa B	NFκ-B
octadodecyl	ODS
oral glucose tolerance test	OGTT
phosphate buffered saline	PBS
(poly)phenolic index	PP-index
polytetrafluoroethylene	PTFE

reagent blank	RB
reagent blank corrected reading	Rbcorr
reactive nitrogen species	RNS
reactive oxygen species	ROS
revolutions per minute	rpm
retention time	Rt
standard and sample blank corrected reading	SBcorr
4-fluoro-7-sulfobenzofurazan	SBD-F
standard deviation	SD
solid phase extraction	SPE
standard and sample blank	SSB
standard and sample blank corrected reading	SSBcorr
standard and sample test	SST
standard and sample test corrected reading	SSTcorr.
Signal transducer and activators of transcription 1	STAT1
systolic blood pressure	Syst.
type 2 diabetes mellitus	T2DM
trichloroacetic acid	TCA
tris (2-carboxyethyl) phosphine hydrochloride	TCEP-HCl
total cysteine	tCys
total cysteinylglycine	tCysGly
total glutathione	tGSH
total homocysteine	tHCys
total ion count	TIC
time after consumption of a product when the metabolites reach maximal concentration in plasma	Tmax
3, 3', 5, 5'-tetramethylbenzidine	TMB
uric acid	UA
volume to volume	v:v
waist-to-hip ratio	WHR

Chapter 1
Literature review and objectives

In the human diet, (poly)phenols represent the most abundant antioxidants and have therefore attracted increasing interest. These plant secondary metabolites are present in all plant-derived foods and beverages (Pandey and Rizvi, 2009). Unlike primary metabolites, (poly)phenols are not essential for photosynthesis, respiration, growth or development of the plant, but they play an important role in its survival, as they are involved in processes such as protection against ultraviolet radiation, infection by pathogens or bacteria, through anti-mutagenic properties, and lignin biosynthesis (Pandey and Rizvi, 2009, Frohnmeyer and Staiger, 2003, Lattanzio et al., 2006). (Poly)phenols are present at a higher concentration in the outer layers and therefore also contribute to the organoleptic properties of the plant (i.e. pigmentation and bitter or astringent taste) (Crozier et al., 2006a). Of particular interest is the study of popular (poly)phenol-rich products such as coffee, tea and cocoa preparations, as well as fruits, vegetables, (poly)phenol-based supplements and some alcohol-containing beverages in terms of their effects on health. The present dissertation will focus on the influence of habitual consumption of (poly)phenol-rich non-alcoholic products, with emphasis on coffee, on human general health. Carefully selected biomarkers are a useful tool to assess a particular health status of a subjects as these are the result from the metabolism and will be used in this context to understand the impact on health of a habitual consumption of (poly)phenol-rich products, with emphasis on coffee.

1.1 Coffee

Formerly consumed as its fruit or cherry for its energizing properties, coffee was later also sold for its believed medicinal qualities, such as clearing out the bowel (Brown et al., 1990). Coffee is now the second most traded commodity in the world after oil and is included amongst the most popular beverages worldwide (http://www.investorguide.com/article/11836/what-are-the-most-commonly-traded-

commodities-igu/), along with water and tea, due to its stimulant properties on the body and mind and increasing public awareness of the bioactive contents of coffee and interest in the health benefits related to its constituent (poly)phenols.

1.1.1 From bean to beverage

Ideal growing conditions for coffee are found between the Tropics of Cancer and Capricorn, where the average annual temperature is about 21°C, the rainfall is abundant and the soils are well drained and nutrient-rich. Although many *Coffea* species have been described, two major species of coffee plant, representing over 60% of the world production, are grown for commercial purposes, namely Robusta (*Coffea canephora*) and Typica (*Coffea arabica*), and to a lesser extent Liberica (*Coffea liberica)* and Excelsa (*Coffea dewevrei*) (www.ico.org).

Coffee beans are contained in a red berry fruit. The first step in coffee processing, after picking, is the isolation of the beans from their involving layers. This can be achieved either using the "wet" or "dry" method. In the "wet" processing method, the coffee beans are sorted in a water-floatation system and sent to a puling machine to remove the outermost epicarp. During fermentation in water, the next layer (mesocarp) is enzymatically dissolved. The beans are then washed and dried in the sun or using mechanical dryers. Endocarp and spermoderm are removed in the final hulling step before storage and shipping. The "dry" processing method is a more economical option, as beans are usually stripped all at once, collected from the ground and sundried before the removal of the hull and most of the silverskin or testa by a coffee huller.

The different stages of coffee processing will influence the chemical composition of the beans, and consequently, the final flavour. Decaffeination, the removal of caffeine, however never absolute (≤ 0.1 % residual caffeine), is one of the processing steps of coffee performed on the unroasted green beans (www.ico.org). Four major commercial decaffeination methods exist and briefly consist of water, ethyl-acetate, methylene chloride and carbon dioxide decaffeination methods. In a water decaffeination, the coffee beans are immersed in a hot water saturated with water-soluble aromatic coffee components to minimize the loss of the

aroma. The saturated water is then passed through a filter of activated carbon that retains the extracted caffeine. The ethyl-acetate (also referred to as "natural decaffeination") and methylene chloride decaffeination methods are comparable, as in both methods, the coffee beans are first softened in hot water or steam. In this process, caffeine and a fraction of the oils and flavour are extracted. The recirculation of ethyl-acetate or methylene chloride in the extracting water removes the caffeine and allows the coffee beans to re-equilibrate and take up the lost aromatics and oils. Finally, in the decaffeination by carbon dioxide, carbon dioxide is added to the extracting water in its supercritical state (pressure of up to 250 atm). Subsequently, caffeine is selectively eliminated with the removal of carbon dioxide. This method does not affect the other coffee constituents, but it is also costly and only worthy for important quantities of coffee (http://antoine.frostburg.edu/chem/senese/101/consumer/faq/decaffeinating-coffee.shtml).

1.1.2 Composition and biologically active components

There are variations in the amounts of chemical contents between *C. arabica* and *C. robusta*, but both have the same complex mixture of compounds. As the green beans undergo processing (e.g. storage and roasting), the original chemical profile of coffee changes as a result of transformation or even progressive destruction of certain components (Ludwig et al., 2014). Roasting particularly affects the constituent profile of coffee, with the production of compounds resulting from the Maillard reaction (Ludwig et al., 2014). Coffee has been reported to contain more than a thousand chemicals, including carbohydrates, vitamins, lipids, amino acids, non-protein nitrogenous compounds, minerals, the diterpenes kahweol and cafestol, alkaloids (e.g. caffeine) and phenolic compounds which are part of the bioactive compounds of coffee and possess various effects on health (Spiller, 1998, Ludwig et al., 2014). The compounds described below constitute only a small fraction of the chemical composition of coffee.

1.1.2.1 Caffeine

The major naturally occurring bioactive constituent of coffee beans, and probably the most well known, is caffeine or 1,3,7-trymethylxanthine. This purine is distributed in the coffee pulp and found free in the cytoplasm of the seed or bound to the cell wall. On a dry basis, there is about twice as much caffeine in *C. robusta* (1.7 - 4.0%) as in *C. arabica* (0.8 - 1.4%). At the concentrations reached in plasma after coffee consumption (i.e. 20 - 40 µM), the principal pharmacological actions of caffeine are, due to structural similarities (Figure 1.1), the antagonism of A_1 and A_{2a} subtypes of the adenosine receptor (Fisone et al., 2004) with the main metabolic activities being the stimulation of the central nervous system and increase of the blood pressure, metabolic rate and diuresis (Carrillo and Benitez, 2000). Relatively recent findings also suggested a beneficial effect of this alkaloid on type 2 diabetes mellitus (T2DM) and obesity (Westerterp-Plantenga et al., 2006, Heckman et al., 2010). Caffeine is rapidly and almost fully absorbed in the stomach and small intestine to then be distributed to all tissues. When ingested at higher doses, the clearance of caffeine is however decreased and the usual 3 - 6 hour half-life is extended (Heckman et al., 2010). Only about 1% of the caffeine is directly excreted in urine without being metabolized (Clifford, 1985), the rest of the caffeine is metabolized in the liver to form three major metabolites, namely 1,7-, 3,7-, and 1,3-dimethylxanthine, or paraxanthine (84%), theobromine (12%) and theophylline (4%), respectively. These will in turn be further metabolized.

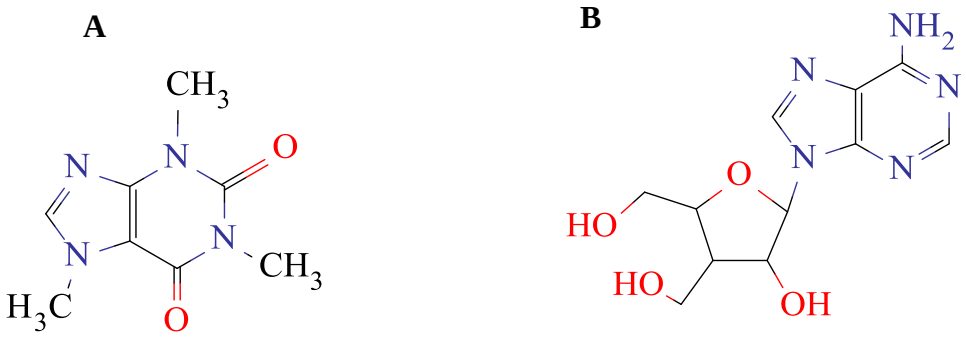


Figure 1.1: Structural similarity between caffeine (a) and adenosine (b). Caffeine binds to the adenosine receptors A_1 and A_{2a} and acts as an inhibitor of these.

1.1.2.2 Diterpernes

Cafestol and kahweol are two diterpenes that can be isolated from coffee oil and identified as the cause of reported elevation of total and LDL cholesterol in serum (Urgert and Katan, 1997). This increase is, however, attenuated when the consumed coffee is filtered, as most of the cafestol and kahweol are retained (Jee et al., 2001). Aiming at understanding the molecular mechanisms behind the cholesterol-raising effect of cafestol consumption, APOE3 Leiden mice, a well-recognized model used for patients with diet-induced hyperlipidemia, were used in a study carried out by Post et al. (2000). In this study, cafestol was shown to act by suppressing the synthesis of bile acids, which are essential for the regulation of dietary lipids and lipophilic vitamins. Besides their cholesterol-raising effect, cafestol and kahweol have the property of decreasing mutagenesis and tumorigenesis in animal models (Arab, 2010).

1.1.2.3 Trace elements

Although micronutrients play key roles in the physiology of plant and animal organisms, these are not secondary metabolites and are therefore not categorised as bioactive compounds. Trace elements, such as minerals, are important in plant nutrition, and sometimes in the defence against disease (Clifford, 1985). In animal organisms, whether they are part of a catalytic centre and facilitating the conversion of substrates to end products (e.g. copper), whether they donate or accept electrons in redox reactions, whether they bind, transport and release oxygen in the body (e.g. iron) or whether they are of structural significance in the stabilization of the tertiary structure of biological molecules (e.g. zinc), micronutrients play an important role in maintaining a healthy and equilibrated metabolism (Shenkin, 2006). Micronutrients consumed through coffee could therefore also contribute to these health aspects in humans.

1.1.2.4 (Poly)phenols

(Poly)phenols are characterized by several hydroxyl groups on aromatic ring(s), but their chemical structure is variable and can reach high levels of complexity (D'Archivio et al., 2007). Their classification is based upon the number of phenol rings and the structural elements holding these together. Five main classes, namely phenolic acids, flavonoids, stilbenes, lignans and phenolic alcohols are recognized and each of them can be subdivided into sub-classes. Of particular interest in the present project are hydroxycinnamic acids, a sub-class of phenolic acids that is abundantly, but not exclusively, present in coffee (Clifford, 1999). The most common hydroxycinnamic acids are caffeic, ferulic, *p*-coumaric, dihydrocaffeic, isoferulic, dihydroferulic and 3, 4-dimethoxycinnamic acid (Figure 1.2, A). In nature and unprocessed foods, hydroxycinnamic acids most often appear as their esters with quinic acid, referred to as chlorogenic acids. An increasing number of chlorogenic acids has been reported (Clifford, 1985, Clifford et al., 2006), but the ones of major interest for the present project chiefly consist of caffeoylquinic acids (i.e. 3-, 4- and 5-*O*-caffeoylquinic acid) (Figure 1.2, B), which can represent > 70 % of the total chlorogenic acids in coffee (Stalmach et al., 2010). The remaining (poly)phenolic fraction is mainly represented by feruloyquinic acids (i.e. 3-, 4- and 5-*O*-feruloylquinic acid), caffeoylquinic acid lactones (i.e. 3- and 4-*O*-caffeoylquinic acid lactone), dicaffeoylquinic acids (i.e. 3, 4-, 3, 5- and 4, 5-dicaffeoylquinic acid) and *p*-coumaroylquinic acids (i.e. 4- and 5-*O*-*p*-coumaroylquinic acid) (Stalmach et al., 2010). The chlorogenic acid composition of coffee is influenced by the species and cultivars (Clifford, 1985), as well as by processing (Crozier et al., 2006b). On a dry matter basis, green coffee beans contain about 5-10% chlorogenic acids (Ky et al., 1997), but a progressive destruction and modification of the chlorogenic acid profile is observed upon roasting (Ludwig et al., 2014). Despite this loss, the amounts of chlorogenic acids present in a regular cup of coffee are significant, and for regular coffee consumers, the daily chlorogenic acid intake can reach 1 g (Stalmach et al., 2009). Stalmach et al. quantified, in a 200 mL coffee beverage prepared from the solubilization of 3.4 g coffee pellets, a chlorogenic acid content of 146 mg, of which 65 % were caffeoylquinic acids (Stalmach et al., 2009). Upon analysis of several espresso preparations, Crozier et al. reported a content of total

caffeoylquinic acids ranging from 24 to 422 mg for an espresso beverage of 23 to 70 mL (Crozier et al., 2012).

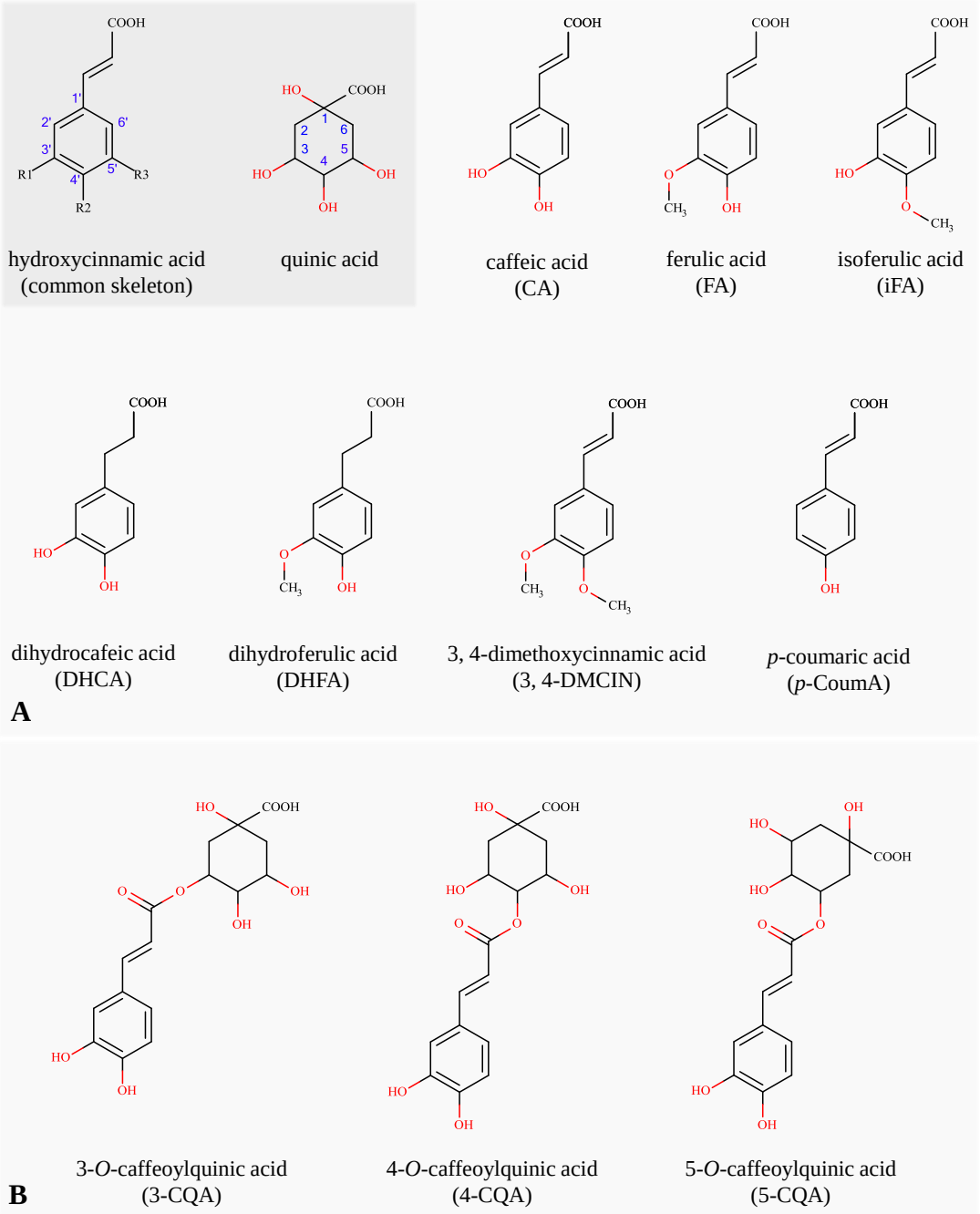


Figure 1.2: Major free hydroxycinnamic acids (A) and chlorogenic acids (B) present in coffee. Hydroxycinnamic acids share a common C6-C3 skeleton structure (structure shown in A). Free hydroxycinnamic acids are mostly present as their ester with quinic acid (structure shown in A), referred to as chlorogenic acids.

1.1.3 Metabolism of chlorogenic acids

Once ingested, chlorogenic acids are exposed to different environments in the digestive system. In the mouth, the combined action of mastication, enzymes present in the saliva and bacteria contributes to the release of (poly)phenols from the food matrix and to their bioaccessibility (Bohn, 2014, Alminger et al., 2014). In the stomach, the low pH of 2 - 4, the gastric enzymes, such as pepsin, and the peristaltic movements further reduce the size of the digest and increase the bioaccessibility of (poly)phenols. In an *in vitro* model of gastric epithelium, upon incubation of coffee solutions (9, 18 or 27 mg/mL) under conditions mimicking the human gastric compartment, an increase in isoferulic, ferulic and dimethoxycinnamic acids, resulting from the hydrolysis of chlorogenic acids, as well as passive and facilitated absorptions of intact chlorogenic acids were observed (Farrell et al., 2011) (Figure 1. 3), suggesting a possible absorption of these compounds in the stomach *in vivo*. In the small intestine the peristaltic movements remain present, however, the pH increases to approximately pH 7, which provides a favourable environment for the enzymes present in pancreatic and bile secretions. By comparing the excreted chlorogenic acid-derived urinary metabolites in ileostomy participants and in subjects with an intact colon, it was possible to assess that about 30 % of the ingested chlorogenic acids are absorbed before reaching the colon, while the remaining 70 % passes to the colon and are exposed to the microbiota activity (Stalmach et al., 2009, Stalmach et al., 2010). Microbiota are important constituents of the gastrointestinal tract and are essential for a balanced metabolic and immune system (Marín et al., 2014, Zoetendal and de Vos, 2014). As the gastrointestinal tract progresses, the microbiota population increases and reaches 10^{12} cells per gram of content in the colon (Sekirov et al., 2010), where these will hydrolyze the glycosides into aglycones, allowing absorption.

Chlorogenic acids and other (poly)phenols are perceived as xenobiotics and are metabolised as such by the human organism. Once absorbed, the compounds are subject to a phase I and II metabolism. Phase I renders the compounds more hydrophilic, by reduction, oxidation, hydrolysis or hydration, thus facilitating the urinary and biliary excretion of the compound (Manach et al., 2004, Bohn, 2014). Phase II metabolism consists mainly of sulfation (by sulfurotransferase, SULT),

methylation (catechol-*O*-methyl transferase, COMT) and glucuronidation (glucuronosyltransferase, UGT) conjugation reactions and further facilitates the excretion. Although the liver is the major organ for phase II metabolism, conjugation by glucuronosyltransferases and sulfurotransferases also occurs in the small intestine where these enzymes have been detected (Manach et al., 2004). A schematic representation of the metabolism and absorption of major coffee chlorogenic acids as suggested by Farrell et al., Stalmach et al. and da Encarnação et al. is shown in Figure 1. 3.

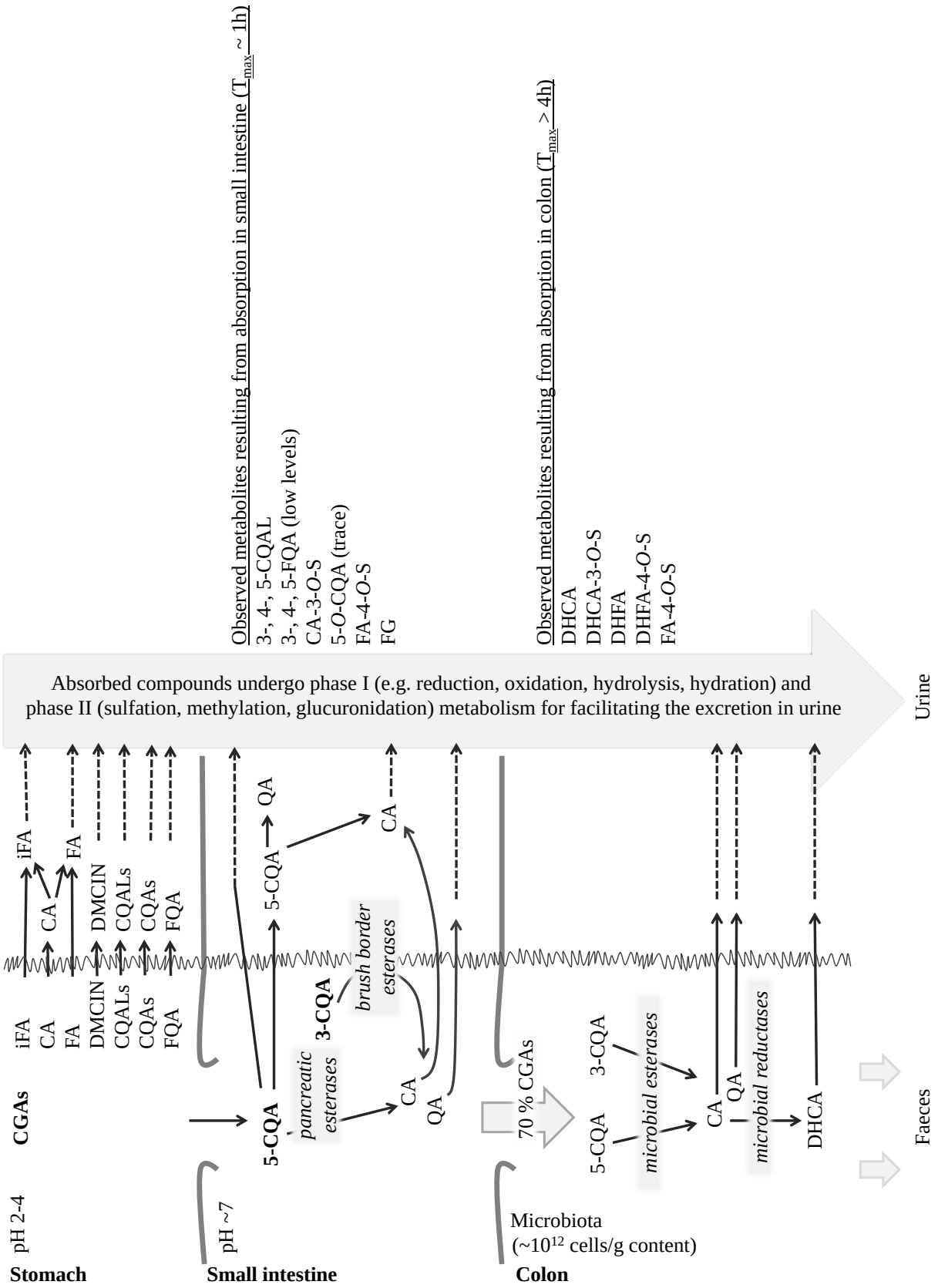


Figure 1. 3: Metabolism and absorption of chlorogenic acids, as suggested by Farrell et al. (2011) and metabolism and absorption of 3- and 5-caffeoylquinic acid in the small and large intestine, as suggested by Stalmach et al. (2010) and da Encarnação et al. (2014).

In order to have any physiological effect (bioefficacy), a bioactive compound first needs to be absorbed and enter the circulation (bioavailable), which, however, does not imply that a compound with high bioavailability will necessarily have a high bioefficacy and vice versa. As previously seen, although chlorogenic acids are abundant in coffee, these have difficulty when passing the epithelial barrier of the gastrointestinal tract in their native form. Free hydroxycinnamic acids pass the epithelial barrier much more easily, and therefore, these aglycones of chlorogenic acids, as well as chlorogenic acid derivatives are more likely to be the compounds which have a higher impact on the metabolism. Although *in vitro* studies are important to understand the molecular aspects of the metabolism of (poly)phenols, they cannot replace human studies and in order to understand the impact of habitual consumption of (poly)phenol-rich products on human health, bioavailability studies are important as these include the analysis of plasma, urine and faecal samples upon consumption of a studied product. The analysis of metabolites in these biological samples improves the understanding of the metabolism. Specifically, in pharmacokinetic studies, the metabolites resulting from a pre-colonic or colonic absorption can be identified. Typically, compounds that are absorbed before the colon (e.g. caffeic, ferulic, isoferulic and 3,4-dimethoxycinnamic acid) appear in plasma approximately up to 3 hours (T_{max} = 0.5 to 2.0 hours) upon consumption of a product, while compounds absorbed in the colon (e.g. dihydrocaffeic, dihydroferulic and free hydroxycinnamic acids resulting from the catabolism of chlorogenic acids) appear in plasma approximately 4 hours (T_{max} = 4.8 to > 12 hours) after consumption of a product (Renouf et al., 2010b, Williamson et al., 2011).

1.2 Coffee and other (poly)phenol-rich products and health

(Poly)phenols have been associated with a decreased risk of some diseases such as cancer, cardiovascular diseases and other chronic diseases (Scalbert and Williamson, 2000). Many earlier conclusions were, however, based on animal or *in vitro* studies, and although this initial research is important to understand the underlying molecular mechanisms of action, the outcomes are difficult to extrapolate to human metabolism. Weichselbaum et al. (2010) reviewed that *in vitro* studies have either shown or suggested several processes that could contribute to the

antioxidant properties of (poly)phenols. (Poly)phenols, such as flavonoids (Garcia-Lafuente et al., 2009) or phenolic acids (i.e. caffeic acid) (Li et al., 2005) have also been shown to improve endothelial function and several epidemiological studies have demonstrated that diets enriched with tea, a flavonoid-rich product, were associated with a lower risk of myocardial infarction, whether in case-control or cohort studies (Peters et al., 2001).

As effective inhibitors of LDL oxidation, certain (poly)phenol-rich products have the potential to reduce the risk of developing certain chronic conditions such as atherosclerosis, and later cardiovascular disease. Aviram et al. (2000) reported upon conduction of an *in vitro* and *ex vivo* study, that pomegranade juice, rich in flavonoids, inhibited peroxidation in plasma and isolated LDL and HDL, *in vitro* and *in vivo*. Inactivation of free radicals, reduction of the oxidative potential of metal ions, inhibition of pro-oxidant enzymes, induction of antioxidant enzymes and possibly the protection of other antioxidants such as ascorbic acid are other potential mechanisms of indirect action that could further contribute to the antioxidant status of (poly)phenols *in vivo***.** Iwai et al. (2004) investigated the *in vitro* antioxidative effects of seven hydroxycinnamoyl derivatives extracted from green coffee beans, namely, 3-, 4- and 5-cafeoylquinic acid, 5-feruloyquinic acid and 3, 4-, 3, 5-, and 4, 5-dicafeoylquinic acid and their ability of inhibiting tyrosinase (Iwai et al., 2004). The free radical and superoxide anion radical scavenging activity was the highest for the dicaffeoylquinic acids, followed by the caffeoylquinic acids and 5-feruloylquinic acid. Similarly, the tyrosinase inhibitory activitiy was the highest for the dicaffeoylquinic acids, but 5-feruloylquinic acid had a stronger inhibition that the caffeoylquinic acids tested. *In vitro* studies frequently test native compounds and as earlier mentioned, the most abundant native forms might not be the most biological active ones and it is more likely that their metabolites are the compounds with the highest impact on the metabolism (Manach et al., 2004, Kroon et al., 2004).

A few mechanisms of action of chlorogenic acids and phenolic acids have been suggested and some of them are described below. In 2000, Hsu et al. (2000) tested caffeic acid for its antihyperglycemic effect, in male Wistar rats. Upon intravenous injection of caffeic acid (0.5 - 3 mg/kg) into two models of diabetic rats (i.e. streptozotocin-induced and insulin-resistant), the glycaemia decreased in a dose-dependent manner, suggesting an insulin-independent action of caffeic acid. This

was not observed in normal non-diabetic rats. When the diabetic rats were pre-treated with caffeic acid (1 mg/kg) and thereafter subject to a glucose challenge test (i.e. intravenous injection of glucose, 60 mg/kg), the elevation of plasma glucose was attenuated. Further investigation indicated that the uptake of glucose by isolated adipocytes was increased in a concentration-dependent manner, suggesting that this would be a plausible mechanism responsible for the lowering of plasma glucose by caffeic acid. 5-Caffeoylquinic acid was reported to have a similar mechanism of action. In an *in vitro* experiment conducted by Alonso-Castro et al**.** (2008), 5-caffeoylquinic acid stimulated, without affecting adipogenesis, the uptake of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose in insulin-sensitive and insulin-resistant murine adepocytes. The uptake of glucose was stimulated by 404 % in insulin-sensitive adipocytes in presence of insulin, by 176 % in insulin-sensitive adipocytes in the absence of insulin, and by 141 % in insulin-resistant adipocytes in the absence of insulin. In the same year, Tousch et al. (2008) reported in an *in vitro* experiment a stimulation of glucose intake by 3-caffeoylquinic acid and chicoric acid in the rat skeletal muscle L6 cells, in presence of insulin. In the same research, 3-caffeoylquinic acid and chicoric acid stimulated the secretion of insulin from the pure β-cells (INS-1E cell line) and from isolated Langerhans islets.

Rodriguez de Sotillo et al. (2006) investigated in the obese Zucker (*fa*/*fa*) rat model (hepatic insulin-resistant), the *in vivo* effect of a 3-week treatment with 3-caffeoylquinic acid (3-CQA) infused daily intravenously (5 mg/kg), on plasma insulin concentrations, liver protein and DNA concentrations, the activity of hepatic glucose-6-phosphatase and the expression of hepatic insulin receptor mRNA. In Zucker (*fa*/*fa*) rats, the plasma glucose levels after a glucose challenge or meal progressively increase with age and the fasting levels increase at fast, after the fourth week of age. In the experiments of Rodriguez de Sotillo et al., the plasma glucose level, 1 h after a glucose challenge, was attenuated in 3-CQA-treated 22 week-old rats, when compared to the same group of rats at 19 weeks of age, prior to the 3-CQA treatment, suggesting an insulin-sensitivity enhancement by 3-CQA. The fasting levels of glucose were, however, similar between 3-CQA-treated 22 week-old rats and the untreated rats of 12, 19 or 22 weeks of age. After 30 min of a glucose tolerance test, the 3-CQA 22 week-old Zucker (*fa*/*fa*) rats had lower plasma insulin levels, when compared to the same group at 19 weeks of age. At fast, in 22

year-old Zucker (*fa*/*fa*) rats treated with 3-CQA the fasting insulin levels were lower, when compared to the same group at 19 weeks of age and to the untreated rats of 22 weeks of age. In 22-week old fasted Zucker (*fa*/*fa*) 3-caffeoylquinic acid-treated rats, the concentration of total protein and DNA increased, when compared to the untreated control group of the same age. The hepatic synthesis of protein is stimulated by insulin. In obese T2DM insulin-resistant animal models, the hepatic protein synthesis is lower. The increase of protein reported in the study by Rodriguez de Sotillo et al. could therefore be due to an increased liver sensitivity to insulin. The DNA increase could be due to the higher zinc and magnesium concentrations, which are important for the synthesis of DNA. The activity of glucose-6-phosphatase was unchanged between the 3-CQA-treated 22-week old rats and the untreated control group of the same age. Although the administered amounts of 3-CQA are plausible in a human diet, the compound was administered intravenously. Once ingested, the majority of chlorogenic acids are hydrolysed from their quinic acid moiety in order to be absorbed and enter the circulation, therefore, the circulating compounds are in majority derived metabolites. The observed effects of 3-CQA may therefore not be significant with a regular diet. A further action of 3-CQA was the inhibition of glucose-6-phosphatase reported by Hemmerle et al. (1997).

In an *in vitro* model, Li et al. (2005) tested the effect of caffeic acid on the angiotensin II-induced proliferation of aortic vascular smooth muscle cells from spontaneously hypertensive rats and from healthy Wistar-Kyoto rats. When compared to the angiotensin II (100 nM) only treatment, an inhibition of intracellular superoxide anion production was observed when the muscle cells of the hypertensive rats were simultaneously exposed to angiotensin II (100 nM) and caffeic acid (100 μM). Also, the tyrosine phosphorylation of Janus kinase 2 (JAK2) stimulated by angiotensin II was inhibited by the simultaneous treatment with caffeic acid, with an optimal inhibition when caffeic acid was used at > 50 μM. Similarly, the tyrosine phosphorylation of STAT1 (α/β) stimulated by angiotensin II was significantly repressed when caffeic acid was added to the treatment. A further effect of caffeic acid was observed on the extracellular signal regulated kinase (ERK) cascade. The phosphorylation of ERK (1/2) observed upon angiotensin II treatment was significantly inhibited when the cells were exposed to caffeic acid.

Finally, the expression of heat shock protein 90, which responds to oxidative stress and is involved in cell proliferation, was stimulated by angiotensin II, which effect was antagonised by the addition of caffeic acid. Of note, the results were less marked in cells of normotensive rats and the caffeic acid concentration used in this study, i.e. 100 μM, were much higher than that observed in the circulation, i.e. 0.16 μM, after the consumption of an instant coffee prepared from 8 g of coffee pellets (Renouf et al., 2013).

In spontaneous hypertensive rats, a reduction of the systolic blood pressure was observed after a single-dose and continuous 8-week administration of approximately 300 mg/kg weight 5-caffeoylquinic acid (Suzuki et al., 2006). This concentration is much higher than that achievable with a normal healthy diet. When administered as a single-dose, 30 mg/kg did not significantly decrease the systolic blood pressure. Associated with the decrease in systolic blood pressure upon the 8-week treatment with 300 mg/kg 5-caffeoylquinic acid, was a decreased production of NADPH-dependent superoxide in the aorta and the inhibition of $p22^{phox}$ gene expression. A major limitation in this study is the administrated dose representing approximately 20 times that of what is estimated to be consumed by humans for the combined chlorogenic acids and therefore, although those benefitial effects were observed, a decreased dose may not result in significant effects.

In a recently published research, Sompong et al. (2015) reported the effect of ferulic acid on protein glycation, lipid peroxidation, membrane ion pump activity and phosphatidylserine exposure in young healthy human erythrocytes, which are important for the transport of oxygen and the elimination of carbon dioxide, exposed to high glucose levels. Glycated haemoglobin was used as an indicator of protein glycation. When compared to the glucose (45 mM) only treatment, the addition of ferulic acid (10 and 100 μM) significantly decreased the measured amounts of glycated haemoglobin by approximately 15 %. At the same concentrations, ferulic acid increased the utilization of glucose by erythrocytes, when compared to erythrocytes subject to the glucose treatment only and it partly reversed the inhibitory effect of glucose (45 mM) on the Na^+/K^+-ATPase activity. When the lipid peroxidation was investigated, a concentration as low as 0.1 μM ferulic acid had significant effects on lowering the lipid peroxidation. The strongest decrease was, however, observed with a concentration of 100 μM ferulic acid. At the same

concentrations (i.e. 0.1, 1, 10 and 100 μM), ferulic acid decreased phosphatidylserine exposure into the outer layer of the erythrocyte membrane, when compared to the glucose (45 mM) only treatment. These data suggest mechanisms on how ferulic acid may lower the circulating glucose levels and prevent cellular dysfunction and vascular complications associated to diabetes.

1.2.1 Fruits and vegetables and health

In 2007, He et al. (2007) carried out a meta-analysis of 13 prospective cohort studies on the effect of fruit and vegetable consumption on the risk of developing coronary heart disease. The median follow up was 11 years and analyses included data from 278,459 adult subjects. Overall, upon adjustment for possible confounding factors (i.e. some anthropometric and physiological variables, lifestyle habits and post-menopausal status), although some studies did not show the same trend, the combined relative risk of the 13 studies indicated a decreased risk of coronary heart disease with a higher consumption of fruit and vegetables. He et al. reported a 17 % decrease in risk for an increase in consumption from < 3 to > 5 servings per day. The outcome was similar in four studies, in which the risk of non-fatal myocardial infarction was also investigated. As for all the meta-analyses, even if the measurements were adjusted for potential confounders, the studies did not adjust for the same counfounders, which could influence the outcome of the individual researches.

Three years later, Carter et al. (2010) carried out a meta-analysis of six prospective cohort studies to investigate the effect of fruit and vegetable consumption on the incidence of T2DM, a strong independent risk factor for cardiovascular disease. All the included studies adjusted for potential confounding factors (e.g. age, BMI and family history of T2DM), although not all for the same. The analysis revealed no significant decrease in the incidence of T2DM with increased consumption of either fruit or vegetables, or a combined consumption of fruit and vegetables, but a 14 % lower incidence of T2DM was observed with higher consumption from 0.2 to 1.35 servings per day of green leafy vegetables. Of note, only two of the analysed studies included male subjects and both indicated a beneficial effect of fruit and vegetable consumption on the incidence of T2DM,

therefore the outcome may have been different if more men had been part of the investigation.

In both He et al. and Carter et al.'s analyses, the consumption of fruit and vegetables was positively associated with physical activity. The latter was not taken into account in the adjusted analyses and therefore a different outcome cannot be excluded if this factor was corrected for.

More recently, Wang et al. (2014) carried out a meta-analysis of 16 prospective cohort studies to investigate the link between the consumption of fruit and vegetables and death from all causes, from cardiovascular diseases or from cancer. In this review, the combined number of subjects was 833,234, of which 56,423 had passed away from either a cardiovascular disease (n = 11,512) or from cancer (n = 16,817). The adjusted analyses indicated a curvilinear relationship between the combined consumption of fruit and vegetables and the risk of death from all causes, with estimated hazard ratios decreasing from 0.92 for 1 serving per day, to 0.74 for 5 servings per day, when compared to no consumption of fruit and vegetables. No further improvement was reported for > 5 servings per day. Similarly for their separate consumption, a higher consumption of fruit or vegetables was associated with a decreased risk in death from all causes, up to 2 and 3 servings per day, respectively. Specifically, there was a 4 to 5 % risk reduction of death from cardiovascular disease for each additional fruit and/or vegetables serving per day. As for the risk of death from cancer, the influence of a higher consumption of fruit and vegetable was not significant.

In regards to other health conditions, such as cancer, the intake of fruit and vegetables has been consistently inversely associated with renal cell cancer (Arab, 2010).

1.2.2 Tea and health

In a prospective cohort study, van Dieren et al. (2009) examined the effect of tea (mainly black) and coffee (section 1.2.3.1) consumption on the incidence of T2DM. The study included 38,176 female and male participants and had a mean follow-up of 10 years. Van Dieren et al. reported a negative relationship between the consumption of tea and the incidence of T2DM in a multivariate model which was

also adjusted for the consumption of coffee. The lowest hazard ratio was obtained for a consumption of > 5 cups per day, which is relatively high. When the relationship between the consumption of tea and biomarkers of T2DM was investigated in a smaller random study subgroup (n = 2,604), no significant association was detected. A major limitation of the study was that no distinction was made between the consumption of black tea or green tea and that the consumption of the latter was assumed to be 4.6 % based on the country statistics.

In a later prospective cohort study, De Koning Gans et al. (2010) investigated the association between the consumption of tea (mainly black) and coffee (section 1.2.3.4), and cardiovascular diseases, with data from 37,514 healthy adult participants obtained during a mean follow-up of 13 years. Upon adjustment for the age, the gender and other potential confounders (e.g. physical activity, waist circumference, smoking status and alcohol intake), De Koning Gans et al. reported a negative linear association between the consumption of tea and the incidence of coronary heart disease, and a U-shaped relationship with the death from coronary heart disease, with the lowest hazard ratios obtained for 1 to 6 cups consumed per day. No association was detected between the consumption of tea and the incidence of stroke or associated death. As for the study of van Dieren et al., the consumption of black tea was assumed to be similar to that of the general population. A further important limitation of this study is the low number of subjects that died from coronary heart disease (n = 123) or stroke (n = 70), reducing the power of the analyses. These analyses, however, are suggestive of a beneficial effect of a long-term consumption of tea in the incidence of coronary heart disease and associated death.

A meta-analysis by Yang et al. (2014) regarding the effect of different *Camellia sinensis* tea preparations on the incidence of T2DM, was recently published. Twelve cohort studies with a combined total of 761,949 subjects were included in the analysis. When compared to subjects consuming no tea or < 0 to 1 cup per day, those consuming ≥ 4 cups per day had a 16 % lower relative risk. When the effect of the consumption of ≥ 3 cups per day on the incidence of T2DM was investigated, the combined result of nine eligible studies indicated a significant beneficial effect, with a 16 % reduction of relative risk.

1.2.3 Coffee and health

In 1985, very little research had been performed on the impact of coffee (poly)phenols or chlorogenic acids on health. The investigations mainly targeted caffeine due to its known pharmacological activities (Clifford, 1985). Although the outcomes of *in vitro* and *in vivo* studies are variable and some studies indicate a detrimental effect on human health (e.g. increase in plasma lipids and/or blood pressure), there is increasing epidemiological evidence for the beneficial effects of regular coffee consumption on the incidence of certain life-threatening chronic and degenerative diseases. Coffee consumption may thus reduce the risk of developing T2DM (Oba et al., 2010), cardiovascular diseases (Mineharu et al., 2011) and certain cancers (Arab, 2010).

1.2.3.1 Type 2 diabetes mellitus

In a cross-sectional epidemiological study with 7,949 initially healthy subjects of both genders, Agardh et al. (2004) investigated the association between the consumption of coffee, the incidence of T2DM and impaired glucose tolerance. Upon adjustment for potential confounders, Agardh et al. reported a decreased relative risk of T2DM and impaired glucose tolerance in healthy subjects with a higher consumption of coffee. In diabetic subjects consuming ≥ 5 cups per day, the relative risk of insulin resistance and of a low β-cell function were decreased, when compared to subjects consuming ≤ 2 cups per day. In subjects with an impaired glucose tolerance, a higher consumption of coffee was associated with a decreased risk of insulin resistance. Additionally, in women with impaired glucose tolerance, but not in men, a decreased risk of low β-cell function was also reported. Due to the cross-sectional design of the study, it is difficult to establish the direction of the causality of associations.

In a later small-scale study of 11 healthy male subjects, an oral glucose tolerance test (OGTT) was performed 1 hour upon consumption of either a caffeine capsule, caffeinated coffee, decaffeinated coffee or a placebo dextrose capsule. The results revealed the highest insulin sensitivity index (indicative of a better response of glucose to insulin) when the same male subjects consumed decaffeinated coffee, when compared to caffeinated coffee and caffeine capsules, suggesting that coffee

compounds, other than caffeine, must account for this observation (Battram et al., 2006). This study, however, only reflect the acute effect of these compounds and the long-term effect may not have a significant effect on the insulin sensitivity. In a later cross-over study with T2DM patients, the insulin sensitivity index upon an OGTT was similar after consumption of caffeinated coffee and decaffeinated coffee (Krebs et al., 2012). In the latter study, the number of participants was very low (n = 18), no adjustment for potential confounders was done and these observations would only apply for T2DM patients. A further OGTT study showed lower fasting glucose levels in subjects consuming ≥ 5 cups per day, when compared to irregular coffee consumers. An inverse association between the habitual consumption of coffee and glucose intolerance was also identified, suggesting that a habitual consumption of coffee may improve the fasting and postprandial glycaemia levels (Yamaji et al., 2004). Limitations of the study include the fact that only male subjects from one ethnicity were recruited and, as previously mentioned, that the cross-sectional nature of the study does not allow the determination of causality of the detected associations. Finally, the Insulin Resistance Atherosclerosis Study, an epidemiological cross-sectional study with 954 healthy adults showed that regular consumption of caffeinated coffee was positively associated with insulin sensitivity and inversely to 2 hour post-load glucose (Loopstra-Masters et al., 2011).

In the previously mentioned prospective cohort carried out by van Dieren et al. (2009), a U-shaped relationship between the consumption of coffee (adjusted for the consumption of tea) and the hazard ratios of T2DM was observed. The lowest hazard ratio was obtained for a consumption of 4.1 to 6.0 cups per day. When the effect of the consumption of decaffeinated coffee was investigated, no association was found. When the relationship between the consumption of coffee and biomarkers of T2DM was investigated, no significant association was detected. The inclusion of blood pressure, the intake of magnesium, potassium and caffeine in the adjusted model did not significantly change the outcome, suggesting that other components in the coffee may explain the negative relationship observed.

Recently, Bhupathiraju et al. (2014) investigated the influence of a change in coffee consumption, over a period of 4 years, on the risk of T2DM in the subsequent 4 years. The data used were obtained from three cohort studies which included both

female and male participants (n = 123,733). An increase in coffee consumption by > 1 cup per day over a 4-year period was associated with a 11 % decrease in the incidence of T2DM in the subsequent 4 years (BMI- and weight-adjusted), when compared to an unchanged consumption of coffee. This decreased risk persisted in the subsequent 12 and 16 years. Inversely, an equivalent decrease in coffee consumption was associated with a 17 % rise in the incidence of T2DM in the subsequent 4 years, but not on a longer term. A similar trend was observed when the consumption of caffeinated coffee was taken into account. A change in the consumption of decaffeinated coffee was not associated with a higher or lower incidence of T2DM, however, the authors claim that the number of subjects that modified their consumption of decaffeinated coffee was very little and therefore the possibility of not detecting a true association may have been present. Also, the adjustment for the waist-to-hip ratio, which was not measured, would have been a better adjustment for the body fat, than the BMI. A further limitation of the study was that the information of the dietary habits was assessed once every fourth year, thus not taking into account any dietary change during the gap.

Overall, while some studies have shown no association between the consumption of coffee and the incidence of T2DM (Reunanen et al., 2003), there is increasing evidence supporting a beneficial effect of a long-term consumption of coffee and the incidence of T2DM (Salazar-Martinez et al., 2004, van Dam and Hu, 2005). Whether caffeine is the cause for the beneficial effect on the glucose-insulin homeostatic system has been a matter of discussion. While it has been recognised that caffeine can improve insulin sensitivity (Yoshioka et al., 2003) and enhance its secretion (Shi, 1997), in a study where decaffeinated and caffeinated coffee consumers were followed, the reduction of the risk was not as significant for decaffeinated coffee consumers, but was still present, suggesting that compounds other than caffeine may also contribute to the protective effects on developing T2DM (Salazar-Martinez et al., 2004). Mechanisms responsible for a reduced risk of developing T2DM could include a reduction of glucose production during gluconeogenesis and glycogenolysis by the inhibitory competition of chlorogenic acid on G-6-phosphate translocase protein (Arion et al., 1997), a reduction of glucose absorption in the small intestine by chlorogenic acid and other coffee

phenolic compounds (Welsch et al., 1989), an increase in the energy expenditure as well as weight loss caused by caffeine.

1.2.3.2 Inflammation

In a normal physiological situation, inflammation is a favourable response of the body to external stress or tissue damage for self-protection and will lead to restoration of a tissue's integrity and function and defence against the infection. A persisting status, however, can have pathological consequences and result in the development of several conditions such as type 1 and type 2 diabetes, atherosclerosis or several neurodegenerative diseases (Nathan, 2002). During inflammation, an increase in superoxide (O_2^-) is observed as a result of the NADPH oxidase activation, in the mitochondrial electron transport chain. In parallel, the induction of the nitric oxide synthases produces the nitric oxide radical (•NO) and subsequent reactive nitrogen species. O_2^- and •NO can lead to further reactive oxygen and nitrogen species respectively. Inflammation is therefore closely related to oxidative stress. Coffee is known to have antioxidants compounds, which include (poly)phenols (Gonthier et al., 2003), caffeine (Nawrot et al., 2003), heterocyclic compounds (Illy, 2002, Yanagimoto et al., 2002) and aromatic compounds (Illy, 2002) and following acute consumption of coffee by humans, a significant 5.5% increase in plasma antioxidant capacity has been reported (Natella et al., 2002).

In a human study with healthy participants investigating the effect of coffee consumption on the circulating levels of homocysteine, a biomarker of inflammation and cardiovascular disease, the results indicated that higher concentrations of the biomarker were positively associated with increased coffee consumption (Grubben et al., 2000). In this study, however, non-filtered coffee was given to the participants, and therefore, the cholesterol-rising diterpenes were still fully present. Also, the results would only apply to heavy drinkers, since the administered dose corresponded to 6 large mugs of strong coffee, equivalent to a daily consumption of 1L of coffee.

In 2004, Zampelas et al. (2004) investigated the effect of coffee consumption on biomarkers of inflammation in a health population of 3042 healthy adults. In analyses adjusted for potential confounders, women and men that had a habitual

coffee consumption of > 200 mL per day had higher levels of C-reactive protein, interleukine-6, serum amyloid A and tumor necrosis factor-α, when compared to subjects consuming no coffee. These results would suggest a detrimental effect of a regular consumption of coffee, when compared to no consumption, however, in this study, no distinction was made between filtered and non-filtered and a different outcome cannot be excluded if the effect of filtered and non-filtered coffee was analysed separately.

In a cross-sectional study by Lopez-Garcia et al. (2006), the effect of a long-term consumption of filtered coffee on various biomarkers of inflammation and endothelial health were assessed in two different female subcohort groups. The control group consisted of 730 women, with no diagnosed cardiovascular disease, cancer or T2DM, and the T2DM group consisted of 663 women, diagnosed with T2DM but no cardiovascular disease or cancer. The age ranged from 43 to 70 years old. Upon multivariate analyses (age-, BMI-, physical activity-, smoking status-, alcohol-, aspirin use-, postmenopausal hormone therapy-adjusted) an inverse association between the consumption of decaffeinated filtered coffee and the concentration of C-reactive protein (CRP) in the control group, and an inverse association with E-selectin and C-reactive protein (CRP) in the T2DM group. These results suggest that filtered coffee does not have a detrimental effect on inflammation or endothelial health, however, the study only included female participants and female hormones may influence the outcome. Another limitation of the study is the cross-sectional deisgn.

In the cohort Iowa Women's Health Study, 27,312 post-menopausal women were followed for 15 years to investigate coffee consumption and the total and cause-specific mortality. Data, adjusted for confounders, showed an inverse association between coffee consumption and death attributed to an inflammatory disease other than CVD and cancer (Andersen et al., 2006). This cohort targeting post-menopausal women, the results cannot be extrapolated to the general population.

In a recent intervention study carried out by Kempf et al. (2010), the association between the consumption of coffee at different doses on selected biomarkers of inflammation was investigated. Forty-seven participants, healthy but at a higher risk of developing T2DM, were recruited. When compared to a 1-month

period of coffee abstention, a 1-month period with a coffee consumption of 8 cups per day was associated with an increase in some markers of inflammation (i.e. IL-18, adiponectin), a decrease in 8-isoprostane, a marker of oxidative stress, and the increase of total cholesterol and apolipoproteins A-I, but did not influence the variables of glucose metabolism. In this study, the design was not randomized and the administered coffee dose of 8 cups coffee per day is very high and only achievable by a small population fraction.

In 2012, Yamashita et al. (2012) reported the data from a cross-sectional study with 3317 female and male participants on the effect of habitual consumption of coffee on inflammation and metabolic biomarkers. Upon analyses adjusted for potential confounders, a higher consumption of coffee was positively associated with adiponectin levels and negatively associated with leptin and liver enzymes, but no association with the biomarker of inflammation C-reactive protein, interleukin-6 or tumor necrosis factor-α was osberved. Having a cross-sectional design, the causality of the association cannot be determined.

In a later randomized control study, Corrêa et al. (2013) investigated the effect of consuming filtered coffee with a different roast level over 4 weeks on biomarkers of inflammation and cardiovascular risk. In the 20 healthy adult subjects recruited, independently of which coffee type had been consumed, the levels of total homocysteine remained unchanged when compared to baseline values. Also relevant for the present dissertation, the serum glycaemic biomarkers, glucose and insulin, remained unchanged. From the other biomarkers of inflammation and cardiovascular risk analysed, Corrêa et al. suggested an inflammatory effect related to the consumption of coffee. The fact that only 20 subjects were subject to analysis, the power is reduced and unlikely to be representative of a healthy adult population.

1.2.3.3 Cardiovascular health

The effects of coffee on cardiovascular health remain controversial. How coffee affects the cardiovascular parameters of health, such as endothelial function, partly depends on how the beverage is prepared. While a filtered coffee retains most of the cholesterol-raising diterpenes, the frequent and long-term consumption of

unfiltered coffee increases the chances of raising the blood cholesterol levels. Consuming caffeinated or non-caffeinated coffee also has an impact on the outcome.

In a double-blinded crossover study with healthy subjects, an acute ingestion of caffeinated, but not decaffeinated, coffee led to a significant decrease in flow-mediated dilation and both systolic and diastolic blood pressures (Buscemi et al., 2010), suggesting a negative effect of caffeine on cardiovascular health. In another study in which caffeinated and decaffeinated coffee was administered randomly to healthy subjects, arterial stiffness was assessed by measuring the carotid femoral pulse wave and the arterial wave reflection at the aorta. Results indicated an increase of these parameters after intake of caffeinated coffee (Mahmud and Feely, 2001). In these two latter studies, the statistical power is reduced by the limited number of study participants (n = 20 and 7, respectively). Also, the parallel analysis of caffeine as a pure compound would have been of interest, as in the study by Battram et al. (2006), to assess the contribution of coffee compounds other than caffeine to the counteraction of the negative effects observed. Although increases in several cardiovascular disease risk factors, such as blood pressure and plasma homocysteine, were detected, and a high coffee consumption (5 to 10 cups/day) has been associated with a significantly increased risk of coronary heart disease and myocardial infarction, the majority of prospective cohort studies and a more moderate consumption of 3-4 cups/day do not show this positive association (Sesso et al., 1999).

In the previously mentioned prospective cohort study carried out by De Koning Gans et al. (2010), a higher consumption of coffee was associated with the incidence of coronary heart disease in a U-shaped relationship, with the lowest hazard ratio for subjects consuming 2.1 to 3.0 cups per day. De Koning Gans et al. did not find any association between the consumption of coffee, the incidence of stroke, and death from coronary heart disease, stroke or from all causes, in a model adjusted for age, gender and other potential confounders (e.g. physical activity, waist circumference, smoking status and alcohol intake). As previously mentioned, the small number of subjects that died from coronary heart disease or stroke reduces the power of the analyses for these associations. These findings, however, are suggestive of a beneficial effect of a moderate long-term consumption of coffee on the incidence of coronary heart disease and associated death.

Recently, Lopez Garcia et al. (2011) published the data from a 24-year follow-up cohort study with female subjects who had suffered a non-fatal cardiovascular event. The association between the consumption of filtered caffeinated coffee and death from all causes or from cardiovascular disease was investigated in 11,697 women, of which 1,159 passed away during the follow-up period. In this follow-up, no association between the consumption of coffee and death from all causes or cardiovascular disease was detected, suggesting that the long-term consumption of filtered coffee does not have a detrimental effect on the incidence of death from cardiovascular disease or from all causes. The fact that the participants had suffered a non-fatal cardiovascular event and that all were healtcare professionals may have influenced the choice of lifestyle for some of them, which may have had an impact on the results observed.

In the same year, Larsson and Orsini (2011) carried out a meta-analysis of 11 prospective studies, aiming at assessing the association between the consumption of coffee and the incidence of stroke. Combined, a total of 479,689 subjects were included, 10,003 of which had a stroke. The analyses indicated a maximal 17 % reduction of the relative risk from no coffee consumption to up to 3 - 4 cups daily, whether the subjects were healthy from the start or whether the analysis included data from diabetic patients or from subjects that had recently had an acute myocardial infarction. These data suggest that a moderate long-term consumption of coffee may reduce the incidence of stroke.

Overall, a higher consumption of fruit and vegetables has mostly been associated with beneficial effects on the incidence of cardiovascular diseases. Characterised by a non-linear relationship, the highest decreased risk was assessed with the consumption of 5 servings per day. In general, when compared to no or very rare consumption, a tea consumption of ≤ 3 cups per day is associated with a lower incidence of T2DM. The results from studies with coffee are somewhat more controversial and the effect of coffee on health remains uncertain. Factors contributing to the variable outcomes observed include the variety of coffee constituents able to influence parameters that in turn influence inflammation, cardiovascular health or other health conditions; the preparation mode of the beverage that has an impact on the quality of the brew and therefore, on the

composition of the coffee; the frequency of consumption; as well as the genetics of a person (Cano-Marquina et al., 2013). The underlying mechanisms of the observed beneficial effects of a higher consumption of coffee and other (poly)phenol-rich products are still unclear, but good candidates are the constituent (poly)phenols. However, in order to have any physiological effects, these first need to be absorbed. To assess whether a better health status is significantly related to a higher consumption of (poly)phenol-rich foods, the links between the habitual consumption of (poly)phenol-rich products, the absorption of (poly)phenols and the health of subjects require investigation.

1.2.4 Biomarkers and their importance

The health status of a subject can be assessed by the measurement of biological markers (biomarkers). These can be measured in different tissues or body fluids, but are frequently measured in blood or urine. An accurate definition of biomarkers was given by the Biomarkers Definitions Working Group, in 2001 as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (BDWG, 2001).

When studying the impact of an external factor on biomarkers of health in a healthy population, unless the healthy range is very broad, one may miss true associations, as extreme values, which are easily obtained in subjects suffering from a particular condition, may be difficult to obtain in healthy subjects. Several epidemiological studies and human studies on the postprandial changes of health biomarkers upon consumption of (poly)phenol-rich products (e.g. coffee, tea, fruits and vegetables) have been conducted, however there is still a lack of information concerning the link between the bioavailability of (poly)phenols and the general health status of subjects. When studying the general health status, it is important to measure the biomarkers of health at fast, in order to avoid any postprandial influence. Glucose and insulin are two good examples of biomarkers that easily fluctuate upon consumption of food. Also, the choice of biomarkers needs to be done carefully, as these should be reliable markers for conditions of interest (e.g. glucose, insulin for diabetes; aminothiols, cholesterol for cardiovascular health;

aminothiols, CRP, cystokines such as IL-10, TNF-α for inflammation). In the present study, the total aminothiols cysteine, homocysteine, cysteinylglycine and glutathione, glucose, insulin and uric acid were subject to analysis. Although these only represent a small fraction of indicators of cardiovascular and inflammation health status, they have been reported to be reliable.

Aminothiols are metabolically related via the transsulfuration pathway, which links the methionine to the biosynthesis of glutathione. Small increases in homocysteine are an independant risk factor for premature cardiovascular conditions (Moat et al., 2001) and a possible explanation is a the lower bioavailability of nitric oxide (NO) and subsequent endothelial dysfunction caused by an increased production of superoxide radicals. The latter may originate from the autoxidation of homocysteine, from uncoupled endothelial nitric oxide synthase (eNOS), or from higher levels of asymmetrical dimethylarginine (Antoniades et al., 2009). As a consequence of higher levels of homocysteine, some mediators of inflammation are activated, including the nuclear factor-kappa B (NFκ-B).

Cysteine, the most abundant aminothiol in plasma, has been suggested to be an antioxidant although contradictory data were reported (Moat et al., 2001). Cysteine is a substrate for the production of NO, an important vasodilator. Lower levels of cysteine may thus decrease the availability of NO, which in turn may lead to endothelial dysfunction, a risk factor for cardiovascular disease (Moat et al., 2001; Hermann M et al., 2006).

Glutathione, a tripeptide of glutamate, cysteine and glycine, plays several key functions in the metabolism. Through its ability to detoxify reactive oxigen species, glutathione plays an important role as a regulator of the redox status and may thus prevent cellular damage from oxidative stress, which may lead to inflammation (Shimizu et al., 2004).

Analysing the aminothiols simultaneously is important, as not only the individual concentration in plasma is important, but the relative amounts to each other is determinant for the health as they may give indication of the enzymatic activities in the transsulfuration pathway.

Glucose and insulin are two determining biomarkers in cardiovascular heatlh. An unbalance in their metabolism may lead to the development of diabetes, which

puts the subjects are higher risk for developing other conditions, such as cardiovascular diseases. In healthy subjects, an increase of glucose (> 3.3 mM) leads to the secretion of insulin by the pancreatic β-cells. When a subject develops insulin resistance, although the glycaemic levels can be normal, the secretion of insulin is increased. Insulin secretion can however decrease with the age, thus raising the glycaemia in these subjects. Subject developing diabetes have a mortality rate of 2 to 4 times higher than that of healthy subjects (Poretsky, 2010). A few mechanisms by which hyperglycaemia may lead to cardiovascular health issues have been described and all of them have in common and increase formation of superoxide (Gleissner et al., 2007). As an example, in case of normal glycaemic levels, toxic aldehydes are reduced to inactive alcohols by the aldose reductase with little effect on glucose (and little production of sorbitol), due to their low affinity for each other. In presence of higher glucose levels, however, the production of sorbitol and subsequently fructose is increased. In this situation the intracellular accumulation of sorbitol and consumption of NADPH required for the production of glutathione leads to an oxidative stress. On the other side, the higher levels of fructose increase the formation of advanced glycation end products, which contribute to increase the oxidative stress and inflammation (Uribarri et al., 2010).

Uric acid, a product of the metabolism of purines, is the cause of gout, an inflammatory condition caused by the deposition of monosodium urate crystal in several anatomical locations (Ghaemi-Oskouie and Shi, 2011). The accumulation of monosodium urate crystals leads to the production of IL-1β and IL-18, two pro-inflammatory cytokines.

The measurement of the biomarkers described above is relevant as changes can be observed at early stages and in healthy stubjects and therefore be targets for the prevention of inflammation and cardiovascular diseases. For this reason, these biomarkers of health were selected to be analysed in the healthy population of the present research. Other biomarkers not investigated due to monetary, equipment and/or time limitation are good reflectors of inflammation and cardiovascular health. Examples are the c-reactive protein, a biomarker of chronic low-grade inflammation, TNF-α, interleukins-1, -6, or -10 (Zakynthinos and Pappa, 2008).

In health, numerous factors can lead to the development towards diseases and information about each factor can be collected in different ways in order to assess

the status of a subject. Figure 1. 4 shows examples of risk factors that may lead to cardiovascular diseases. Only the factors that were of interest in the present study (gray boxes) and how information about each of them was collected (dashed boxes) are illustrated.

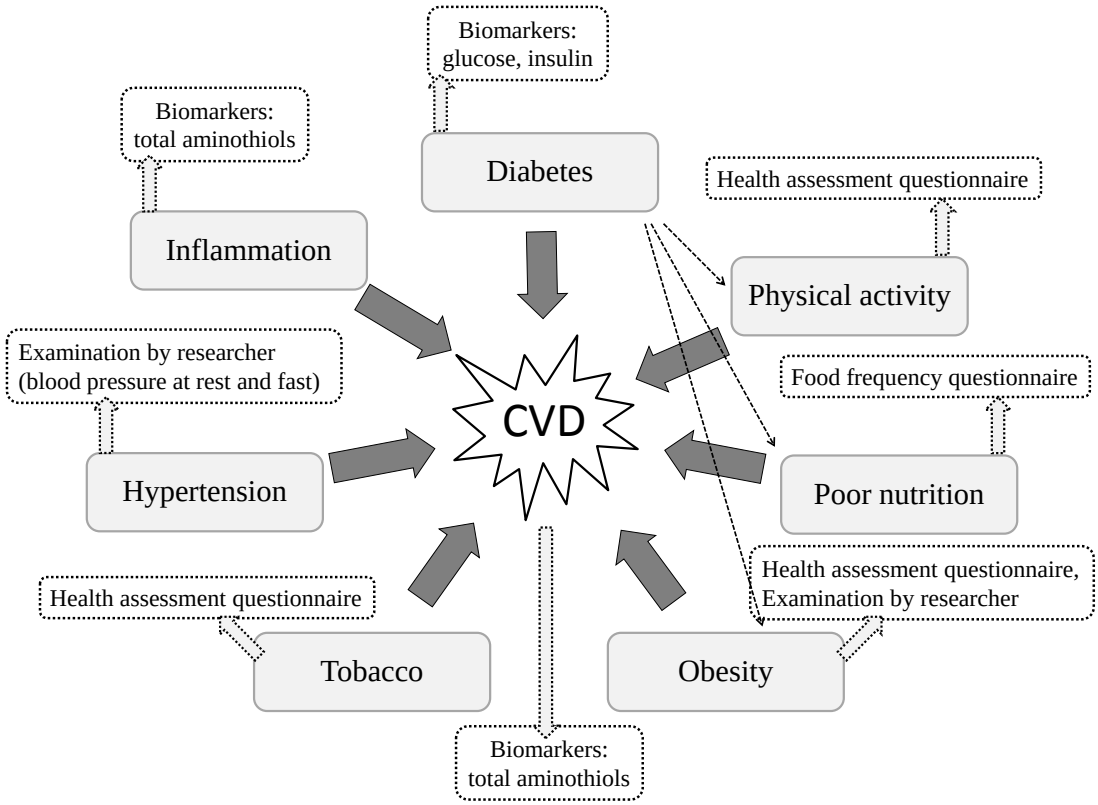


Figure 1. 4: Risk factors contributing to the development of cardiovascular disease (full boxes) analysed in the present study, and possible ways of assessing a status of a subject in regards to a particular factor (dashed arrows and boxes). Other biomarkers and assessment methods, not presented in this diagram, exist.

1.3 Objectives

The questions raised and aimed at to be answered with the present PhD thesis were the following:

1. Does roasting or decaffeination have an impact on the free phenolic acid content (i.e. caffeic, dihydrocaffeic, ferulic, isoferulic, dihydroferulic and 3,4-dimethoxycinnamic acid) of soluble coffee?

2. Chlorogenic acids are present in significant amounts in coffee, however, although some absorption of native esters was reported (Farrell et al., 2011,

Renouf et al., 2013), these first need to be cleaved into their derivative aglycones. As observed in previous pharmacokinetic studies (Renouf et al., 2013), the chlorogenic acid-derived metabolites, caffeic, ferulic, isoferulic and 3,4-dimethoxycinnamic acid, appear in plasma mainly between 1 and 3 hours upon consumption of coffee indicating that these mainly result from a pre-colonic absorption. A further aim was thus to investigate whether native aglycones that are present in a regular cup of coffee can significantly contribute to the appearance of respective metabolites observed in plasma between 1 and 3 hours, and resulting from a pre-colonic absorption, following the consumption of the beverage.

3. Are subjects with a higher consumption of particular (poly)phenol-rich products, targeting non-alcoholic beverages (i.e. coffee, tea, cocoa) and common solid products (i.e. fruits, vegetables, herbs and spices and supplements), more likely to also have a higher intake of other (poly)phenol-rich products?

4. Based on methods available in the literature, a selection of methods was improved, mainly for time efficiency, and validated. Using these, it was assessed whether selected inflammation and cardiovascular health biomarkers in plasma are stable over a period of at least 8 weeks, in healthy human adults.

5. Do healthy human adults with higher habitual consumption of (poly)phenol-rich products have a better profile of selected inflammation and cardiovascular health biomarkers in plasma?

6. Does the absorption and metabolism of coffee chlorogenic acids have an impact on selected inflammation and cardiovascular health biomarkers in the plasma of healthy human adults?

7. Does the habitual consumption of coffee and some non-dietary daily habits have an impact on the absorption of coffee chlorogenic acids in healthy human adults?

Chapter 2
Free phenolics in soluble coffee and their contribution to the pool of metabolites derived from a pre-colonic absorption

2.1 Abstract

Coffee is a rich source of chlorogenic acids, but also contains free phenolics. Upon ingestion of coffee, phenolic acids have been identified in plasma approximately up to 3 hours (early appearance) or approximately 4 hours (late appearance) after the consumption of the beverage (Renouf et al., 2010b). These values are typical for compounds resulting from a pre-colonic and colonic absorption, respectively. No publication was found on the possible contribution of free phenolic acids present in a consumed coffee beverage to the early appearance of derived metabolites in plasma. The present chapter thus reports the analysis of major free phenolics in various commercially available soluble coffees and the subsequent assessment of their possible contribution to the early appearance of derived metabolites in plasma, using pharmacokinetic modelling based on previously published data. In all the coffees analysed, caffeic acid was present in higher amounts, but overall, the phenolic acids were present at low levels, when compared to the chlorogenic acids. The results suggest that roasting and decaffeination significantly reduce the content of free phenolic acids. When present in amounts found in a regular cup of coffee, the contribution of the analysed free phenolic acids to the early appearance of derived metabolites was assessed to be non-significant. Other colleagues (Mr. Nicolai U. Kraut, Dr. Tracy Farrell and Ms. Alexandra Ryder) conducted experiments to indicate possible alternative mechanisms of contribution. These are the hydrolysis by small intestine and pancreatic esterases of certain chlorogenic acids, particularly of 3- and 5-*O*-caffeoylquinic acids, respectively, as assessed by *in vitro* enzymatic experimentation.

2.2 Introduction

Coffee is a rich source of chlorogenic acids, a diverse family of esters formed between quinic acid and certain phenolic acids, mainly hydroxycinnamic acids, such as caffeic, ferulic and *p*-coumaric acids (structure presented in Figure 1.2 of chapter 1) (Clifford, 1999). Variations of chlorogenic acid levels amongst coffee types have been reported as a consequence of different bean quality and climates (Vaast et al., 2006). Further contributing to the variation is processing, such as decaffeination and roasting, which has also shown to extensively modify the chemical composition and final chlorogenic acid content (Clifford, 1999, Perrone et al., 2010, Ludwig et al., 2014). A limited number of studies have identified small amounts of free phenolic acids in green coffee beans and commercially available instant coffee (Alonso-Salces et al., 2009, Farrell et al., 2011) and very little is known about the impact of processing of coffee on these.

Phenolic acids were identified in plasma approximately up to 3 hours or after 4 hours following the consumption of coffee (Renouf et al., 2010b, Williamson et al., 2011). These values are typical for compounds derived from a pre-colonic and colonic absorption, respectively. Although intact chlorogenic acids have been identified in plasma at nM levels, these do not easily pass the gastrointestinal epithelium barrier without prior cleavage of the native form and about 70 % passes through to the colon, where they are subject to the hydrolytic action of the abundant microbiota population (Plumb et al., 1999, Stalmach et al., 2009). The occurrence of free phenolic acids in coffee may thus explain the early appearance of metabolites in human plasma, but to date, no study has elucidated the contribution of these to the pool of metabolites identified after coffee consumption. Supporting this hypothesis are previous investigations using cell models of the intestinal epithelium (Caco-2 cells) indicating that phenolic acids are rapidly transported into the blood. Also, human intervention studies investigating the appearance of phenolic acids in human plasma have indicated that free phenolic acids, especially dimethoxycinnamic acid, are highly bioavailable after coffee consumption (Renouf et al., 2010b).

A significant amount of the phenolic acids appear at earlier time points in the blood (1-3 hours), especially for metabolites such as ferulic acid sulphate and dimethoxycinnamic acid (Renouf et al., 2010b), but it is not known if these are

derived from free phenolic acids already present in the coffee, or from the hydrolysis of chlorogenic acids by enzymes present in the small intestine. The latter could include pancreatic secretions and brush border enzymes. A previous study on human tissues indicated no hydrolysis upon incubation of chlorogenic acid (mainly 5-*O*-caffeoylquinic acid) with extracts of human intestinal tissue, liver or plasma. On the other hand, chlorogenic acid was efficiently converted into caffeic acid when incubated with faecal extracts (Plumb et al., 1999). There is also some evidence for a small amount of gastric absorption of intact chlorogenic acids in animal and cell models (Lafay et al., 2006, Farrell et al., 2011).

Phenolic acids may be the principal bioactive compounds responsible for the observed beneficial health effects associated with regular coffee consumption (Huxley et al., 2009). There is growing evidence that chlorogenic acid metabolites may contribute to improved intestinal health by increasing mucosal membrane integrity (Bergmann et al., 2009). Additionally, studies with animal models of type 2 diabetes mellitus indicated favourable antiglycemic effects and modulation of oxidative enzymes following consumption of caffeic acid (Jung et al., 2006) and ferulic acid (Balasubashini et al., 2004). It is now widely appreciated that chronic low grade inflammation plays a key role in development of degenerative diseases (Baker et al., 2011), and the reported bioactivity of phenolic acids may in part contribute to their control or prevention.

The aim of the analyses reported in the present chapter was to first verify the presence of major free phenolic acids, namely caffeic (CA), ferulic (FA), isoferulic (iFA), dihydrocaffeic (DHCA), dihydroferulic (DHFA) and 3,4-dimethoxycinnamic acid (3,4-DMCIN), in a variety of commercially available instant coffees and estimate the impact of roasting and decaffeination on the phenolic acid content. Pharmacokinetic modelling was then used to assess whether the amounts of free phenolic acids, present in a regular coffee preparation, could account for the levels of derived metabolites observed in human plasma after coffee consumption. *In vitro* experiments using pancreatic secretions (carried out by Mr. Nicolai U. Kraut) and preparations of Caco-2 cells monolayers (carried out by Dr. Tracy Farrell and Ms. Alexandra Ryder) were used to investigate the hydrolysis of individual caffeoylquinic acids and chlorogenic acids, as present in coffee, to assess the

potential alternative contributing mechanisms. The work reported in the present chapter has been recently published (da Encarnação et al., 2014).

2.3 Materials and Methods

2.3.1 Chemicals and materials

All chemicals were purchased from Sigma-Aldrich (Berkshire, UK) unless otherwise stated. Acetonitrile (HPLC-MS grade) was purchased from Fisher Scientific Ltd (Leicestershire, UK). Ferulic acid (≥ 99 %) and sinapic acid (≥ 99 %, HPLC) were purchased from Fluka Analytical; dihydroferulic acid (97 %) and dihydrocaffeic acid (> 98 %) were obtained from Alfa Aesar (Lancashire, UK); isoferulic acid was from Extrasynthèse (Genay France) and DMSO from Riedel-de Haën.

The instant coffees used were purchased from a local supermarket: A coffee made from 35 % unroasted and 65 % roasted green coffee beans (GrC), a golden roast with caffeine (GoC), a decaffeinated golden roast (GoD), a medium-dark roast with caffeine (MC), and a decaffeinated medium-dark roast (MD).

2.3.2 Sample preparation

Standards: For the analysis of free phenolic acids in coffee solutions, calibration curves with concentrations ranging from 0.05 to 100 μM for caffeic acid, dihydrocaffeic acid, ferulic acid, dihydroferulic acid, isoferulic acid and 3,4-dimethoxycinnamic acid were prepared in premixed 5 % acetonitrile and 94.9 % water acidified with 0.1 % formic acid (solvent A). Sinapic acid was used as internal standard at a final concentration of 100 μM.

Preparation of coffee solutions: Triplicates of spiked and non-spiked coffee samples were prepared from two different batches of commercially available instant coffees. Phenolic extraction was done by adding boiling deionized water to the coffee granules to a final 50 mg/mL concentration. This first extracted stock was then diluted to 5 mg/mL in solvent A. Spiked samples were supplemented with free

phenolic acids to a final concentration of 100 µM and sinapic acid was used as internal standard at a final concentration of 100 µM.

2.3.3 Sample analysis

The analysis of the coffee solutions was performed on an HPLC-DAD-ESI-QQQ. Dihydrocaffeic and dihydroferulic acids were analysed at 280 nm, and caffeic, ferulic, isoferulic and 3,4-dimethoxycinnamic acids at 325 nm. Upon optimization of the standards, the mass spectrometric variables shown in Table 2.1 were used.

Table 2.1: Optimization variables for phenolic acids of interest used for the analysis on HPLC-DAD-ESI-QQQ. CA, caffeic acid; DHCA, dihydrocaffeic acid; FA, ferulic acid; DHFA, dihydroferulic acid; iFA, isoferulic acid; 3,4-DMCIN, 3,4-dimethoxycinnamic acid; Rt, retention time (min); λ, wavelength of maximal absorbance (nm); [M-H$^-$], mass-to-charge ratio (m/z) of negatively charged molecular ion; Fragm., fragmentor voltage (eV); MS^2, mass-to-charge ratio of product ion produced by fragmentation of [M-H$^-$]; CE1 and CE2, collision energy for first or second transition.

	Rt (min)	**λ (nm)**	**[M-H$^-$] (m/z)**	**Fragm. (eV)**	**MS^2 (m/z)**	**CE1 (eV)**	**CE2 (eV)**
DHCA	12.45	280	181	90	137, 59	8	15
CA	15.77	325	179	90	135, 89	12	28
DHFA	33.60	280	196	90	136, 121	12	25
FA	36.31	325	193	90	178, 134	8	12
SA	38.33	325	223	100	208, 164	5	10
iFA	38.60	325	193	80	178, 134	5	12
3,4-DMCIN	45.95	325	207	80	103, 163	8	10

Five µL of coffee solution was injected. Chromatographic separation was achieved on an Eclipse plus C18 column (30 °C, 2.1 mm x 100 mm, 1.8 µm pore size; Agilent Technologies) using a 61-min gradient of solvent A (premixed 94.9 % water in 5 % acetonitrile modified with 0.1 % formic acid) and B (premixed 5 %

water in 94.9 % acetonitrile modified with 0.1 % formic acid), based on a previously established gradient (Farrell et al., 2011).

2.3.4 Recoveries

Recovery rates of phenolic acids extracted from coffee were estimated from the comparison of triplicate samples of non-spiked 5 mg/mL coffee solutions with 100 µM-spiked 5 mg/mL coffee solutions. The increase of the peak area in spiked samples (AUC_{sp}) was corrected for the peak area in non-spiked samples ($AUC_{non\text{-}sp}$). Recovery was calculated by comparing the concentration detected to the spiking concentration expected (AUC_{std}) for a particular compound, using the relevant calibration curve and the following formulas:

$$AUC_{sp} - AUC_{non-sp} = \Delta AUC \qquad \frac{\Delta AUC}{AUC_{std}} = recovery(\%)$$

2.3.5 Pharmacokinetic calculations

To estimate the possible contribution of unmodified free phenolic acids to the early plasma pool, pharmacokinetic curves of 4 g of GrC were computed assuming the following variables: 100 % bioavailability (F), a volume of distribution measured for another (poly)phenol, (-)-epigallocatechin gallate, equivalent to that of 200 mg in healthy human (V_β/F 2009 +/- 1514 L), an administration dose of free phenolic acid as quantified in 4 g GrC (FA: 310.2 µg, iFA: 7.2 µg, DHFA: 17.2 µg, CA: 1116 µg, DHCA: 173 µg, 3,4-DMCIN: 149 µg), a maximal concentration (C_{max}) and half-life ($t_{1/2}$) taken from existing pharmacokinetic curves (Renouf et al., 2013), an elimination constant (ke) calculated from $t_{1/2}$ ($ke = \ln(2)/ t_{1/2}$) and a ka calculated using the following formula: $ka * t_{max} - \ln(ka) - ke * t_{max} + \ln(ke) = 0$.

2.3.6 Statistical analyses

The statistical software R was used for analyses of data. Each free phenolic acid was compared between coffees, using the Welch two-sample t-test. Only values of $p < 0.05$ were considered statistically significant.

2.4 Results

2.4.1 Free phenolic acid content of coffee

By using a chromatographic detection with simple diode array detection, the identity of the peak could hardly be attributed and was based on the comparison with the chromatographs of spiked samples. With the simultaneous use of mass spectrometry, each phenolic acid of interest was attributed the corresponding peak with certitude. The conditions established for the analysis of free phenolic acids by HPLC-DAD-ESI-QQQ are shown in Table 2.1 and Figure 2.1 and a typical diode array detector chromatographic profile is shown in Figure 2.2. The limit of quantification per injection was 7.5, 45, 5.6 and 4.8 pg for CA, DHCA, FA and DHFA respectively. Using this method, the content of 6 free phenolic acids was determined in 5 different instant coffees (Table 2.2). All compounds were above the limit of detection. Amongst the 5 coffees analysed, the coffee originating from a mixture of roasted and unroasted beans (GrC) had the highest content of CA and FA ($p < 0.001$ and $p < 0.05$ respectively). On the other hand, the medium dark roast caffeinated coffee (MC) contained the highest content of DHCA, DHFA and iFA, and the golden roast caffeinated coffee (GoC) contained the highest level of 3,4-DMCIN. Finally, the two decaffeinated coffees tested had, when compared to their corresponding roast caffeinated coffee, lower levels of all free phenolic acids. These observations suggest that not only roasting, but also decaffeination, may affect the content of free phenolic acids. The levels of free phenolic acids are > 2 orders of magnitude lower than that of chlorogenic acids typically present in the GrC coffee, as reported by Hoelzl et al. (2010) (. In the latter report, the content of chlorogenic acids was: 3-CQA: 42,000 μg/g coffee; 4-CQA, 49,600; 5-CQA, 162,400; 4,5-diCQA, 23,200; 3,5-diCQA,16,000; 3,4-diCQA, 22,800; 4-FQA, 8,800; 5-FQA, 31,600.

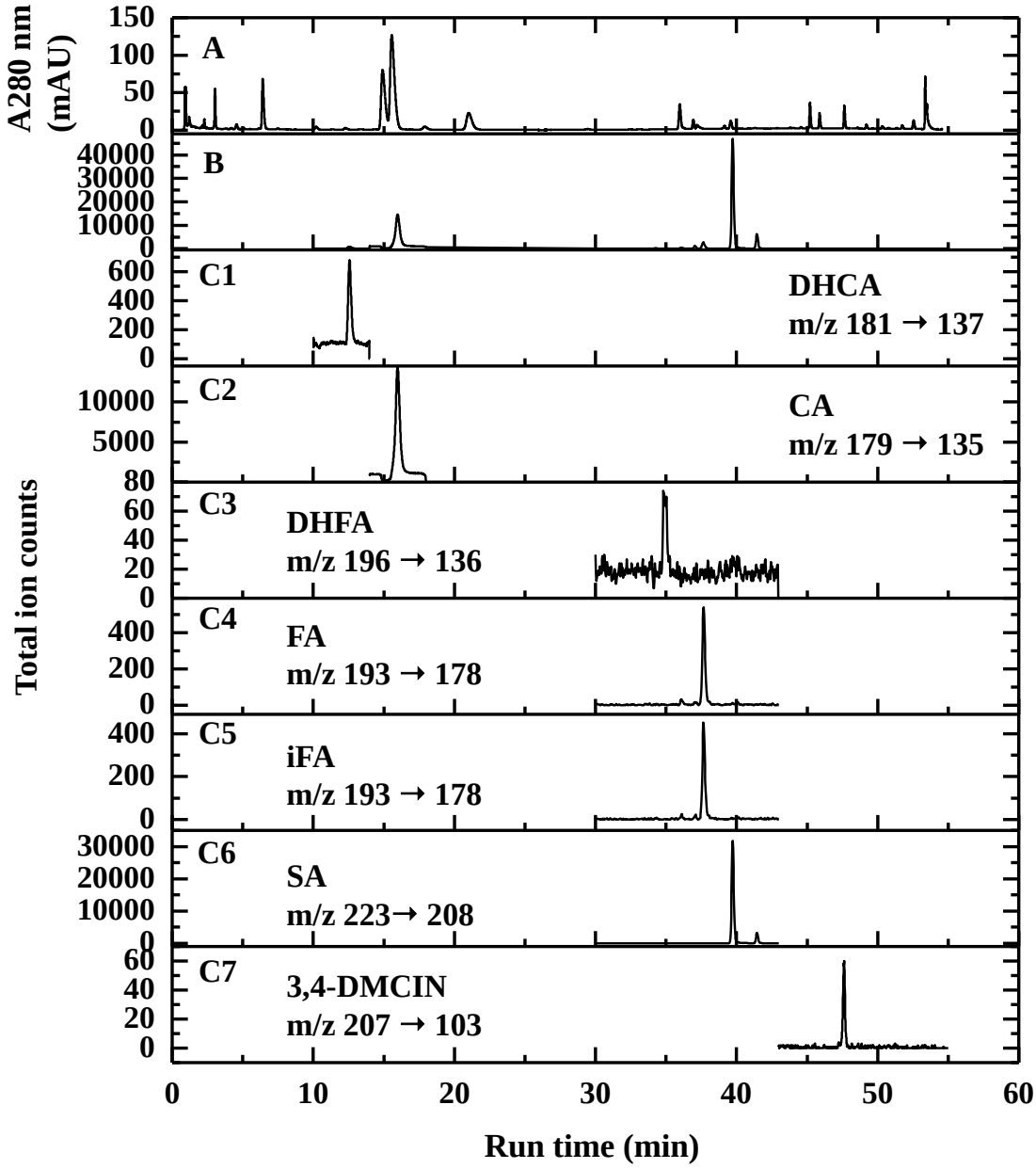


Figure 2.1: Typical diode array detector and total ion count chromatograms of the coffee sample containing 35 % of green coffee beans. Absorbance profile at 280 nm (A), Total ion counts from mass spectrometry (B, C), Individual transitions for indicated mass-to-charge ratio (m/z).

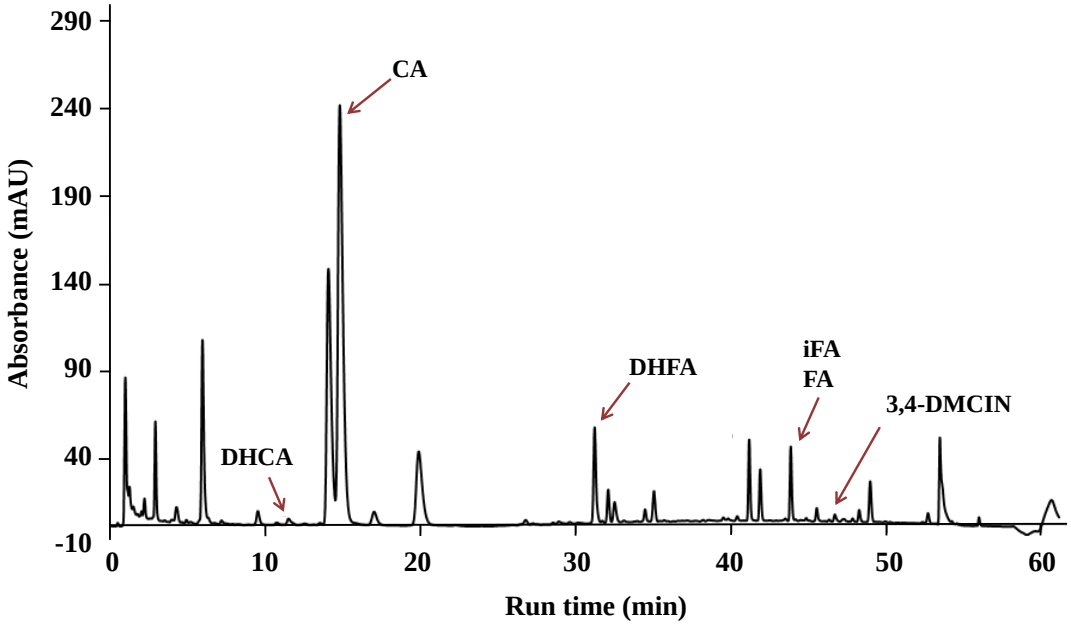


Figure 2.2: Typical diode array detector chromatographic profile of a coffee sample, at 280 nm. Profile of a 5 µL injection of the coffee originating from a mixture of roasted and unroasted beans (GrC).

Table 2.2: Free phenolic acid content in 5 instant coffees. Values are given in µg phenolic acid per gram of dry weight of instant coffee. Standard deviations are for the two batches of coffee analysed, with three biological replicates, each with two technical replicates.: GrC, 35 % unroasted and 65 % roasted coffee caffeinated; GoC, golden roast caffeinated; GoD, golden roast decaffeinated; MC, medium roast caffeinated; MD, medium roast decaffeinated.

µg phenolic acid/g coffee	DHCA	CA	DHFA	FA	iFA	3,4-DMCIN
GrC	43 ± 1	279 ± 16	4.3 ± 0.2	77 ± 5	1.8 ± 0.1	37 ± 0
GoC	56 ± 1	107 ± 3	4.1 ± 0.2	23 ± 1	2.7 ± 0.4	48 ± 1
GoD	44 ± 2	72 ± 4	3.6 ± 0.2	22 ± 2	1.7 ± 0.6	19 ± 0
MC	71 ± 1	87 ± 2	6.7 ± 0.2	29 ± 4	3.2 ± 0.3	32 ± 2
MD	46 ± 1	75 ± 4	4.6 ± 0.2	22 ± 1	1.7 ± 0.0	15 ± 0

2.4.2 Estimation of the contribution of free phenolic acids to the early appearing phenolic acid metabolites in blood

Phenolic acid metabolites were reported in plasma in a previous human study where subjects consumed a cup of GrC coffee beverage at different concentrations (Renouf et al., 2013). To ascertain the contribution of phenolic acids present in coffee to the early appearance of phenolic acids metabolites in plasma, the contents in the GrC coffee from the current investigation were compared to computed pharmacokinetic curves (Figure 2.3) and to the maximum plasma values previously reported obtained after consumption of a cup of 4 g GrC coffee (Renouf et al., 2013). Assumptions made are shown in section 2.3.5. However, these values are the maximum estimates and real values are likely to be less. Even in the unlikely event that these assumptions are underestimated by 10-fold, the amount of free phenolic acids in coffee is still not enough to account for the early appearing blood peaks by at least an order of magnitude, and more for some compounds. As an example, the free ferulic acid fraction in coffee would give rise to a peak of 0.5 nM in blood assuming 100 % absorption, compared to an obtained value of 200 nM after drinking a cup of coffee, i.e. approximately 400-fold higher. From this calculation, most of the early appearing phenolic acids is estimated to be due to hydrolysis of the constituent chlorogenic acids, which must occur either in the stomach or in the small intestine.

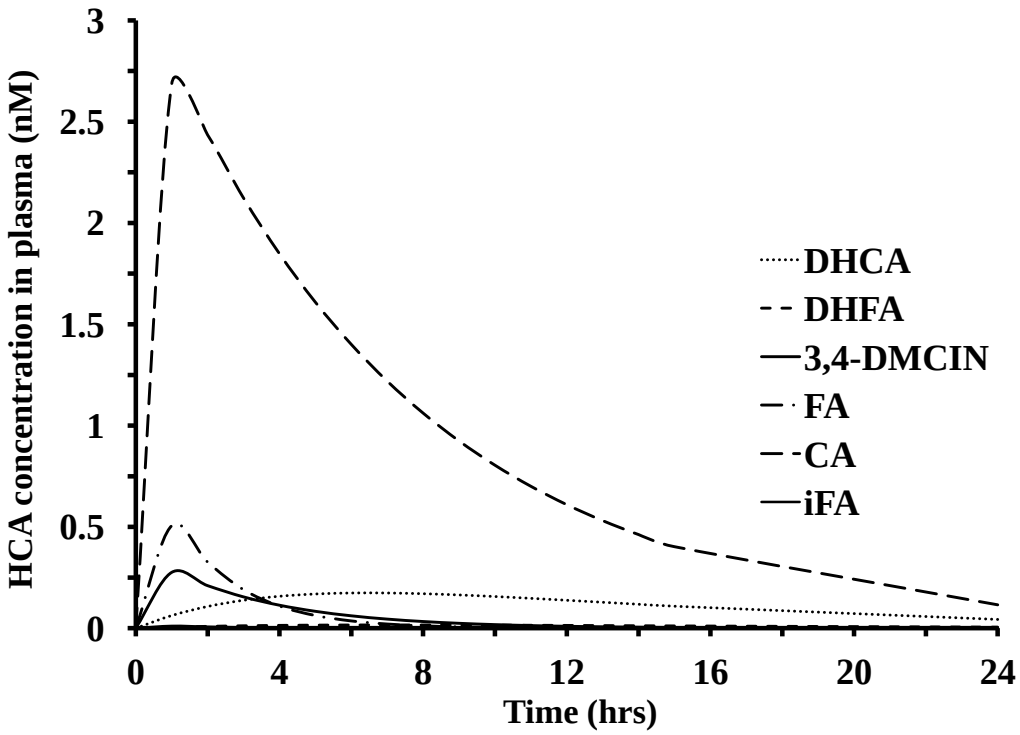


Figure 2.3: Theoretical pharmacokinetic curves for phenolic acids in plasma based on free phenolic acid content in 4 g of GrC and assuming a 100 % bioavailability. From published data, the small intestinal C_{max} values (i.e. < 3 hours) after consumption of this amount of coffee were approximately: DHCA, 50 nM; DHFA, 60 nM; 3,4-DMCIN, 500 nM; FA, 210 nM; CA, 180 nM; iFA, 80 nM.

Some possible sources of esterase in the small intestine were thus tested by former colleagues for the ability to hydrolyse the various chlorogenic acids present in coffee. As intestinal and liver tissue extracts did not hydrolyse 5-*O*-caffeoylquinic acid by Plumb et al. (1999), the pancreatic secretion, which is secreted into the small intestine, was tested (by Mr. Nicolai U. Kraut). Incubation of the coffee solution with porcine pancreatin led to the formation of free caffeic acid, but not ferulic acid. Further investigations with pure chlorogenic acids as the substrates for pancreatin indicated linear rates over 60 min, at increasing rates for 3-, 4- and 5-caffeoylquinic acid. When 3-, 4- or 5-feruloylquinic acids were incubated with the pancreatin solution, no significant increase in ferulic acid was observed. These data suggest that caffeoylquinic acids and more specifically 5-*O*-caffeoylquinic acid is a better substrate for pancreatin. *In vitro* experiments with Caco-2 cells (carried out by Dr. Tracy L. Farrell and Ms. Alexandra Ryder) confirmed to possess an esterase activity,

showed a distinctly lower hydrolysis of the caffeoylquinic acid isomers when compared to that of the methyl ester analogue used for testing the esterase activity of the monolayers. Interestingly, 3-*O*-caffeoylquinic acid was hydrolysed approximately 10-fold more rapidly than 5-*O*-caffeoylquinic acid.

2.5 Discussion and conclusion

Amongst a variety of commercially available instant coffees, the caffeinated instant coffee made from 35 % green and 65 % roasted beans water extract (GrC) had the highest content of free CA and FA. The medium dark roast caffeinated coffee (MC) had the highest content of free DHFA, iFA and DHCA and the golden roast caffeinated coffee (GoC) had the highest content of 3,4-DMCIN. Additionally, when comparing caffeinated and decaffeinated coffees of a same roast, the decaffeinated coffees contained less of all free phenolic acids tested. These data suggest that not only chlorogenic acids (Clifford, 1999), but also some free hydroxycinamic acids decrease with roasting intensity and decaffeination.

As expected, when compared to the amount of intact chlorogenic acids previously reported by Hoelzl et al. (2010), the amounts of free phenolic acids present in GrC are substantially lower (Table 2.2). However, free CA and FA are readily absorbed through the intestinal wall without further modifications (Stalmach et al., 2010) and less abundant compounds could have the potential to contribute significantly to the pool of metabolites measured in human fluids. The pharmacokinetic simulation suggests that the free phenolic acids are not present in sufficient quantities to make a significant contribution to the derived metabolites in plasma, even if 100% absorption is assumed. The *in vitro* esterase experiments using pancreatic secretions and small intestinal cells suggest, however, that the hydrolysis of chlorogenic acids into free phenolic acids are major contributing mechanisms to the early appearance of free phenolic acids in plasma, with a hydrolysis of 5-*O*-caffeoylquinic acid by pancreatic enzymes up to 5-fold higher when compared to 3- and 4-*O*-caffeoylquinic acid. The preparation of pancreatic secretions used being a complex mixture of lipases, trypsin, peptidases, proteases, RNAses, DNAses, amylases and unspecific carboxylesterases, it is not possible at this stage to attribute

the hydrolysis to a single enzyme. The hydrolysis of methyl esters in Caco-2 cells has been previously reported (Kern et al., 2003) and was used to verify the presence of esterase activity in the Caco-2 cell model. The expression of carboxylesterase(s), which is believed to be localized on the endoplasmic reticulum (Imai, 2006) has been previously characterized in both the Caco-2 cell model and human intestinal tissue (Sun et al., 2002).

The very low rate of hydrolysis of 5-*O*-caffeoylquinic acid by the intestinal model, differentiated Caco-2 cells, is consistent with previous reports, where intestine, liver and tissue extracts were unable to hydrolyse 5-*O*-caffeoylquinic acid (Plumb et al., 1999). However, 3-*O*-caffeoylquinic acid was hydrolysed at a much higher rate (~ 10-fold). After hydrolysis, the product caffeic acid, and its methylated derivative ferulic acid, are transported differently, probably due to the differing lipophilicity of the methyl esters and caffeoylquinic acids, Log D ~ 2 and – 3.5 respectively (reference: MarvinSketch, 2012; version 5.3.1, ChemAxon). The hydrophilic nature of the caffeoylquinic acids and paracellular permeation (Farrell et al., 2012) may explain the lower rates of hydrolysis compared to the methyl esters. Steric hindrance in the active site of human carboxylesterase-2, the predominant isoform in the small intestine (Imai, 2006), has been reported to occur with substrates containing a bulky acyl-moiety (representing the phenolic acid part of the quinic acid ester) which interfere with the formation of the enzyme-acyl intermediate during the first stage of hydrolysis. Steric interference may in part explain the differential hydrolysis of caffeoylquinic acids, and the lack of hydrolysis of the feruloylquinic acids. Interestingly, these findings may support data from human bioavailability studies *in vivo* which showed that only 5-*O*-caffeoylquinic acid could be detected in plasma after coffee consumption, perhaps suggesting that the susceptibility of the 3-acyl isomer to hydrolysis and relative resistance of the 5-acyl may contribute to the differential amounts of chlorogenic acids observed *in vivo.* An additional site of absorption is the stomach; cultured gastric epithelial cells have the capacity to hydrolyse chlorogenic acids into phenolic acids, with the esterase action favouring hydrolysis of caffeic and dimethoxycinnamic acid-containing substrates (Farrell et al., 2011). Several publications have also shown that intact chlorogenic acids can pass across the rat stomach and Caco-2 cell monolayers,

but only minor hydrolysis into phenolic acids occurred in the rat stomach (Lafay et al., 2006, Scherbl et al., 2014).

In conclusion, these experiments demonstrate that free phenolic acids quantified in coffee are not present in sufficient amounts to account for the appearance of pre-colonic derived metabolites following the consumption of a regular coffee beverage. Further *in vitro* investigation indicated that the hydrolysis of 5-*O*-caffeoylquinic acid by pancreatic esterases and 3-*O*-caffeoylquinic acid by brush border esterases in the small intestinal could be major alternative contributing mechanisms to the early peak of phenolic acids derived metabolites in plasma. A summarizing diagram of sites where chlorogenic acids are hydrolysed is presented in Figure 2.4.

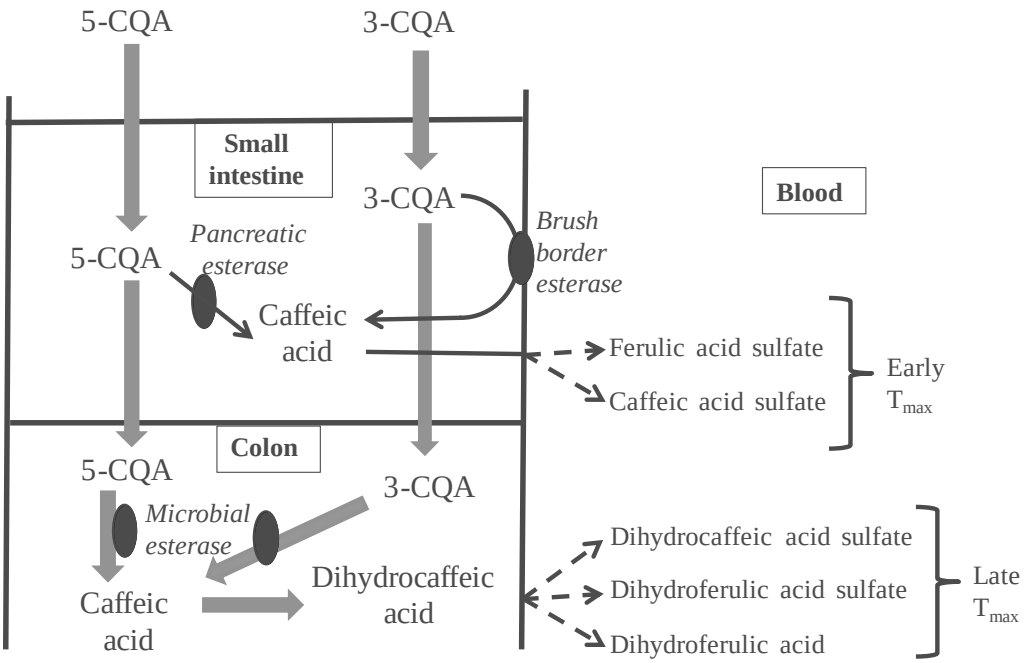


Figure 2.4: Sites of hydrolysis of chlorogenic acids derived from this and previous studies. Figure taken from (da Encarnação et al., 2014)

Chapter 3
Validation of methods used for the measurement of health biomarkers in human plasma and development of questionnaires

3.1 Abstract

Biomarkers are useful tools to assess the health status of a subject. These can be measured in different biological samples, which include tissues or body fluids such as urine or blood. Commercially available analytical methods often target the measurement of biomarkers in persons at risk of particular diseases and therefore the same methods may not be ideal for the measurement of the same biomarkers in healthy subjects, as the concentrations may be outside the range provided. For the present human study, total aminothiols, glucose, insulin and uric acid concentrations were measured in the fasting blood of healthy adults. In order to ensure reliable results within the range of interest, the use of reproducible methods guaranteeing the accurate quantification of the above-mentioned biomarkers was sought. Therefore, based on reliable procedures published in literature, a selection of methods were tested. In order to minimize the sample loss, the least plasma pre-treatment was wished. The present chapter reports the improvement and validation of the methods used for the quantification of the above biomarkers of health, used in later chapters to describe the study population recruited. Total aminothiols were measured using a chromatographic method with fluorescence detection, glucose and uric acid were measured using enzymatic colorimetric methods and insulin was measured by a commercial enzyme-linked immunosorbent assay. The plasma pre-treatment was limited to a centrifugation with no further need of deproteination or defatting. The improved and optimised methods showed a high reproducibility, good linearity within the range of interest, good recoveries and were time-efficient.

3.2 Introduction

The health status of a subject can be assessed by the measurement of biological markers (biomarkers). An accurate definition of biomarkers was given by the Biomarkers Definitions Working Group, in 2001 as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (BDWG, 2001). Biomarkers can be measured in diverse biological fluids and tissues, but for the current research, the measurements were done in human plasma. Commercially available analytical methods such as immunoassays are valuable tools for the detection of specific biomarkers of health, however, assays are often developed for the analysis of biomarkers in biological samples of people at risk of developing a particular condition and therefore the detection range may not be appropriate for healthy subjects. In the present study, total aminothiols, glucose, insulin and uric acid were measured in fasting plasma samples of a healthy adult population, as biomarkers of their general inflammation and cardiovascular health status. Although chromatographic, enzymatic and/or colorimetric techniques for these biomarkers have been published and some of them validated, not all achieve satisfactory results. Particularly for aminothiols, the chromatographic separation is often not satisfactory (Jacobsen et al., 1994, Minniti et al., 1998, Ferin et al., 2012, Frick et al., 2003). Therefore, in the present chapter, improved methods were sought, when possible, for the analysis of the above-mentioned biomarkers, based on the literature.

3.2.1 Total aminothiols

During normal metabolic processes, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced. In order to be maintained at physiological levels, these need to be neutralized or cleared. For this, scavengers are important to maintain a balanced redox status and prevent oxidative stress. Aminothiols are thiol-containing amino acids present in cells, but also found in plasma unbound (free), as a homodisulfide (e.g. glutathione-glutathione) or as a heterodisulfide (e.g. protein-bound aminothiol) and playing an important role in the balance of the redox status and hence, in human health. Aminothiols are involved in

the transsulfuration pathway, which links methionine to the biosynthesis of glutathione (Belalcazar et al., 2014) (Figure 3.1). Malfunction or deficiency of cystathione β-synthase, highly abnormal levels of homocysteine in plasma and urine, coronary artery disease and other cardiovascular events (Dudman et al., 1996, Verhoef et al., 1996, Durand et al., 2001, Mangoni et al., 2013, Robinson et al., 1994), pancreatitis (Rahman et al., 2009), neurodegenerative diseases (Sabens Liedhegner et al., 2012, Tchantchou, 2006) and inflammation (Mangoni et al., 2013) are some of the disorders that have been linked to an altered transsulfuration pathway. During pregnancy, the aminothiol redox status is also modified, however temporarily. Although the affected tissues are numerous in case of pathology, the transsulfuration pathway seems to be mainly localized in the liver, pancreas, certain lymphoid cells and brain (Rahman et al., 2009, Vitvitsky et al., 2006, Mudd et al., 1965, Sturman et al., 1980), and recently detected in an *in vitro* study, in mammary epithelial cells (Belalcazar et al., 2014). Levels of aminothiols have been reported to vary with the age, and an increase in total homocysteine and cysteine, but not cysteinylglycine, was observed in older healthy subjects (Özkan et al., 2002). Amongst the factors influencing the plasma concentration of aminothiols is diet. A protein-rich meal led to fluctuations of some aminothiols, however, outcomes vary. While Ubbink et al. (1992) observed a decrease in plasma homocysteine, Guttormsen et al. (1994) and Verhoef et al. (2004) reported a rise of homocysteine, in a healthy young adult population. Additionally, Guttormsen et al. reported an accompanying decrease in concentrations of cysteine and cysteinylglycine .

Although these postprandial variations have been demonstrated and some metabolic studies have been done, although mainly focusing on homocysteine, it is still not fully understood how the habitual consumption of (poly)phenol-rich foods influence the metabolism of aminothiols in healthy adults, and no investigation has been done on how these are related to the bioavailability of phenolic acids present in coffee. Due to their implication in health and the lack of information, aminothiols are good candidates to assess the metabolic redox status by the analysis of plasma.

The most frequently studied aminothiol that has been linked to diet and to health is homocysteine, but a number of chromatographic methods exist for the simultaneous detections of homocysteine, cysteine, cysteinylglycine and glutathione. These four were also selected as being of particular interest for the present research

to investigate the impact of habitual consumption of (poly)phenol-rich products, with emphasis on coffee and other daily habits on the inflammation and cardiovascular health status.

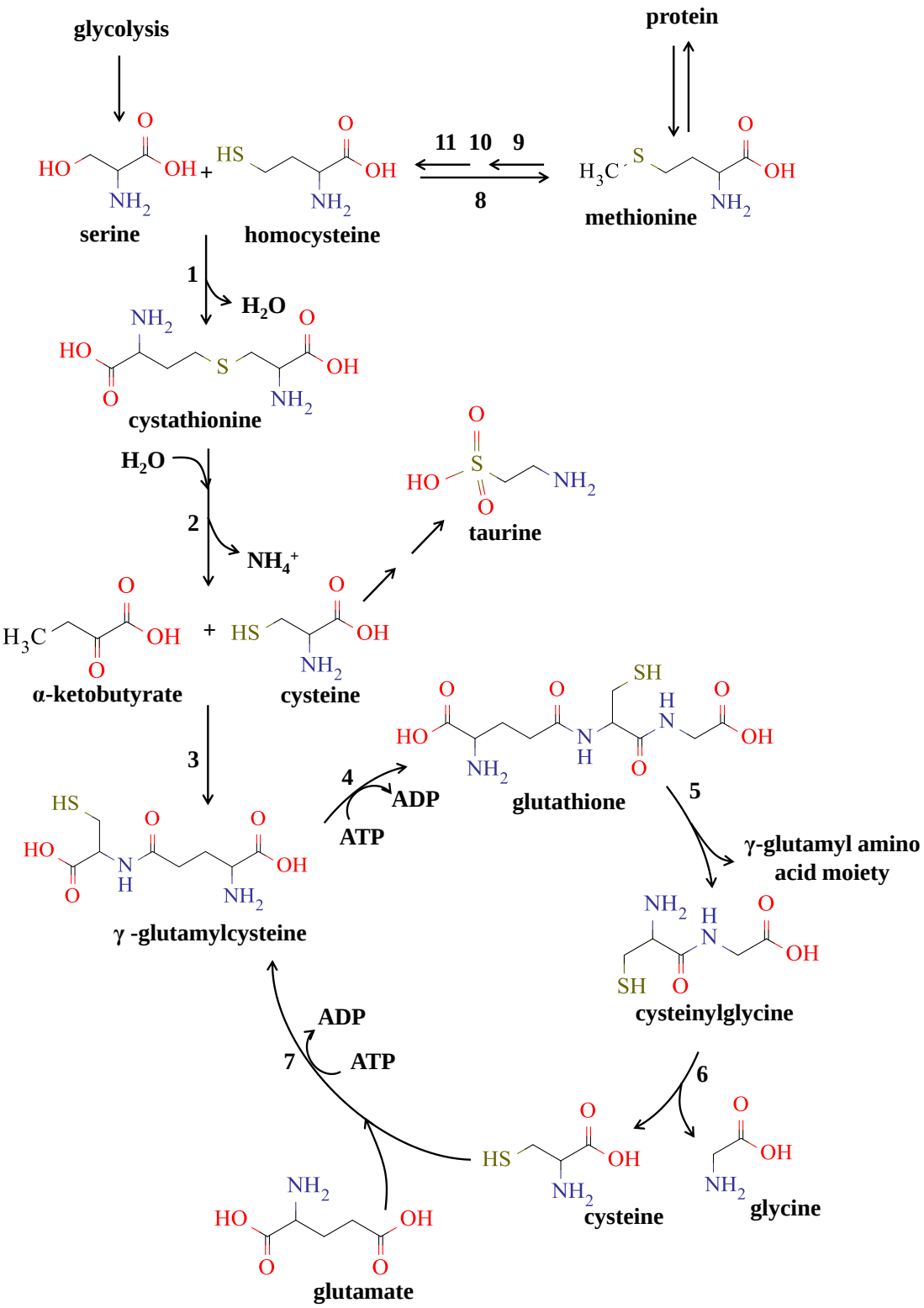


Figure 3.1: Transsulfuration pathway. The aminothiols of interest for the present study are linked together via the transsulfuration pathway, which occurs intracellularly. The enzymes involved in the reactions of the pathway are the vitamin B6-dependent *cystathionine β-synthase* (1) and *cystathionine γ -lyase* (2), *γ-glutamylcysteine synthetase* (3, 7), *glutathione synthetase* (4), *γ-glutamyltranspeptidase* (5), *dipeptidase* (6), *methionine synthase* (8), *methionine S-adenosyltransferase* (9), *glycine N-methyltransferase* (10) and *S-adenosylhomocysteine hydrolase* (11).

3.2.1.1 Homocysteine

Homocysteine is the starting point in the transsulfuration pathway (Figure 3.1). It has the ability to inhibit the synthesis of nitric oxide and to promote oxidative stress, leading to inflammation (Mangoni and Jackson, 2002). As it has been recognized as an independent risk factor for cardiovascular morbidity, the clearance of homocysteine in the transsulfuration pathway is important to avoid its accumulation. In two randomized cross-over studies, heavy consumption of coffee led to increased levels of circulating homocysteine (Grubben et al., 2000, Urgert et al., 2000). Later, Olthof et al. (2001) tested in a random order the effect of a 7-day exposure of 2 g chlorogenic acid, 4 g black tea solids and 440 mg quercetin-3-rutinoside on the plasma concentration of homocysteine. When compared to the placebo treatment, fasting and 4-5 h postprandial levels of homocysteine significantly increased after the period of exposure to each supplement. Along with the studies of Urgert et al. and Grubben et al., the results of Olthof et al. suggest that chlorogenic acid partly contributes to the observed increase. The administered doses of supplement are equivalent to that found in 1.5 L strong coffee (2 g chlorogenic acid), 2 L strong black tea (4 g black tea solids) and 13 L black tea (440 mg quercetine-3-rutinoside) and are unlikely to be reached by the average population. Stolzenberg-Solomon et al. (1999) also reported an increase in fasting serum total homocysteine with higher coffee consumption, and a lower fasting total homocysteine with increased dietary protein intake, however, the study population consisted of elderly subjects with a narrow age range and therefore the influence of age could be masked.

The effect of tea consumption on homocysteine is more inconsistent, with both positive and negative correlations being reported (Jacques et al., 2001, Olthof et al., 2001). In a study of Hodgson et al. (2003), a regular moderate to high black tea consumption did not induce significant overall changes in serum total homocysteine, but increased the urinary excretion of 4-*O*-methylgallic acid. Subjects with an increase in 4-*O*-methylgallic acid in the lowest quartile had a significant decrease in total homocysteine, while those for which the increase was in the upper quartile had a significant increase. These results are consistent with the hypothesis that dietary (poly)phenols can elevate homocysteine concentrations by increased methylation reactions where a methyl group is transferred from S-adenosylmethionine to (poly)phenols (Olthof et al., 2001). Although data from the Hordaland homocysteine study reports interesting relations between coffee and tea consumption and other lifestyle habits, the analyses were performed on non-fasting blood and therefore the previous consumption of food could influence the homocysteine levels (Nygard et al., 1997, Nygard et al., 1998). The same observation applies to a few other studies (de Bree et al., 2001, Rasmussen et al., 2000).

In a study from Edgar et al., fasting total homocysteine in a mid-age male population was positively associated with total cysteine and total cysteinylglycine, and negatively associated with fruit and vegetable intake (Edgar et al., 2008).

3.2.1.2 Cysteine

Cysteine is part of the essential amino acids and is the most abundant aminothiol in plasma, with concentrations of 321 μM having previously been reported in healthy subjects (Andersson et al., 1999). Cysteine can be obtained from the diet and from homocysteine in a two-step transsulfuration process catalyzed by two vitamin B6-dependent enzymes, and is a precursor and a rate limiting factor for the synthesis of glutathione (Figure 3.1). Results for cysteine are contrasting and have led to divergent opinions in regards to its status in regards to the development of cardiovascular diseases. In a case-control study, higher levels of total cysteine were reported in atherosclerotic vascular disease patients, when compared to the control group, and a positive association between cysteine and total homocysteine was identified. Total cysteine was also positively associated with age, gender, BMI,

cholesterol, apolipoprotein, creatinine, triglycerides and folic acid, and negatively associated with smoking. It should be noted that medication and modifications in the lifestyle and dietary habits of case subjects may influence the measurements of biomarkers in plasma (El-Khairy et al., 2001, Graham et al., 1997). In accordance with this outcome, after adjustment for conventional risk factors of coronary heart disease, a more recent study reported no association between total cysteine and the increase in rate of myocardial infarction amongst postmenopausal women. After adjusting for age, fasting total cysteine was correlated with total homocysteine and BMI, and inversely with alcohol, in control women (Page et al., 2010). *In vitro* studies have also reported similar results, associating cysteine and homocysteine with cytotoxicity and the promotion of the detachment of human arterial endothelial cells in culture (Dudman et al., 1991).

Moat et al. reported different outcomes in an *in vivo* study where plasma levels of total cysteine and homocysteine were compared in hyperhomocysteinaemic patients, their heterozygous parents and healthy subjects. The analyses revealed that in case of hyperhomocysteinaemia, the oxidative stress could result not only from a direct cytotoxicity from homocysteine, but also from the associated decrease of plasma cysteine, and plasma total cysteine was suggested to be an independent predictor of plasma total antioxidant capacity in healthy and homocysteinaemic subjects (Moat et al., 2001).

When compared to homocysteine, little research has focused on the influence of dietary habits and lifestyle on plasma total cysteine. Analysis of plasma in the Hordaland homocysteine study suggested that coffee consumption was one of the main determinants of plasma cysteine, along with age, gender, BMI, diastolic blood pressure and total cholesterol. However, as earlier highlighted for homocysteine, analyses were done with non-fasting blood samples and previous consumption of food may influence the relationships. From the same study, no association was found between total cysteine and the risk of hospitalizations and death due to cardiovascular disease (El-Khairy et al., 2003). The various outcomes of studies on cysteine may be explained by the inconsistent adjustment for confounders.

3.2.1.3 Glutathione

Glutathione is a major intracellular antioxidant which assists in detoxification processes of xenobiotics and/or their metabolites amongst other functions (Wu et al., 2004). Although it is present in mM ranges in all cells, reaching up to 10 mM in the liver, the concentrations found in plasma remain within the μM ranges (Fraga, 2010). Glutathione plays an important role in the antioxidant defence, the metabolism of nutrients and in the regulation of cellular events. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), which comprise radical and non-radical species, are products of normal metabolic processes, such as aging, immune response or inflammation, but can also result from the exposure to external stresses such as radiation, smoking, pollutants, excessive exercise some drugs (Pham-Huy et al., 2008, Lobo et al., 2010). Most free radicals are unstable and very reactive, but when present at low concentrations, these are not harmful and are even helpful in the defense of the organism for the destruction of pathogens. However, their accumulation can lead to a state of oxidative stress, which plays a key role in the development of chronic and degenerative diseases, such as diabetes, inflammation or cardiovascular diseases (Wu et al., 2004, Pham-Huy et al., 2008). Characteristic oxidative stress processes include lipid peroxidation and oxidative DNA damage. An example of neutralization of a non-radical species by glutathione ($C_{10}H_{17}N_3O_6S$) is the formation of two water (H_2O) molecules from one molecule of hydrogen peroxide (H_2O_2). In this reaction, two molecules of reduced glutathione are oxidized, linked through a disulfide bond and two hydrogen (H^+) molecules are lost and become available for the formation of water, thus avoiding the formation of 2 hydroxyl radicals (•OH) from H_2O_2. The reduced form of glutathione is thus important to the neutralization or removal of ROS and RNS contributing to the balance of the redox status and thus, lower concentrations of glutathione would lead to a higher risk of oxidative stress and derived conditions.

In a study with male adolescents, serum total glutathione, along with total homocysteine, but not total cysteine, predicted parental coronary heart disease (Morrison et al., 1999). In an intervention study reported by Esposito et al. (2003), the consumption of 5 cups per day coffee (italian style) over a period of 7 days significantly increased the total plasma glutathione concentration. Although plasma samples were obtained 4 to 5 hrs following the breakfast, which could have an

impact on the aminothiol concentration, a subsequent period of coffee abstention of 7 days did not restore the glutathione concentrations measured before the exposure to coffee. This suggests that a coffee, when regularly consumed in moderate to high doses, has the potential of increasing the general plasma glutathione when regularly consumed. The increase in glutathione was, however, not accompanied by an increase in oxidative stress, implying that this was not part of a stress response.

3.2.1.4 Cysteinylglycine

Cleavage of glutathione by γ-glutamyltransferase leads to the formation of cysteinylglycine (Figure 3.1). Very scarce information is available on this aminothiol as a biomarker of health. So far, it has been positively correlated to oxidized LDL and myocardial infarction after controlling for some risk factors (Drogan et al., 2010). Only one publication, which studied the relation of cysteinylglycine, along with cysteine and homocysteine, with diet and some cardiovascular disease risk factors, was found. In this study, total cysteinylglycine was positively correlated with total homocysteine and total cysteine, as well as with diastolic blood pressure, and BMI. Contrary to total homocysteine, habitual coffee consumption and smoking did not seem to affect the baseline cysteinylglycine (Edgar et al., 2008).

Most studies with aminothiols have focused on homocysteine and glutathione and less or scarce information is known on the relationship of cysteine and cysteinylglycine with inflammation, cardiovascular health, diet and other lifestyle habits. Due to their implication in inflammation and cardiovascular health, aminothiols were interesting compounds to be included in the analyses of this research project. As extracellular concentrations of aminothiols may be affected by confounders, the identification of these and adjusting for the analysis is important to avoid drawing premature conclusions. In the transsulfuration pathway, the substrate-to-product ratios have been said to be a useful tool to study enzymatic activity, therefore the simultaneous analysis of several members of the transsulfuration pathway is advised (Mangoni et al., 2013). Unbalances in the transsulfuration pathway, as for other conditions, could be partly explained by reduced enzymatic activities that accompany aging, as well as an impaired renal function (Wilcken and

Gupta, 1979). In the study by Mangoni et al. (2013), the homocysteine-to-cysteine and glutathione-to-cysteinylglycine ratios were positively and negatively associated with the cardiovascular risk score, respectively, suggesting that a lower conversion of homocysteine to cysteine and a higher conversion of glutathione to cysteinylglycine may be important in the mechanisms leading to cardiovascular diseases.

3.2.2 Glucose

Normal standards of glycaemia vary slightly depending on the country, however, in 2003 the American Diabetes Association, often taken as a reference, redefined normal glucose levels. A healthy glycaemia is considered to be < 5.6 mM at fasting and < 7.8 mM two hours following a 75 g oral glucose load; this is also the cutoff adopted by the World Health Organization (WHO, 2006). Fasting levels of 5.6 to 7.0 mM typically reflect a pre-diabetic or impaired glucose tolerance state while values equal or above 7.0 mM are already defined as diabetes.

Circulating glucose derives endogenously from glycogenolysis and gluconeogenesis, and exogenously from the intestinal absorption of dietary sugars. Although blood glucose fluctuates upon ingestion of food, the range of circulating glucose remains relatively narrow as a consequence of the homeostatic events to counteract hyper- and hypoglycaemia. Glucose represents the principal source of energy for the brain and its homeostasis is thus essential to provide sufficient energy and prevent impaired cerebral function. On the other hand, prolonged elevated glucose concentrations may lead to cerebral damage and represent a risk for cardiovascular morbidity (Shrayyef and Gerich, 2010).

The homeostasis of glucose depends on the balance of the rates of increase of circulating glucose and the rate of clearance from the circulation. The two main, but not only, regulator hormones of blood glucose are glucagon and insulin. In the case of low blood glucose (e.g. fasting, physical activity, hypoglycaemia), the secretion of glucagon from the pancreatic α-cells into the bloodstream is stimulated by Ca^{2+} signals. The glucagon present in the bloodstream binds to receptors at the cellular surface of target organs, mainly the liver but also in the adipose tissue, triggering a series of intracellular reactions that result in an increased production of glucose,

through the breakdown of glycogen (glycogenolysis) and the *de novo* synthesis from non-carbohydrate carbon compounds (gluconeogenesis), and subsequent secretion into the circulation. Animal studies with dogs, often used due to the high level of translation to the human, have demonstrated that the compensatory glucose rise was mainly explained by glycogenolysis, rather than through gluconeogenesis (Ramnanan et al., 2011). At fast, the liver is the main organ where glycogenolysis and gluconeogenesis occur, but in case of extreme starvation, kidneys are also able to compensate for the disappearing glucose (Aronoff et al., 2004). Skeletal muscle also hosts glycogenolysis, however, due to the high activity of hexokinase present in muscle, glucose rapidly undergoes glycolysis to provide the muscle with the necessary energy, resulting in little glucose exiting into the circulation.

During the postprandial rise in blood glucose, a series of events are triggered to help maintain glucose at physiological levels. The increased sensing of glucose by pancreatic β-cells stimulates the release of insulin into the bloodstream. The latter inhibits the secretion of glucagon and consequently the glycogenolysis and gluconeogenesis (Aronoff et al., 2004). Induced by the insulin binding to insulin-receptors at the surface of skeletal muscles cells and adipose tissue, glucose is incorporated into these tissues, further contributing to the regulation of glycaemia. Once in the tissues, glucose is either stored as glycogen (glycogenesis) or undergoes glycolysis with the production of energy in form of ATP (Shrayyef and Gerich, 2010).

3.2.3 Insulin

Insulin belongs to the hormones regulating circulating glucose levels, and its production and secretion by the pancreatic β-cells are stimulated upon ingestion of food. Insulin is able to lower blood glucose by the combined action of the inhibition the secretion of glucagon by the pancreatic α-cells, the stimulated uptake of circulating glucose by insulin-sensitive tissues such as skeletal muscle cells and adipocytes, and the induction of glycogenesis in the liver.

At fasting, concentration of 12 - 150 pM have been measured in healthy subjects (Mercodia) and insulin is only secreted once glycaemia reaches values > 3.3 mM. Although insulin secretion is mainly controlled by the circulating glucose

concentration, plasma concentrations of glucagon like protein-1, gastric inhibitory polypeptide or particular amino acids, and parasympathetic stimulation via the vagus nerve can also stimulate insulin secretion.

Insulin is a relatively small protein constituted of two polypeptide chains held together by two disulfide bonds. Methods for analysing insulin comprise liquid chromatography linked or not to mass spectrometry, radioimmunoassay, enzyme linked immunosorbent assays, luminescent immunoassays, capillary electrophoresis.

3.2.4 Uric acid

Uric acid is a heterocyclic organic compound naturally present in the circulation. It is the end metabolic product in the catabolism of purine nucleoside and free bases in animals. Due to the lack of uricase in humans, the enzymatic conversion of uric acid into the highly soluble allantoin is absent and, versus those of other animals, high baseline levels of serum uric acid are observed (0.2 – 0.4 mM) (Sautin and Johnson, 2008), with males having generally higher levels of uric acid (normal high cutoff: < 0.416 mM) than females (< 0.297 mM). Uric acid predominantly circulates as monosodium urate due to the high levels of sodium in the extracellular fluid. The consequence is a low solubility of approximately 0.4 mM, when compared to that of uric acid of 7.1 mM (Terkeltaub et al., 2006). Above 0.4 mM and at 37 °C, the risk of deposition of monosodium urate crystals in the joints increases and may chronically lead to the development of gout, a risk factor of cardiovascular disease (Richette and Bardin, 2012, Roddy and Doherty, 2010).

The plasma concentration of uric acid is determined by the balance of its endogenous hepatic and to a lesser extent small intestinal synthesis, by its production from the reutilization of purine compounds and by the consumption of purine-rich diet (i.e. animal-based foods particularly organ meats, anchovies, mackerel, seafood), and its clearance (i.e. renal and intestinal uricolysis by gut bacteria). The major cause of hyperuricemia and gout is the lower level of uric acid renal elimination (Snaith and Scott, 1971, Terkeltaub, 2010, Sautin and Johnson, 2008, Terkeltaub et al., 2006). Clearance of uric acid is in turn modulated by the genetics, aging, medication and by the consumption of certain foods, particularly alcohol (Bieber and Terkeltaub, 2004). Little research has been done in regards to

coffee consumption and uric acid in plasma and outcomes have been variable depending on the type of study conducted. In a study by Natella et al. (2002), an acute increase in plasma uric acid was reported after coffee and tea consumption. In a more recent research using data from a cross-sectional study with a representative sample of US adults (Third National Health and Nutrition Examination Survey, NHANES-III), a significant inverse relation was observed, after adjustment for age, gender and other covariates, between coffee (but not tea) intake and circulating uric acid. In the same study, this relation was not associated with the intake of caffeine (Choi and Curhan, 2007), suggesting that compounds present in coffee, other than this methylxanthine, would be responsible for this inverse association. Similar observations were reported in a study by Kiyohara et al., with a middle-aged Japanese male population (Kiyohara et al., 1999).

Much of the research has focused on uric acid and gout. However uric acid is also associated with the metabolic syndrome and some other risk factors for cardiovascular disease (Roddy and Doherty, 2010, Kim et al., 2009, Richette and Bardin, 2010, Ruggiero et al., 2006, Gagliardi et al., 2009). The double bonds in the structure of uric acid (Figure 3.2) make it a compound with high antioxidant capacity but research indicates that it may act as both pro-oxidant and anti-oxidant.

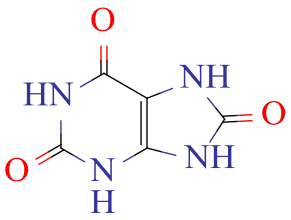


Figure 3.2: Structure of uric acid. Structural characteristics of a purine compound (i.e. a pyrimidine and an imidazole ring). The double bonds make uric acid a compound with high antioxidant capacity.

Uric acid has been associated with and has been said to predict the development of conditions that may lead to cardiovascular events (Johnson et al., 2003, Alderman et al., 1999), such as hypertension (Johnson et al., 2003, Johnson et

al., 2005, Alper et al., 2005), visceral obesity (Masuo et al., 2003, Ogura et al., 2004), insulin resistance, T2DM (Samant et al., 2012), dyslipidemia (Matsubara et al., 2002). High but still physiologically possible concentrations of uric acid increased the oxidation of low density lipoproteins and liposomes by peroxynitrite (Sautin and Johnson, 2008). Uric acid has also been said to act as an anti-oxidant. In a study with rats, uric acid had a protective effect on neuronal cells and decreased the brain injury when administered 24 hours prior an acute ischemia induced by occlusion of the middle artery. Similar results were observed *in vitro*, by the same group (Sautin and Johnson, 2008). Further i*n vitro* studies have demonstrated the ability of uric acid for scavenging the singlet oxygen, peroxyl radicals ($RO^{\cdot}_2$) and hydroxyl radicals ($^{\cdot}OH$) (Ames et al., 1981). Several researches have also proposed anti-oxidant effects in the central nervous system (Duan et al., 2002, Hooper et al., 1998, Spitsin et al., 2002). In an animal model, physiological concentrations of uric acid improved the impaired functional responses and functional stability of the heart due to oxidants (Sautin and Johnson, 2008).

The environment and interactions with certain compounds can influence whether uric acid has or not a detrimental effect on the organism. Scavenging of lipophilic radicals by uric acid is for example impeded in a hydrophobic environment, such as within lipid membranes (Muraoka and Miura, 2003), while in the hydrophilic environment of plasma, the anti-oxidant ability of uric acid is favoured (Frei et al., 1988). As for the interacting compounds, ascorbic acid and aminothiols were shown to be necessary to the scavenging activity of uric acid on peroxynitrite ($ONOO^{-}$), in the extracellular space (Kuzkaya et al., 2005). Tyrosine nitrosylation, a post-translational modification of proteins leading to oxidative damage of proteins in the cell, can be inhibited by uric acid. This process is however inhibited (Whiteman et al., 2002) in presence of physiological amounts of bicarbonate.

Overall, research suggests that chronic elevations of uric acid are associated with a pro-oxidant property of it and with increased stroke risk (Mazza et al., 2001) and acute elevations of the uric acid are associated with its anti-oxidant properties, by preventing acute activation of proinflammatory cells in the blood by oxidants. Also, its effect will depend on the environment in which it is present and which other present compounds it interacts with.

For the measurement of total aminothiols and glucose, improved methods were developed based on existing methods (Frick et al., 2003, Pfeiffer et al., 1999, Sigma-Aldrich, 2004). For the analysis of insulin, however, a commercially available method was used unchanged and tested for its repeatability and recovery. The method used for uric acid measurement was improved in the lab (Ms Y. Shi, unpublished results), and some modifications were made and reproducibility further tested here. The use of fasting plasma for the analyses of the current research reflects the general status of a subject, and also eliminates any postprandial influence on the measured biomarkers that are known to fluctuate after a meal. Altogether, improved method for the analysis of biomarkers in plasma were sought, aiming at a good separation (applying to aminothiols, only), repeatability, good recovery and time efficiency of analysis.

3.3 Material and methods

3.3.1 Chemicals

The water used was deionized Millipore water, purified in a filtration system from Millipore UK Ltd. (Hertfordshire, UK).

For the quantification of total thiols, cystamine dihydrochloride, cysteamine (≥ 98 %), L-cysteine (≥ 98 %), cysteinylglycine (≥ 85 %), reduced L-glutathione (≥ 98 %), D,L-homocysteine (≥ 95 %), acetic acid (99 - 100 %), sodium acetate (BioXtra, ≥ 99.0 %), boric acid (≥ 99.5 %), trichloroacetic acid, EDTA and PBS were purchased from Sigma-Aldrich. Sodium hydroxyde (50 % concentrated solution) and SBD-F were obtained from Fluka-analytical, analytical reagent-grade hydrochloric acid (~ 36 %) and HPLC-grade methanol from Fisher Scientific, and TCEP-HCl from Thermoscientific.

For the quantification of glucose in plasma, the glucose hexokinase reagent, formic acid, glyceraldehyde and sodium fluoride were purchased from Sigma. D-glucose, HPLC-grade methanol, HPLC-grade ethanol and Decon 90 were from Fisher Scientific.

For the quantification of insulin in plasma, the insulin ELISA assay for 10 x 96 determinations (10-1113-10) and the diabetes antigen controls (10-1134-01) were

purchased from Mercodia, HPLC-grade ethanol and Decon 90 were from Fisher Scientific.

For the quantification of uric acid, potassium monohydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), potassium hexacyanoferrate (II) trihydrate (98.5 - 102.0 %), 4-aminophenazone (4-aminoantipyrine), horseradish peroxidase, ascorbate oxidase from *Cucurbita* sp., sodium 3, 5-dichloro-2-hydroxybenzenesulfonate, lithium carbonate (≥ 99.0 %), uricase from *Candida* sp. and uric acid were all purchased from Sigma. Triton X-100, ethanol and Decon 90 were from Fisher Scientific.

3.3.2 Methods

Prior to each experiment, the working space was covered with a protective benchcote, and at the end of each experiment, the working area was cleaned with diluted Decon 90 (1:9, V:V) followed by 70 % EtOH.

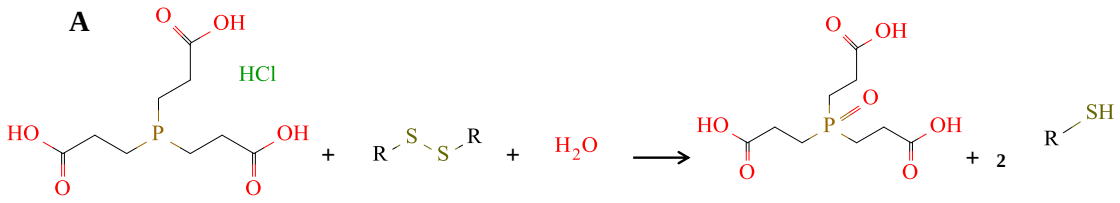
3.3.2.1 Blood collection and preparation of plasma aliquots

To optimise the sample analysis, the procedures were first tested with standards in water and later in plasma samples. All blood samples were drawn into EDTA-containing vacuum tubes (Vacuette, Greiner Bio-One) by venepuncturing the antecubital vein of healthy adult subjects that had fasted for at least 10 hours. Ten milliliters of whole blood was collected from each subject. To reduce the ongoing metabolism of cells, the blood collection tubes were placed on ice as soon as the blood samples were withdrawn. Samples were then rendered acellular within 30 min through a 10 min centrifugation at 3,000 g and 4 °C in a *5810R* eppendorf centrifuge, as frequently done (Ubbink et al., 1992). For the analysis of glucose, 500 µL EDTA-whole blood was transferred to a microcentrifuge tube containing glyceraldehyde and sodium fluoride (see section 3.3.3.4 for the preparation of these antiglycolytic agents) (le Roux et al., 2004), within 15 min of venepuncture, well mixed by inversion and centrifuged for 10 min, at 3,000 g and room temperature. The plasma was carefully separated without disturbing the buffy coat (leucocytes and platelets) and the erythrocytes. Two aliquots for the measurement of each

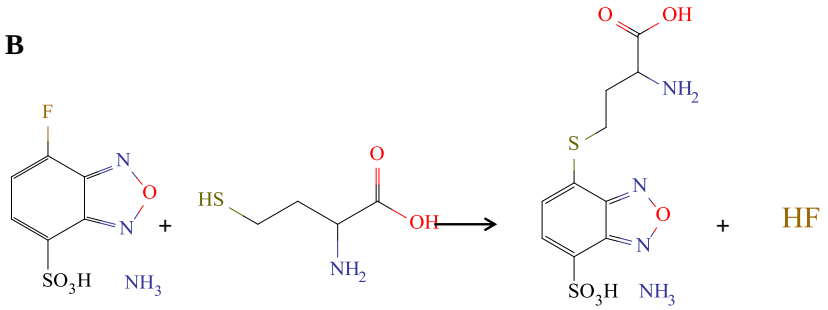
biomarker were prepared and each contained the sufficient volume necessary for running one assay in triplicate. All aliquots were then stored at -80 °C until analysis.

3.3.2.2 Total aminothiols

Principle of the method: Sample processing and aminothiol analysis in this research were based on a previously described method by (Pfeiffer et al., 1999), however some optimization was done to adapt to the equipment available and improve the aminothiol chromatographic separation. In plasma, aminothiols occur as a free form or disulfide-bound to other (amino)thiols or proteins, and so, for the quantification of total aminothiols in plasma, an initial reduction of the disulfide bonds is required. This critical step was achieved by incubating the samples with the water-soluble tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCL) (Figure 3.3, A). The latter is a thiol-free stable reducing agent having the advantages of being odorless, selective to disulfide bonds, irreversible and efficient at room temperature (Krijt et al., 2001, Scientific, 2013). Proteins were then acid-precipitated and removed from the reduced sample and aminothiols were derivatized with 4-fluoro-7-sulfobenzofurazan (SBD-F) (Figure 3.3, B). For a better separation and specificity, analysis using HPLC with fluorescence detection was chosen.



TCEP-HCl



SBD-F **homocysteine** **fluorescent adduct**

Figure 3.3: Reduction of bound aminothiols by tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCL) (A) and derivatization reaction of thiols by 4-fluoro-7-sulfobenzofurazan (SBD-F) (B).

Preparation of reagents and solutions: All the reagents other than the aminothiols standards were prepared and able to be kept at their ideal storage temperature for up to 2 weeks, after which they were discarded, if unused. Aliquots and reagents stored at -20 °C were kept for a longer period of a few months. Solutions of PBS (9.55 g/L, pH 7.4); borate buffer (0.125 M, pH 9.5); borate buffer (0.125 M) containing EDTA (4 mM), pH 9.5; TCA (100 g/L) containing EDTA (1 mM); HCl (1 M); NaOH (1.55 M); NaOAc (0.1 M) and AcOH (0.1 M) were prepared in milliQ water. NaOAc (0.1 M) and AcOH (0.1 M) were used to prepare NaOAc/AcOH (0.1 M, pH 4.99) buffer solution, which in turn was used to prepare the mobile phase for the chromatographic analysis, NaOAc/AcOH (0.1 M) containing MetOH (0.125 %), pH 4.99. EDTA (250 mM) was prepared in NaOH (1.5 M). The PBS, EDTA, borate buffers and TCA solutions were stored at 4 °C, while NaAc, AcOH, NaAc/AcOH, NaOH and HCl solutions were kept at room temperature. TCEP-HCl (100 g/L) aliquots of 1 mL were prepared in milliQ water and stored at -20 °C. Aliquots of 50 µl SBD-F (1 g/L) were prepared in borate buffer (0.125 M), pH 9.5 and stored at -20 °C until needed for derivatization. Concentrated stocks of aminothiol standards were freshly prepared on each day of analysis and used for the calibrator curves and plasma samples preparation. For the calibrator curves prepared in plasma, stocks of L-cysteine, cysteinylglycine, L-glutathione and cystamine dihydrochloride, used as the internal standard, were prepared in milliQ water at 37.6, 18.8, 9.4 and 1 mM respectively, while a 9.4 mM stock of D, L-homocysteine was prepared in HCl (1 M). These stocks, except for the cystamine dihydrochloride, were further diluted in either milliQ water or 1 M HCl to final concentrations of 18.8 mM L-cysteine, 9.4 mM cysteinylglycine, 4.7 mM L-glutathione and 4.7 mM D,L-homocysteine. These later stocks were used for the preparation of the calibration curves in milliQ water and spiking samples. Cystamine dihydrochloride was diluted to a 225.6 µM stock, which was used for the standards

and samples preparations. The final concentration of cystamine dihydrochloride in the samples was kept constant at 6 µM.

Preparation of standard mixes: For each experiment, aminothiol calibration curves were prepared in milliQ water as well as in the biological matrix (plasma). For the preparation of the calibration curve in plasma, standard mixes were prepared by serial dilutions as follows. An intial combined aminothiols stock Mix_{2x} 4 was obtained by mixing 100 µL of each previously described 37.6 mM L-cysteine, 18.8 mM cysteinylglycine, 9.4 mM L-glutathione and 9.4 mM D,L-homocysteine stocks. From it, serial dilutions with PBS (9.55 g/L, pH 7.4) were done to achieve the desired concentrations of Table 3.1. Briefly, Mix_{2x} 3, Mix_{2x} 2 and Mix_{2x} 1 were prepared by diluting 30 µL Mix_{2x} 4 with 90 µL PBS (9.55 g/L, pH 7.4), 20 µL Mix_{2x} 3 with 980 µL PBS (9.55 g/L, pH 7.4) and 10 µL Mix_{2x} 2 with 90 µL PBS (9.55 g/L, pH 7.4), respectively. For the preparation of the calibration curve in milliQ water, the earlier described Mix_{2x} 4 was diluted 1:1 (v:v) with PBS (9.55 g/L, pH 7.4) to obtain the initial combined aminothiol stock Mix_{1x} 4. The latter was serially diluted for the preparation of Mix_{1x} 3, Mix_{1x} 2 and Mix_{1x} 1 by mixing 30 µL Mix_{1x} 4 with 90 µL PBS (9.55 g/L, pH 7.4), 20 µL Mix_{1x} 3 with 980 µL PBS (9.55 g/L, pH 7.4) and 10 µL Mix_{1x} 2 with 90 µL PBS (9.55 g/L, pH 7.4), respectively. Mix_{2x} 0 and Mix_{1x} 0, which contained no aminothiol standard, consisted of milliQ water and were used for the null concentration calibrator point in both types of calibration curve. Mix_{1x} 4 was used for spiking the plasma samples. Final concentrations of each aminothiol standard (Std 0 - Std 4) are summarized in Table 3.1).

Table 3.1: Concentrations of aminothiol standards in the different mixes (Mix_{2x} 0-4 and Mix_{1x} 0-4) used for the preparation of the calibration curves in milliQ water and in the biological sample (plasma). The aminothiol concentrations obtained in the calibration curves are described in the rows Std 0 to Std 4. L-Cys, cysteine; D,L-HCys, homocysteine; CysGly, cysteinylglycine; L-GSH, glutathione.

	L-Cys (µM)	D,L-HCys (µM)	CysGly (µM)	L-GSH (µM)
Mix_{2x} 0/ Mix_{1x} 0	0	0	0	0
Mix_{2x} 1	4.7	1.175	2.35	1.175
Mix_{2x} 2	47	11.75	23.5	11.75
Mix_{2x} 3	2350	587.5	1175	587.5
Mix_{2x} 4	9400	2350	4700	2350
Mix_{1x} 1	2.35	0.5875	1.175	0.5875
Mix_{1x} 2	23.5	5.875	11.75	5.875
Mix_{1x} 3	1175	293.75	587.5	293.75
Mix_{1x} 4	4700	1175	2350	1175
Std 0	0	0	0	0
Std 1	0.1	0.025	0.05	0.025
Std 2	1	0.25	0.5	0.25
Std 3	50	12.5	25	12.5
Std 4	200	50	100	50

Sample preparation and treatments: For the calibration curve in milliQ water, 35 µL PBS (9.55 g/L, pH 7.4), 25 µL cystamine dihydrochloride (225.6 µM), 40 µL standard mix (Mix_{1x} 0-4) and 10 µL TCEP-HCl (100 g/L) were mixed in five microcentrifuge tubes. A pool of combined plasma, prepared by mixing an equal volume of each plasma sample to be tested in a batch, was used for the preparation of the calibration curve in plasma and for the spiked plasma samples. For the calibration curve in plasma, 5 µL PBS, 25 µL cystamine dihydrochloride, 50 µL combined plasma, 20 µL standard mix (Mix_{2x} 0-4) and 10 µL TCEP-HCl were mixed. The spiked plasma samples were prepared by mixing 5 µL PBS, 25 µL cystamine dihydrochloride, 50 µL combined plasma, 20 µL standard mix (Mix_{1x} 4) and 10 µL TCEP-HCl. Finally, non-spiked plasma samples were prepared by mixing 25 µL PBS, 25 µL cystamine dihydrochloride, 50 µL plasma and 10 µL TCEP-HCl. All samples were briefly vortex-mixed, spun at 13,000 g for up to 3 seconds and

then reduced for 15 min in a 37 °C and 130 rpm shaking waterbath incubation (GLS Aqua Plus Grant). Samples were cooled to room temperature, and deproteination was achieved by addition of 90 µL TCA (100 g/L) containing EDTA (1 mM) and vortex-mixing each sample for 1 min. Precipitated proteins were pelleted in a 10 min, 13,000 g centrifugation at 4 °C, in a *5810R* eppendorf centrifuge. Carefully avoiding disturbance of the pellet, 50 µL from the supernatant was added to 50 µL SBD-F (1 g/L) and 127 µL borate buffer (0.125 M) containing EDTA (4 mM), pH 9.5 and 8 µL NaOH (1.55 M). Five randomly picked samples were used to certify that the pH for derivatization was between 9 and 10. Samples were subsequently briefly vortex-mixed, spun and incubated in the dark for 60 min, at 60 °C and 130 rpm, in a shaking waterbath. Samples were then allowed to cool down to room temperature, centrifuged for 10 min, at 4 °C and 13,000 g. Finally, derivatized samples were filtered through a 0.2 Å PTFE filter, into amber vials for HPLC analysis.

Sample analysis: HPLC analysis was carried out using an Agilent 1200 series HPLC equipped with a fluorescence detector. Separation of the fluorescent SBD-F-aminothiol adducts was achieved on a Spherisorb, ODS1 (C18), 5 µm particle size, 4.6 mm internal diameter and 250 mm length, 8 µm pore size analytical column from Waters. A volume of 10 µL sample was injected and run at 0.6 mL/min using a 22.5 min isocratic gradient of 0.1M NaOAc/AcOH containing 0.125 % MetOH, pH 4.99, with the column compartment temperature maintained at 29 °C. Detection of the adducts was at the excitation wavelength of 385 nm and emission wavelength of 515 nm. The chromatographed standard samples were used to construct the calibration curves from which the concentrations of each aminothiol were calculated according to the normalized areas under the curve.

Limits of detection were calculated as the amount of SBD-F-aminothiol adducts for which the signal to noise ratio equals 10. Limits of quantification were the amounts of SBD-F-aminothiol adducts for which the signal to noise ratio equals 3. Recoveries were calculated as the percentage of the ratio of the obtained normalized area under the curve to the expected normalized area.

Repeatability: The inter-day variability was tested with a same single standard sample prepared in milliQ water and run on three consecutive days. To keep the conditions identical to those of an analytical run, the samples were stored

between the repeatability tests in amber vials and in the HPLC autosampler compartment set to 4 °C. As no run lasted more than 48 h, testing the repeatability and stability for three days was considered to be sufficient.

3.3.2.3 Glucose

Principle of the method: The measurement of glucose was one of the first clinical tests available. Techniques such as the analysis of capillary blood collected by finger prick analysed on glucose oxidase/peroxidase strips, or an electronic device allow oneself to monitor glycaemia. Other methods run in laboratories take advantage of different properties of the analyte and include chromatographic, reducing, condensation, enzymatic or electrochemical methods (Noiphung et al., 2013, Schierbeek et al., 2009, Carroll et al., 1970). The method using hexokinase is a reliable enzymatic technique often used not only for food, but has also been used with biological samples (Giampietro et al., 1982). For the analysis of D-glucose in fasting plasma, the glucose hexokinase reagent mix from Sigma was used (Sigma-Aldrich, 2004). This commercial method is described for the usage of cuvettes, requiring a relatively large plasma volume. The method was thus adapted for the usage of 96-microwell plates. The hexokinase method was developed by the American Association of Clinical Chemistry and Centres for Disease Control and has been accepted as the reference method for glucose determination (Schaffer, 1976). The method is based on the detection of NADH produced at the end of a series of reactions, starting with the phosphorylation of glucose into glucose-6-phosphate, which is subsequently oxidized by glucose-6-phosphate dehydrogenase. In this final reaction, an equimolar amount of NAD^+ is reduced into NADH which is then detected at 340 nm (Figure 3.4). The provided hexokinase reagent is available as a lyophilized mixture of non-reactive stabilizing agents (i.e. sodium benzoate, potassium sorbate), fillers and buffer (pH 7.5), as well as a mixture of 1 mM ATP, 1,000 U/L yeast hexokinase, 1.5 mM NAD^+, 1,000 U/L bacterial glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*), 2.1 mM Mg^{2+} and sodium azide (0.05 %) when reconstituted in the correct volume of milliQ water.

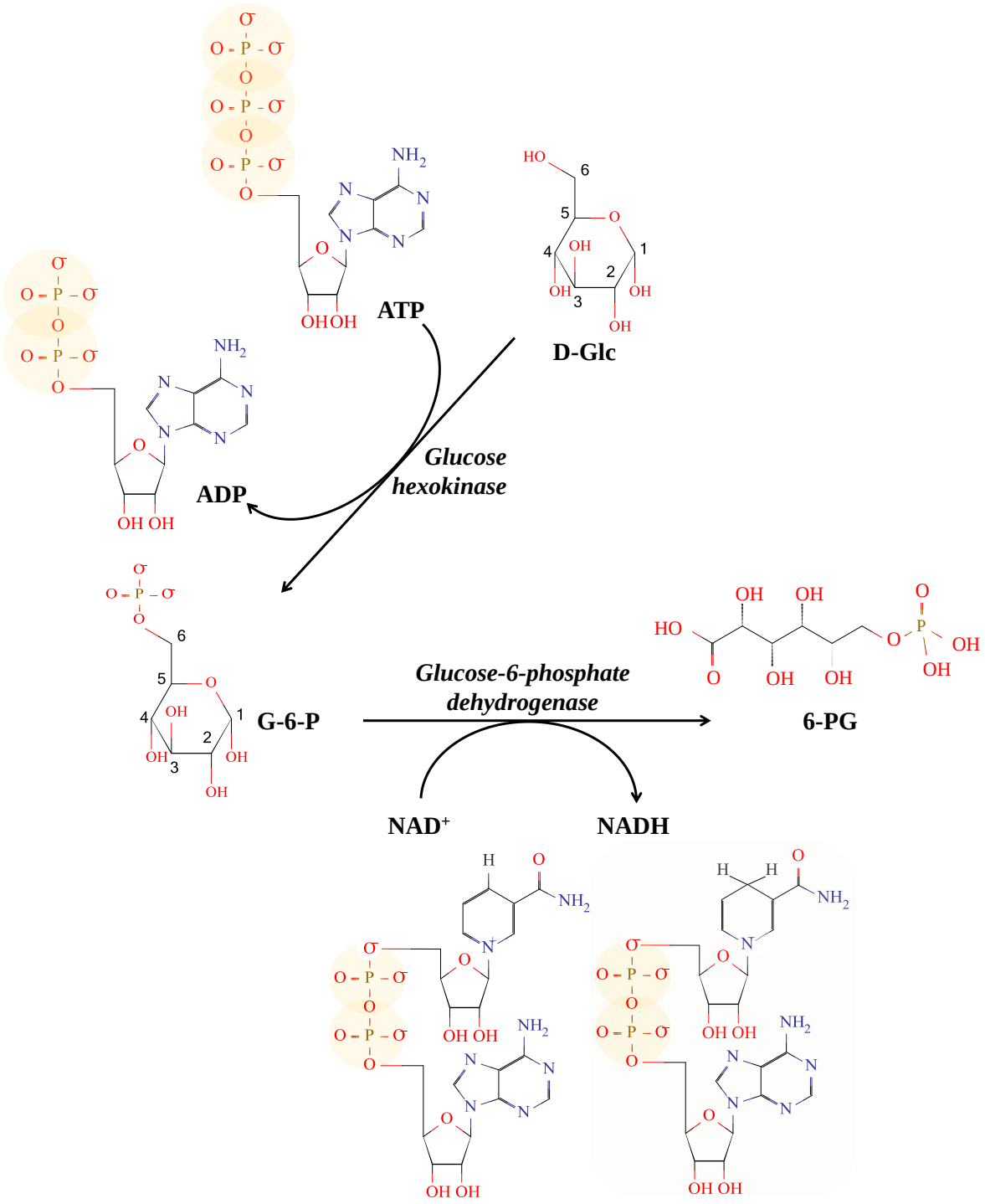


Figure 3.4: Reactions involved during the incubation of the glucose-containing sample with the hexokinase reagent. ATP, adenosine triphosphate; G-6-P, glucose-6-phosphate; 6-PG, 6-phosphogluconate; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide.

Antiglycolytic agents: Once blood is collected, glucose continues undergoing glycolysis, unless the reaction is inhibited. A loss of 5 to almost 14 % per hour has been measured in whole blood (McMillin, 1990). An underestimation of the glycaemia can result from measuring glucose in a plasma sample which has not been treated with antiglycolytic agents or not immediately rendered acellular. The use of sodium fluoride has long been shown to be inefficient within the first hour as an antiglycolytic agent on its own (Chan et al., 1990, de Pasqua et al., 1984). In 2004, Le Roux et al. tested a few antiglycolytic agents, and data indicated that the use of 11 mM glyceraldehyde with 119 mM sodium fluoride, but not either of them alone, appeared to give the best antiglycolytic results. With this combination, uncentrifuged blood samples did not show any glucose loss due to glycolysis, even after 24 hours incubation at room temperature (le Roux et al., 2004). All the blood for this study was drawn into EDTA-vacutainer tubes, but no antiglycolytic agent was present (section 3.3.3.1). For this reason, aliquots of glyceraldehyde together with sodium fluoride were prepared and dried, so that upon addition of 500 µL whole EDTA-blood, 11 mM glyceraldehyde and 119 mM sodium fluoride were present in the sample. To minimize the error by weighing small amounts, both antiglycolytic agents were weighed out in a quantity that was sufficient to prepare 200 aliquots. With a solubility of 50 mg/mL (0.55 M) in water, 99.1 mg glyceraldehyde were weighed and reconstituted in 6 mL milliQ water. With a solubility of 41.99 mg/mL (1 M) in water, 499.7 mg sodium fluoride were weighed and reconstituted in 20 mL milliQ water. From these glyceraldehyde and sodium fluoride stocks, 30 µL and 100 µL respectively were mixed into microcentrifuge tubes and dried in an EZ2+ centrifugal evaporator from Genevac (HCl compatible) using the aqueous setting and 35 °C until total evaporation.

Original method and applied modifications: The commercially available assay from Sigma using hexokinase recommends the usage of cuvettes and a spectrophotometer, however, a few trials (data not shown) using the original protocol by Sigma (Sigma-Aldrich, 2004) rapidly lead to the conclusion that the use of cuvettes was not ideal, due to the duration of the experiment, the irreproducibility of the readings as well as the high volumes of sample and reagent required. In order to overcome the difficulties encountered with the use of cuvettes, an improved method using 96-microwell plates was sought. Running the glucose hexokinase

assay in a UV-compatible 96-microwell plate would allow the simultaneous analysis of numerous samples. In order to run the hexokinase analysis in a 96-microwell plate, a few modifications were done to the procedure recommended by Sigma. The latter does not include specifications for the analysis of plasma, however, the sample to be analysed should have a glucose concentration comprised between 0.27 to 27.75 mM (Sigma-Aldrich, 2004). The healthy fasting glucose concentration ranges from 3.87 to 7.22 mM, therefore no dilution would be required for the analysis of fasting plasma from healthy subjects. However, in order to include spiked samples in the analyses, a 1:1 (v:v) dilution with milliQ water was implemented. This would still allow the diluted samples to be higher than the lower healthy limit, and the spiked samples to be lower than the higher healthy limit.

If performed in cuvettes, 1 mL hexokinase reagent (or milliQ water, for sample blanks) is added to 10 to 200 µL sample (Sigma-Aldrich, 2004). The 96-microwell plates used (Greiner UV-Star®) had a maximal well volume of 392 µL, and the hexokinase reagent and milliQ water volume was reduced to 250 µL, which would allow plate shaking avoiding spillage. Due to the limited plasma volume available, 10 µL sample volume was used for each reaction. This would correspond to using 40 µL sample with 1 mL hexokinase reagent or milliQ water, if cuvettes were used, which is within the sample volume required.

Plasma pre-treatment: A few preliminary tests were done to determine the ideal sample processing leading to the best results with the hexokinase assay. These were conducted with D-glucose standard solutions as well as with plasma samples. Plasma contains proteins and fatty constituents and, depending on the target analytes, the analytical equipment and the chemical reactions, these may interfere with some methods of analysis. In the present experiments, the wish was thus to precipitate proteins and defat the plasma samples.

Protein precipitation: A few methods for precipitating proteins are known, including the use of ice cold ethanol, trichloroacetic acid and perchloric acid. For the precipitation of proteins, ice cold ethanol was used. It leads to aggregation and subsequent precipitation of proteins by increasing the ionic attraction to each other (Sedgwick et al., 1991). In addition to being relatively harmless, it can easily be eliminated by evaporation, thus avoiding further sample dilution an associated measurement errors. The reference method for protein precipitation by ethanol was

adapted from a method for DNA cleanup from the International Genetically Engineered Machine (IGEM, 2011). Nine volumes of ice cold ethanol (4,500 µL) were added for each volume of sample (i.e. plasma) (500 µL), samples were then vortex-mixed for 1 min, incubated for 45 min at - 20 °C and centrifuged 15 min, at 13,000 g and room temperature. The supernatants were carefully recovered and dried in an EZ2+ centrifugal evaporator from Genevac (HCl compatible) using the HPLC fraction setting until total evaporation. The dried samples were reconstituted in a quarter of the original plasma volume (125 µL), centrifuged 15 min at 13,000 g and room temperature to remove any particulate matter, and transferred to clean microcentrifuge tubes for comparative analysis with and without solid phase extraction.

Defat: Solid phase extraction was tested as a method for sample defatting. Ten milligrams sorbent/mL strata-X 33 µm polymeric reversed phase columns from Phenomenex were used. Prior to each experiment, the columns were conditioned following the recommendations with 1 mL MetOH (100 %) followed by 1 mL milliQ water. A volume of 250 µL sample (5.55 mM D-glucose solution or plasma sample) was passed through the SPE column and the fractions of 1 mL MetOH (60 %) and twice 500 µL formic acid (2 % in MetOH) were collected and analysed.

Assessment of sample loss: In order to assess any sample loss and determine the best sample processing, a 5.55 mM D-Glc standard solution and a plasma sample were subject to SPE. The glucose concentration of all collected SPE fractions was measured and compared to that of an untreated 5.55 mM D-Glc standard solution and untreated plasma sample used as a reference.

Improved modified method: The lyophilized hexokinase reagent was reconstituted in the appropriate volume of milliQ water. Each reconstituted reagent was stored at 4 °C and used within one week. On the experiment day, the reagent was brought to room temperature before preparing the samples in the 96-microwell plate.

D-glucose standard samples preparation: Aliquots of 8.33 mM D-glucose stock solution in milliQ water were prepared and stored at -20 °C until analysis. These were thawed once and used to prepare a 6-point standard curve freshly for

each analysis. The final concentrations of 8.33, 5.55, 2.78, 1.39, 0.14 and 0 mM were prepared as described on Table 3.2 by serial dilutions with milliQ water.

Table 3.2: Protocol for the preparation of the glucose standard solutions by serial dilutions.

Final glucose concentration		**Concentration of the glucose solution used (mM)**	**Volume of the glucose solution used (mL)**	**Volume of milliQ water added (mL)**
(mM)	**(mg/mL)**			
8.33	1.50	8.33	1.00	0.00
5.55	1.00	8.33	1.00	0.50
2.78	0.50	5.55	0.50	0.50
1.39	0.25	2.78	0.50	0.50
0.14	0.025	1.39	0.05	0.45
0.00	0.00	0.00	0.00	1.00

Plasma samples preparation: Plasma aliquots containing a combination of 11 mM glyceraldehyde and 119 mM sodium fluoride were thawed and centrifuged for 10 min, at 3,000 g and 4 °C. The spiked and non-spiked samples used for the calculation of recovery were prepared from a pool of combined plasma from the samples analysed in a same batch. A volume of 20 µL from each plasma sample was combined after thawing, gently but well mixed, and centrifuged as described above. Sixty microliters of the combined plasma was then diluted with an equal volume of either milliQ water or D-glucose standard solution (2.75 mM) for the preparation of the non-spiked and spiked plasma sample respectively.

Analytical procedure: Each plasma sample from the human study was analysed in quadruplicate. Duplicates were prepared for each sample on each plate and two analyses were done on separate days. Each reaction was prepared directly in the 96-microwell plate. A volume of 260 µL milliQ water was used as the blank (Bl). The reagent blank (RB) was prepared by adding 250 µL HK reagent to 10 µL

milliQ water and the standard and sample blanks (SSB) were prepared by adding 250 µL milliQ water to 10 µL glucose standard solution or 10 µL plasma, respectively. Standard and sample tests (SST) were prepared by adding 250 µL hexokinase reagent to either 10 µL glucose standard solution or plasma sample. The 96-microwell plate was covered with an adhesive sealing membrane, to minimize the sample loss by evaporation, and incubated at 30 °C for 15 min. The sealing membrane was carefully removed, the plate shaken for 15 sec under medium speed and a double-orbital movement setting, and readings were taken at 340 nm in a PHERAstar® *FS* microplate reader.

In order to determine the final glucose concentration from each sample, a few corrections were made. The reading of the water blank (Bl) was first subtracted from all reagent, standard and sample blank as well as standard and sample test readings to obtain the corrected values of these, described respectively as RB_{corr}, SSB_{corr} and SST_{corr}. The total blank ($Blank_{tot}$) resulting from the sum of both SSB_{corr} and RB_{corr} was subtracted to all SST_{corr} values to obtain the final corrected reading (Δ A 340 nm). The final Δ A 340 nm values from the D-glucose standard solutions were used to create the calibration curve, and the equation from the linear regression line was used to calculate the sample concentrations taking into account any dilution (1:1, V:V for all plasma samples). As the spiked samples were spiked with an equal volume of 2.78 mM D-glucose standard, the expected concentration increase was equivalent to 1.39 mM.

The inter-assay variability was assessed using the coefficient of variation calculated from the average reading and the standard deviation obtained for one same plasma sample assayed on three separate days.

3.3.2.4 Insulin

Principle of the method: Due to their specificity and relatively short time of analysis, the most commonly used methods for the analysis of insulin are enzyme linked immunosorbent assays (ELISAs). For this reason, a commercially available and already validated ELISA method was used for the analysis of insulin in the fasting plasma samples. Although minimal lot-to-lot variation is guaranteed by Mercodia, all the assay kits purchased for the analyses were from the same batch.

The detection of insulin in this method is based on the recognition of particular antigens specific to insulin. In the sandwich ELISA simultaneous assay used, the sample and enzyme-linked detection antibody are incubated simultaneously. The insulin-containing samples are first added to a 96-microwell plate precoated with primary, coating antibodies, specific to an antigen on the insulin molecule. A peroxidase-linked secondary antibody is then added and binding to a different antigen on the insulin occurs. The subsequently added 3, 3', 5, 5'-tetramethylbenzidine reacts with the peroxidase and the resulting chromogenic signal is monitored spectrophotometrically at 450 nm.

Both the primary and secondary antibodies used in this assay were monoclonal, which generally guarantees an analysis of high accuracy and avoids cross-reactions with similar molecules, such as pro-insulin, its biosynthetic precursor.

The reproducibility between assays was monitored by the inclusion of high and low concentration insulin controls in the analysis. The limit of detection was determined by calibrator 0 and values between calibrator 0 and 1 were described as being equal or below the concentration of calibrator 1, rather than given a concentration.

Preparation of reagents and solutions: The entire ELISA assay kit was stored at 4 °C. Except for the enzyme conjugate and the wash buffer, all the reagents and solutions were provided as ready-to-use. The enzyme conjugate was prepared freshly each time an assay was to be run. After being brought to room temperature, a volume of 1 mL of the enzyme conjugate (11 X) stock solution was diluted with 10 mL ready-to-use enzyme conjugate buffer. Wash buffer was prepared in quantities of 735 mL, which was sufficient for the analysis of four complete 96-microwell plates. A volume of 35 mL of the room temperature wash buffer (21 X) stock solution were mixed with 700 mL milliQ water. At the end of the analysis, the remaining 1 x wash buffer was kept at 4 °C and used within two days.

Sample preparation and procedure: The frozen plasma aliquots were thawed, hand mixed and centrifuged for 10 min at 4 °C and 3,000 g in a *5810R* eppendorf centrifuge. In parallel to the centrifugation, calibrators 0 to 5 were allowed to equilibrate to room temperature.

The required number of 96-microwell plates was taken out of the 4 °C environment, after preparation of the solutions and sample centrifugation. A volume of 25 µL calibrators (0, 18, 60, 180, 600, 1200 pM), high and low insulin controls, or plasma was pipetted in duplicate into each corresponding well, followed by 100 µL enzyme conjugate (1 X). The plate was covered with an adhesive film and incubated at room temperature (19 - 21 °C) for 1 hour, at 300 rpm on a Stuart, mini orbital shaker. This first step allows the insulin present in the sample to bind to the antibodies coating the wells and the enzyme conjugate to bind to a different epitope on the insulin molecule. The sealing film was then removed and the plate manually washed to remove any unbound material and reduce the background signal. The content of the wells was discarded and 350 µL wash buffer (1 X) pipetted into each well. The plate was inverted to empty the wells and the procedure repeated five times. 200 µL 3, 3', 5, 5'-tetramethylbenzidine substrate was then added and allowed to react with the enzyme conjugate for 15 min at room temperature and 250 rpm, after which the reaction was stopped with 50 µL H_2SO_4 (0.5 M) -containing STOP solution and the optical density monitored at 450 nm, within 10 min after stopping the reaction.

The duplicate reading of calibrators 1 to 5 were averaged and used for the construction of a calibration curve and plotted against the respective concentration in a double logarithmic scale chart.

3.3.2.5 Uric acid

Principle of the method: The quantification of uric acid in fasting plasma was based on a procedure adapted by a colleague, Ms Yuanlu Shi, adapted from a previously published method by Fossati et al. (1980). This assay method relies on the measurement of a quinoneimine dye resulting from a series of oxidative reactions. Uric acid present in the sample is first oxidized in a reaction catalyzed by uricase and giving rise to allantoin, carbon dioxide and a molecule of hydrogen peroxide. In presence of the latter and in a reaction catalyzed by the horseradish peroxidase, 3, 5-dichloro-2-hydroxybenzene-sulfonic acid, a substituted phenol, oxidatively couples to 4-aminophenazone to form the red-coloured N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinone-monoimine that absorbs at 520 nm (Figure

3.5). To minimize the interference of bilirubin and ascorbic acid present in plasma, ferrocyanide and ascorbate oxidase respectively are added to the reagents in the assay.

This assay is run in a 96-microwell plate allowing the simultaneous analysis of 48 duplicate samples. These include a 6-point calibration curve, spiked and non-spiked combined plasma samples and 40 plasma samples to measure the uric acid.

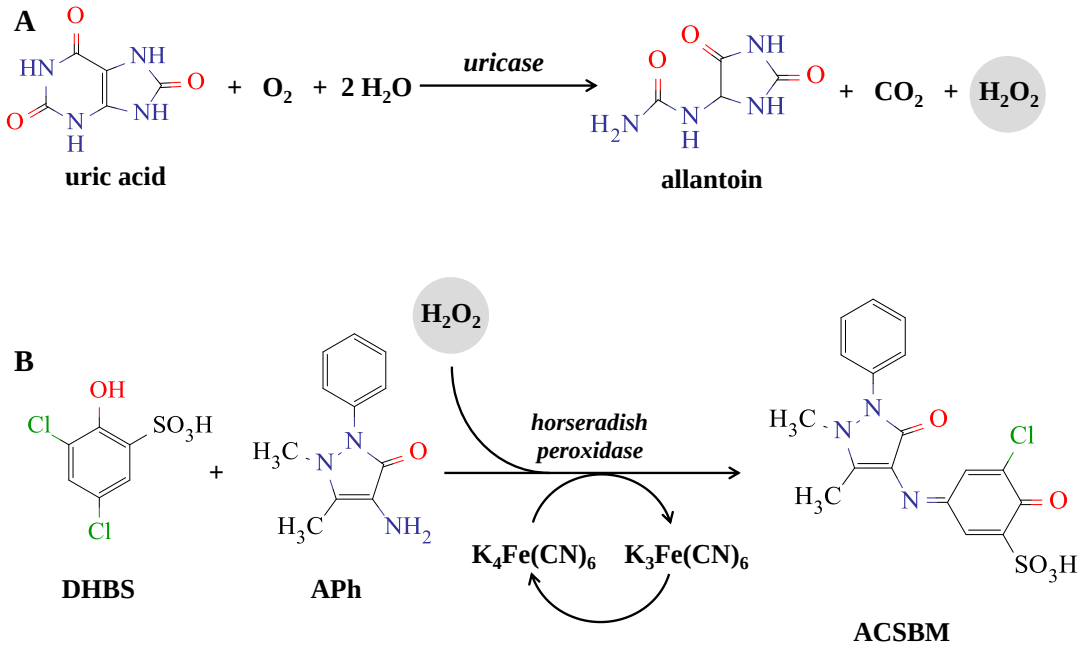


Figure 3.5: Series of reactions involved in the detection of uric acid. The uricase first catalizes the oxidation of uric acid into allantoin (A). The formed H_2O_2 is then used in the following reaction catalyzed by the horseradish peroxidase (B) to couple 3, 5-dichloro-2-hydroxybenzene-sulfonic acid (DHBS) to 4-aminophenazone (APh). The resulting N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinone-monoimine (ACSBM) is detectable at 530 nm.

Reagents: To minimize the error due to the repeated preparation of the different reagents and solution, each of them was prepared in sufficient amounts to allow the analysis of all samples, if the stability of the compounds allowed.

Uric acid stock and standard curve preparation: The solubility of uric acid in water being lower than the highest concentration desired in the calibration curve, a

stock of uric acid (10 mM) containing lithium carbonate (13.8 mM), to increase its solubility, was prepared. A 10 min incubation in a 60 °C waterbath ensured complete dissolution of the uric acid. Aliquots of 5 mL were prepared and stored at -20 °C. To avoid degradation of the uric acid, the aliquots were protected from light in aluminium foil and thawed only once. The samples for a 6 point-calibration curve were prepared by diluting 100, 80, 60, 40 and 20 µL of the uric acid (10 mM) containing lithium carbonate (13.8 mM) standard solution in 900, 920, 940, 960 and 980 µL milliQ water, respectively. Final concentrations ranged from 0.0 to 1.0 mM uric acid with milliQ water being used as the null concentration.

Uricase: A solution of uricase was prepared in milliQ water at a concentration of 6 kU/L. The original bottle containing 50 mg of solid at 5 U/mg solid, 12 mg of the enzyme powder were dissolved in 10 mL milliQ water and stored at 4 °C until needed.

Potassium phosphate buffer: Solutions of K_2HPO_4 (1 M), KH_2PO_4 (1 M), potassium hexacyanoferrate (II) trihydrate (4 mM), 4-aminophenazone (0.33 M), horseradish peroxidase (> 100 kU/L) and ascorbate oxidase (> 100 kU/L) were prepared in milliQ water and used for the preparation of a buffer/enzyme/4-aminophenazone (BEA) buffer solution. 17.41 g K_2HPO_4 and 13.60 g KH_2PO_4 were dissolved each in 100 mL milliQ water in separate recipients and potassium phosphate buffer was prepared by mixing 100 mL K_2HPO_4 (1 M) with 60 mL of KH_2PO_4 (1 M). The pH was then adjusted to 7.0 by further adding KH_2PO_4 (1 M) dropwise.

Potassium hexacyanoferrate (II) trihydrate and 4-aminophenazone: Potassium hexacyanoferrate (II) trihydrate (4 mM) and 4-aminophenazone (0.33 M) were prepared by dissolving 7.0 mg in 24.91 mL milliQ water and 134.1 mg in 2 mL milliQ water, respectively.

Horseradish peroxidase: The original bottle of horseradish peroxidase contained 96.2 mg of solid at 52 purpurogallin U/mg solid and 4 mg of the enzyme were dissolved in 2 mL milliQ water for a final concentration of at least 100 kU/L.

Ascorbate oxidase: The original bottle of ascorbate oxidase contained 6.9 mg of solid at 161 U/mg solid and the entire content was therefore dissolved in 10 mL

milliQ water to achieve a final concentration of at least 100 kU/L. Aliquots of 1.5 mL ascorbate oxidase (≥ 100 kU/L) were stored at -20 °C.

Buffer/enzyme/4-aminophenazone (BEA) buffer solution: A volume of 600 mL of the BEA reagent was prepared and stored for 7 days at 4 °C. This was sufficient for the analysis of the totality of the plasma samples, including that of some test samples for validation. 120 mL potassium phosphate buffer (1 M, pH 7.0), 6 mL potassium hexacyanoferrate (II) trihydrate (4 mM), 0.6 mL 4-aminophenazone (0.33 M), 1.2 mL horseradish peroxidase (> 100 kU/L) and 1.2 mL ascorbate oxidase (≥ 100 kU/L) were mixed and supplemented with 471 mL milliQ water. The final BEA reagent composition was 0.2 M potassium phosphate buffer, 0.04 mM potassium hexacyanoferrate (II) trihydrate, 0.33 mM 4-aminophenazone, > 0.2 kU/L horseradish peroxidase and > 0.2 kU/L ascorbate oxidase. Although it is stable for 6 weeks if stored between 2 and 8 °C, the BEA reagent was used within 6 days and stored at 4 °C.

3, 5-dichloro-2-hydroxybenzene-sulfonic acid (DHBS) reagent: The DHBS reagent consisted of 212.3 mg sodium 3, 5-dichloro-2-hydroxybenzenesulfonate and 500.3 mg Triton X-100 mixed with milliQ water to a final 100 mL volume. To avoid the formation of bubbles due to Triton X-100 and later interference in the optical density reading, the reagent was mixed gently.

Sample preparation and procedure:

Sample preparation: To evaluate the strength of the method, uncentrifuged, centrifuged, non-spiked, spiked, non-fasting and fasting plasma samples were compared to ensure that the calibration curve would include concentrations that were expected and to test whether or not centrifugation would significantly influence the results. Uncentrifuged samples were used for the assay once thawed, without centrifugation and centrifuged samples were subject to a 10 min centrifugation at 3,000 g and 4 °C prior to being assayed. A "combined" pool of plasma made from an equal volume of each plasma sample to be analysed was used for the preparation of non-spiked and spiked plasma samples. Spiked samples were prepared by mixing 40 µL "combined" plasma with 20 µL uric acid standard (0.6 mM) or 18 µL "combined" plasma with 6 µL uric acid standard (0.8 mM).

For the measurement of uric acid in the fasting plasma of the human study, the following samples were included in each 96-microwell plate; a 6-point calibration curve, including the null concentration used as the blank, a non-spiked and a spiked "combined" plasma sample prepared by mixture of either 40 µL "combined" plasma with 40 µL milliQ water or 40 µL "combined" plasma with 40 µL 0.4 mM uric acid standard solution, respectively; and 40 plasma samples. Each plasma sample was analysed twice in duplicate so that averages were calculated from 4 replicates. The inter-assay variability was assessed using the coefficient of variation calculated from the average reading and the standard deviation obtained for the 0.4 mM uric acid standard from the nine standard curves prepared for the measurements of the human study.

Prior to setting up the 96-microwell plate, a working reagent containing 24 mL BEA reagent, 8 mL DHBS reagent and 320 µL uricase solution was prepared freshly (prior to each set of analysis). The mixture was protected from light with aluminium foil and brought to 25 °C in a 130 rpm shaking waterbath.

Analytical procedure: The assay was initiated by adding to each corresponding well, 8 µL of sample (blank, calibrators, spiked or non-spiked plasma) followed by 320 µL working reagent. The plate was incubated at 20 °C for 15 min and the absorbance was monitored at 520 nm in a PHERAstar® *FS* microplate reader.

In order to determine the final uric acid concentration in each sample, the absorbance reading of the water blank was first subtracted from the other sample readings to obtain the corrected values which were used to construct a calibration curve. The corresponding equation from the linear regression line was used to calculate the sample concentration taking into account any dilution (2:1, V:V for all plasma samples). For the calculation of uric acid in the fasting plasma of the participants taking part in the present study, the individual calibration curves of each assay were averaged and plotted into a single calibration curve.

For all the biomarkers studied, the recovery was estimated as the percentage, upon comparison of obtained and expected concentration of spiked plasma samples. The

obtained concentration was then divided by the recovery to take the latter into account for the calculation of the final concentration of each biomarker in plasma.

3.4 Results

3.4.1 Total aminothiols

3.4.1.1 Repeatability

An excellent peak separation was achieved with the chromatographic conditions used. The chromatographic profile illustrated on Figure 3.6 does not only indicate a good repeatability of the method in term of the retention times of each aminothiol, but also that the SBD-F-aminothiol adducts were stable for the maximal analytical time (i.e. 48 h). The coefficients of variations presented in Table 3.3 were calculated with data from 6 standard curves prepared and chromatographed on different days. The values indicate a decrease in repeatability towards the lower concentrations, but overall, considering the amount of sample processing, these coefficients are regarded as indicating a good inter-day repeatability of the method.

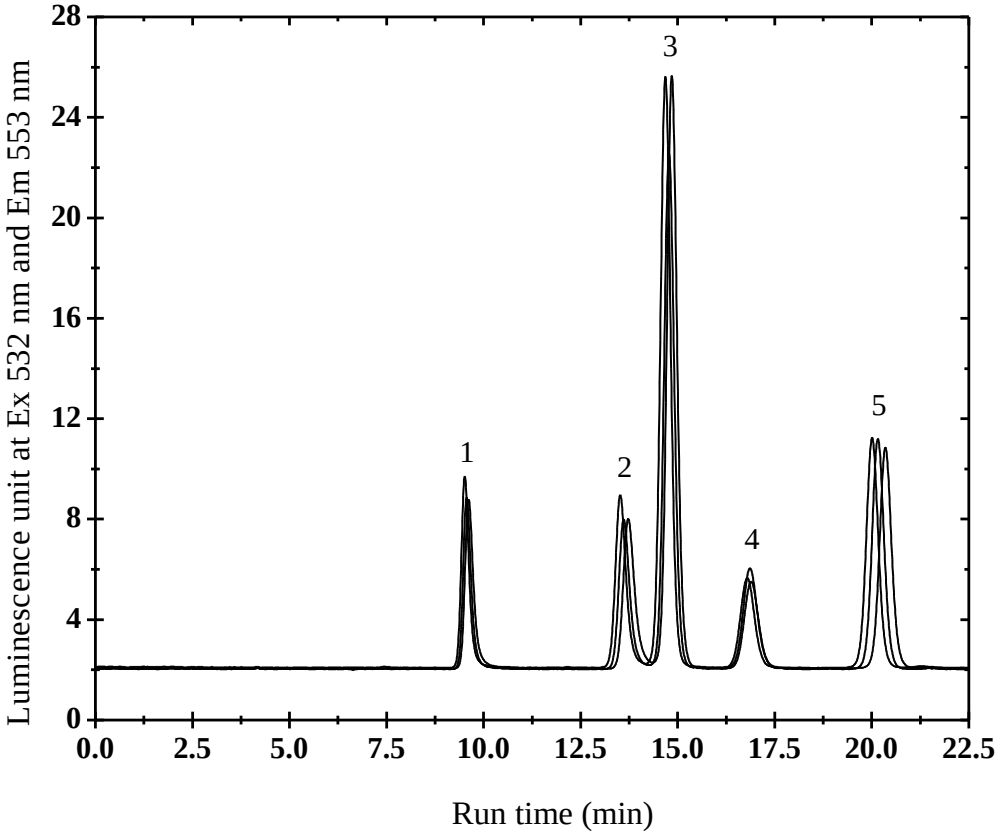


Figure 3.6: Chromatographic profile of aminothiols standards on three different days. Excellent peak separation was achieved. In order of retention time, the peaks represent 1, L-cysteine (50 μM); 2, D,L-homocysteine (12.5 μM); 3, cysteinylglycine (25 μM); 4, L-glutathione (12.5 μM) and 5, cystamine dihydrochloride (6 μM) as the internal standard.

Table 3.3: Inter-assay coefficients of variation for each aminothiol analysed. Data from 6 standard curves prepared and analysed on different days were used for the calculation of the coefficients. The coefficients of variation are estimated low with higher values for the two lowest amounts of glutathione injected in the chromatographic system.

Aminothiol	Aminothiol injected (nmol)	Inter-assay CV % (n = 6)
L-Cys	2.0	14.44
	0.5	15.27
	0.01	16.20
	0.001	17.10
D,L-HCys	0.5	21.47
	0.125	21.06
	0.0025	13.68
	0.00025	22.16
CysGly	1.0	17.91
	0.25	16.34
	0.005	13.48
	0.0005	14.19
L-GSH	0.5	19.64
	0.125	20.09
	0.0025	37.16
	0.00025	35.47

A typical chromatographic profile of SBD-F-aminothiol adducts in fasting plasma of a healthy subject is represented in Figure 3.7. Cysteine (peak 1) and cysteinylglycine (peak 3) are most abundantly present (Figure 3.7, A) and easily visible, however after magnification, homocysteine (peak 2) and glutathione (peak 4) are clear and integration of the peaks can be done precisely (Figure 3.7, B).

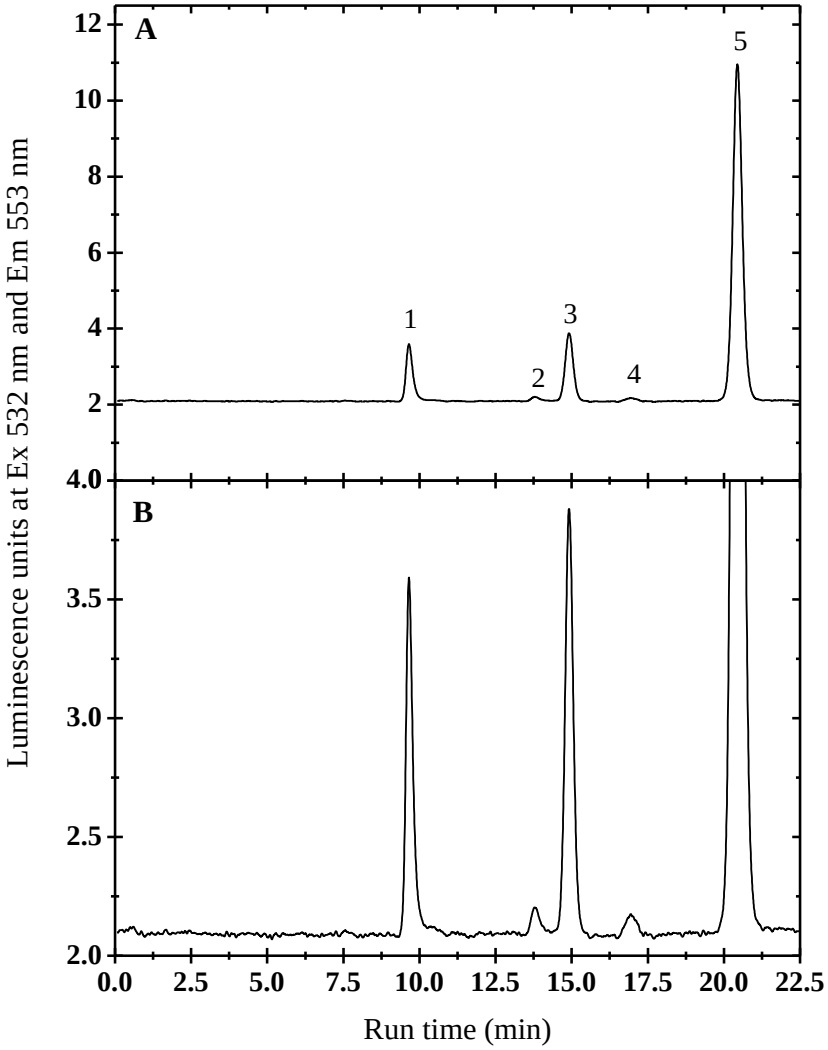


Figure 3.7: Typical chromatographic profile of aminothiols in 0.53 µL of fasting plasma in a healthy adult. Window B represents an amplified section of the chromatogram in window A, for better visualization of D,L-homocysteine (2) and L-glutathione (4). Peaks 1, 3 and 5 are the L-cysteine, cysteinylglycine and cystamine dihydrochloride, respectively.

3.4.1.2 Standard curves

The equations for the regression lines were very similar for the calibration curve prepared in milliQ water and in plasma (Figure 3.8). The calibration curves prepared in milliQ water were compared to the calibration curves prepared in plasma and used to verify the quality of the chromatography. To avoid influence of the biological sample background, the equations from calibration curves prepared in plasma were used for the calculation of plasma total aminothiol concentrations.

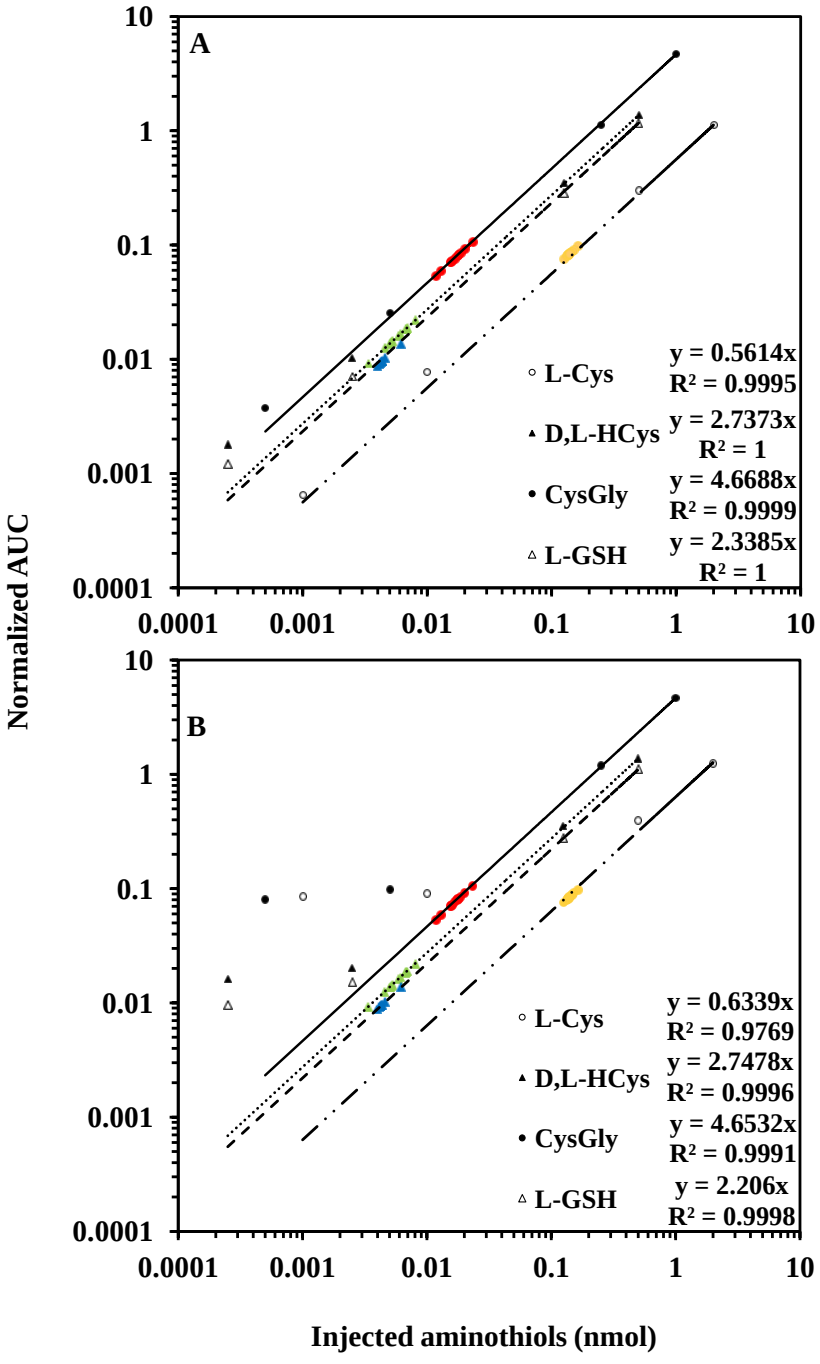


Figure 3.8: Total aminothiols calibration curves prepared in milliQ water and PBS (A), and prepared in plasma (B). The calibration curves prepared in both environments are very similar and the R^2 values of both types of calibration curves are indicative of a good linearity within the range of interest. Coloured marks represent measurements of twelve participants for cysteinylglycine (red), D,L-homocysteine (green), L-glutathione (blue) and L-cysteine (yellow). AUC, area under the curve.

3.4.1.3 Limits of detection and quantification

Details of the obtained limits of detection (LOD) and quantification (LOQ), recoveries, average total aminothiol concentrations and example of healthy concentrations, as reported in the literature, are presented in Table 3.4.

Table 3.4: Limits of detection (LOD) and quantification (LOQ) for each aminothiol of interest. Recoveries and averages obtained were calculated with the samples from all the human study participants (n = 62). L-Cys, cysteine; D,L-HCys, homocysteine; CysGly, cysteinylglycine; L-GSH, glutathione. §, (Pimková et al., 2014); †, (Guttormsen et al., 1994); ††, (Andersson et al., 1999); ‡, (Christensen et al., 2001); ‡‡, (Wu et al., 2004).

	LOD (nmol)	LOQ (nmol)	Recovery (% ± SD)	Mean concentration in 62 subjects (µM ± SD)	Reported healthy concentrations (µM ± SD)
L-Cys	0.002	0.007	93 ± 6	257 ± 25	219 ± 14 §, 256 ± 17 †, 321 ± 50 ††
D,L-HCys	0.001	0.0036	94 ± 4	9.4 ± 3.5	9.45 ± 0.43 §, 9.9 ± 2.4 ‡, 14.1 ± 4.9 ††
CysGly	0.0006	0.002	94 ± 4	30.5 ± 5.6	24.4 ± 4.5 ††, 39.71 ± 4.86 §
L-GSH	0.001	0.0058	67 ± 13	7.7 ± 1.5	2-20 ‡‡, 5.69 ± 1.33 ††, 9.07 ± 1.55 §

3.4.2 Glucose

3.4.2.1 Sample pre-treatment

In order to have an overview of the best sample processing with the least sample loss, the glucose concentration was measured in the samples presented in

Table 3.5 and Table 3.6. Data presented in part A of Table 3.5 are from an untreated plasma sample, an SPE-treated (30 mg sorbent/mL) non-spiked plasma sample with corresponding collected SPE-fractions, and an SPE-treated spiked plasma sample with the corresponding collected SPE-fractions. All three samples were prepared from the same plasma aliquot. The untreated sample was used as a reference to have an indication of the glucose loss in the SPE column. These data are suggestive of a loss of material when the sample is subject to solid phase extraction.

To determine whether this could be explained by a loss of glucose, the experiment was repeated with a standard D-glucose solution. Results presented in part B of Table 3.5 are from an untreated 5.55 mM D-glucose solution and an SPE-treated 5.5 mM D-glucose solution with the corresponding collected SPE-fractions. Data confirm that there is some loss of glucose during the passage through the solid phase extraction column. The reading obtained with the 60 % MetOH fraction was not explained by the fact that it is an organic solvent, because 100 % and 60 % MetOH samples read at 340 nm gave null readings after correction for blanks. This however suggests that some glucose is retained in the column and is eluted with MetOH.

Table 3.5: Concentration of plasma (A) and D-glucose standard samples (B) subject to different pre-treatments. The glucose concentrations are duplicate measurements of single samples.

A	**Sample type and description**	**D-Glc concentration (mM)**
	Untreated, non-spiked plasma (200 µL plasma + 50 µL milliQ water)	5.61
	SPE-treated, non-spiked plasma, flow-through fraction (200 µL plasma + 50 µL milliQ water)	2.28
	60 % MetOH fraction from SPE-treated, non-spiked plasma	- 0.28
	2 % formic acid fraction 1 from SPE-treated, non-spiked plasma	- 0.56
	2 % formic acid fraction 2 from SPE-treated, non-spiked plasma	- 0.44
	SPE-treated, spiked plasma, flow-through fraction (200 µL plasma + 50 µL D-Glc (27.75 mM))	8.05
	60 % MetOH fraction from SPE-treated, spiked plasma	0.44
	2 % formic acid fraction 1 from SPE-treated, spiked plasma	- 0.56
	2 % formic acid fraction 2 from SPE-treated, spiked plasma	- 0.61
B		
	Untreated 5.55 mM D-Glc standard solution	5.83
	SPE-treated, 5.55 mM D-Glc standard solution, flow-through fraction	4.88
	60 % MetOH fraction from SPE-treated, 5.55 mM D-Glc standard solution	0.22
	2 % formic acid fraction 1 from SPE-treated, 5.55 mM D-Glc standard solution	0
	2 % formic acid fraction 2 from SPE-treated, 5.55 mM D-Glc standard solution	0

Data presented in Table 3.6 shows the effect of deproteination and defatting through SPE passage. In the same experiment, non-spiked plasma samples; SPE-treated, non-spiked plasma samples; protein-precipitated, non-spike plasma samples; and SPE-treated, protein-precipitated, non-spiked plasma samples were compared (Table 3.6). When comparing the non-spiked samples, the untreated, non-spiked plasma gave the highest concentration of glucose (5.44 mM), as expected. The plasma sample with either SPE cleaning or protein precipitation, only, showed similar values of 4.44 mM and 4.55 mM, while the samples with the combined SPE passage and protein precipitation resulted in the highest loss (89 % recovery) and lowest glucose concentration (3.83 mM). The repetition of this experiment confirmed the outcome. All the subjects recruited for the study were in general good health and therefore, glycaemia values within the normal range (3.9 - 5.6 mM) were expected. Performing a protein precipitation and or defating the sample would thus result in an underestimation of the effective glycaemia of the participants.

Table 3.6: Samples compared simultaneously in order to determine the best sample pre-treatment for D-glucose analysis by the hexokinase assay.

Sample type and description	D-Glc concentration (mM)
Untreated, non-spiked plasma (30 µL original plasma + 30 µL milliQ water)	5.44
SPE-treated, non-spiked plasma (62.5 µL original plasma + 62.5 µL milliQ water + SPE treatment)	4.44
Protein-precipitated, non-spiked plasma (30 µL precipitated plasma + 30 µL milliQ water)	4.55
SPE-treated, protein-precipitated, non-spiked plasma (62.5 µL precipitated plasma + 62.5 µL milliQ water + SPE treatment)	3.83

Deproteination and defatting plasma samples presented numerous disadvantages. The loss of glucose following a sample pre-treatment by ethanol precipitation and solid phase extraction would underestimate the glycaemia measured. The loss in the SPE method may have been reduced with higher sample volumes, however, little plasma was available for the glucose analysis and increasing the sample volume was not an option.

As in the hexokinase assay by 96-well microwell plate the plasma sample volume required is very little (< 2 % of the reaction volume), and proteins and fat are unlikely to interfere with the hexokinase reagent, the decision was taken not to proceed to any further sample pre-treatment other than a centrifugation following sample thawing. This approach was also supported by another Hexokinase-based method not tested in the present PhD experiments, namely the *Infinity*^{TM} Glucose Hexokinase Liquid Stable Reagent from ThermoScientific, in which it is recommended to use plasma without protein removal for routine laboratory use (ThermoScientific, 2008).

3.4.2.2 Improved method for the glucose quantification

The analysis of plasma samples, with no sample pre-treatment other than a centrifugation, gave results that were comparable to those from glucose samples that were analysed by HPLC, by a former colleague (Dr. Samantha Sutulic, data not shown). The inter-assay variation was 1.01 %, wich indicates a very good repeatability. Recoveries ranged from 93 % to 101 % with an average ± standard deviation of 98 ± 2 %. A good linearity was obtained up to 8.33 mg/mL, as shown in Figure 3.9. A calibration curve combining duplicate measurements of 25 calibration curves is presented in Figure 3.10. This was also the calibration curve used for the quantification of glucose in all the plasma samples from the human study.

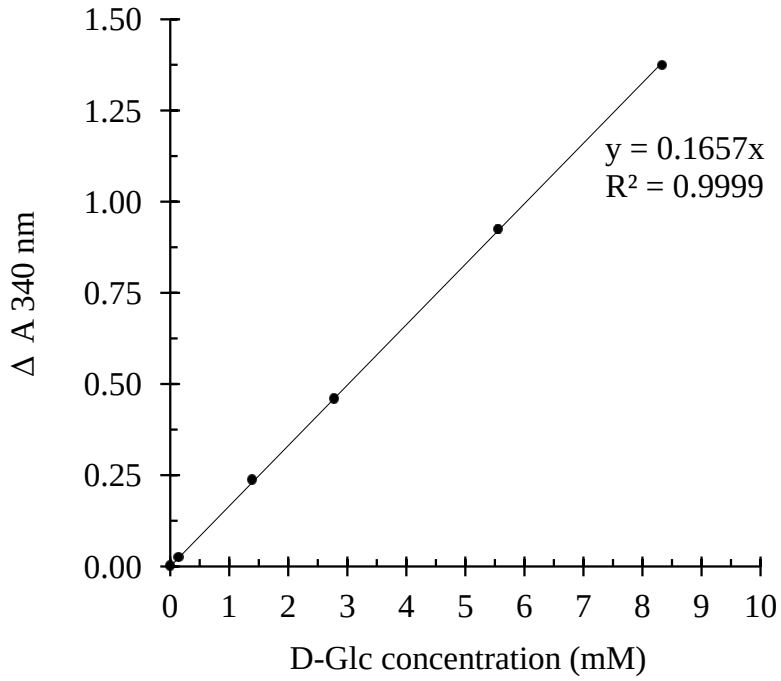


Figure 3.9: D-glucose calibration curves showing the linearity up to 8.33 mM (1.5 mg/mL). Healthy fasting glucose concentrations are expected to be between 3.9 and 5.6 mM and < 7.8 mM following 2 hours after a 75 g oral load of glucose. Therefore, calibration curves were prepared to include 8.33 mM as the highest standard point.

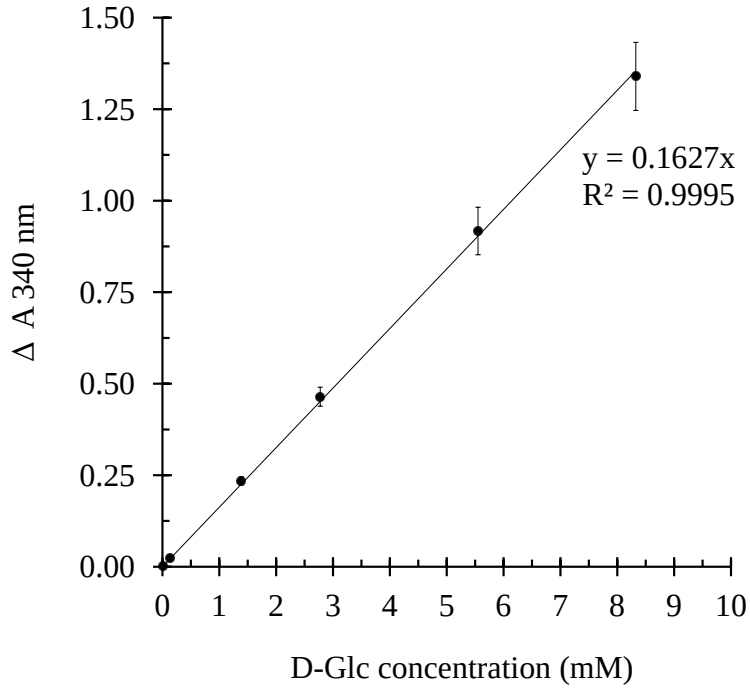


Figure 3.10: Representative combined calibration curve of 25 duplicate measurements. The derived equation was used to calculate the concentrations of glucose in the fasting plasma of the participants that took part in the human study.

3.4.3 Insulin

As for the D-glucose analysis, the standard curves between assays were very close and therefore combined to do the analysis of the human study plasma samples. The inter- and intra-assay repeatability is described by the coefficients of variation obtained with the readings of the low and high insulin controls, between and within assay, respectively. The average ± SD coefficients of variation within assays were 2.9 ± 2.8 % for the low insulin control, and 1.4 ± 0.8 % for the high insulin control. The coefficients of variation between assays were 7.6 % for the low insulin control and 8.0 % for the high insulin control. These values indicate a good repeatability achieved with this method. The combined standard curve obtained from the 9 measurements is illustrated in Figure 3.11. A good linearity was confirmed for the range of interest (2 - 25 mU/L). The small error bars are further indicative of a good reproducibility.

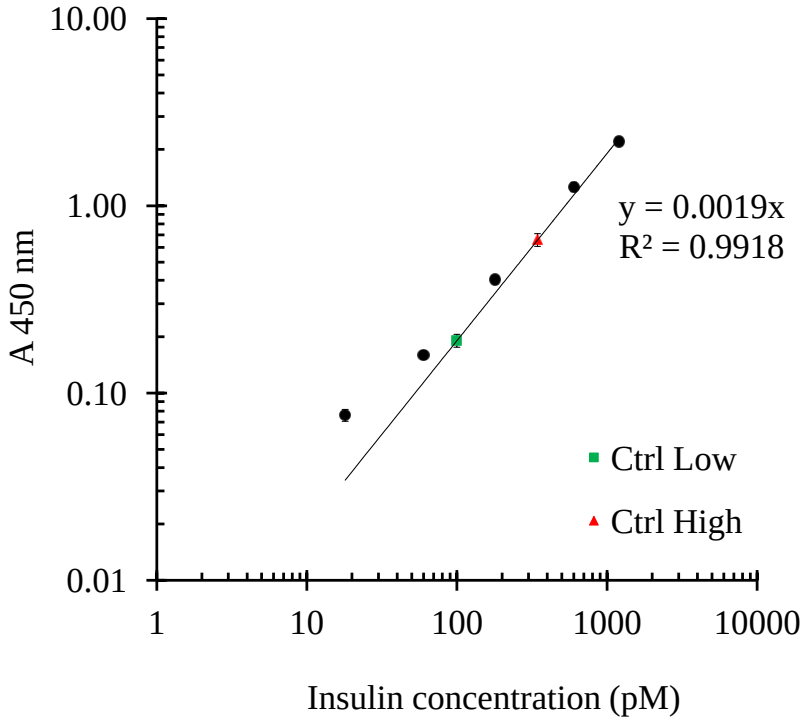


Figure 3.11: Calibration curve used for the calculation of insulin in fasting plasma. The measurements of 9 separate calibration curves were averaged and combined into one curve. The reproducibility between assays was monitored by the inclusion of high (red triangles) and low (green squares) concentration insulin controls in the analysis.

3.4.4 Uric acid

Upon analysis of samples used for validation, no significant difference was measured between uncentrifuged and centrifuged samples. In order to maintain the same sample pre-treatment as for the analysis of total aminothiols, glucose and insulin, all subsequent samples were only subject to centrifugation prior to analysis. To reduce the manipulation of the pipetting volumes and have a consistent spiking value, the spiked samples were thereafter prepared with 40 µL combined plasma spiked with 40 µL uric acid standard (0.4 mM). An increase of absorbance equivalent to that of 0.2 mM was thus expected. The inclusion of the non-fasting samples in the tests confirmed that the range of the calibration curve was sufficient in case of higher concentrations of uric acid (Figure 3.12).

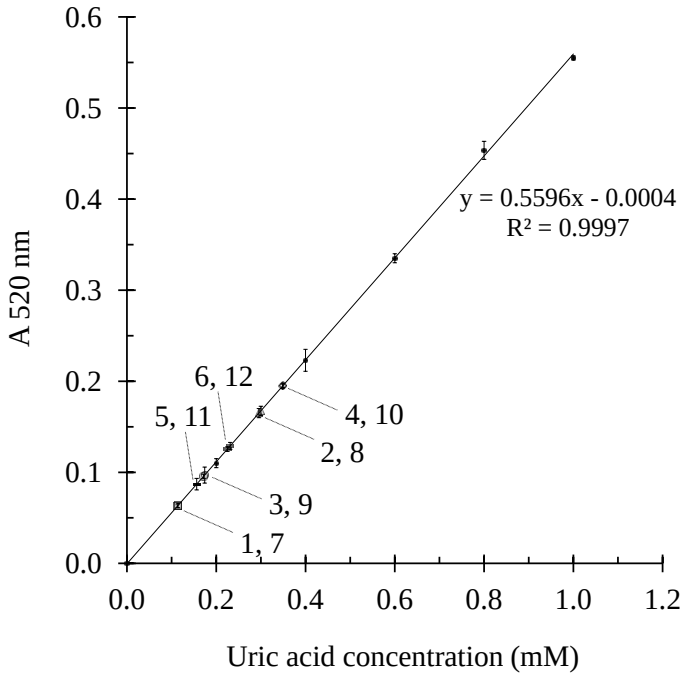


Figure 3.12: Comparison of different samples tested to verify the strength of the method and whether centrifugation influences the concentration of uric acid in plasma. All samples tested were within the calibration curve range. The sequence of samples in the observed range was as suspected: uncentrifuged and centrifuged fasting diluted plasma (1, 7) were at the lower end of the concentration range, followed by uncentrifuged and centrifuged fasting non-diluted plasma (5, 11), uncentrifuged and centrifuged non-fasting diluted plasma (3, 9), uncentrifuged and centrifuged non-fasting non-diluted plasma (6, 12), uncentrifuged and centrifuged fasting spiked plasma (2, 8) and finally uncentrifuged non-fasting spiked plasma (4, 10).

As for the analyses of glucose and insulin, the standard curves between assays were very close and therefore combined to do the analysis of the human study plasma samples (Figure 3.13). The standard deviations of standard curve were very low, ranging from 0.00 to 0.01 and the coefficient of variation between assays was 1.8 %, supporting a good repeatability of this method. The average recovery of 95 % was obtained indicating little sample loss. Also, as seen on the chart, excellent linearity (R^2 = 1) was achieved within (0.2 - 0.4 mM) and beyond the range of analysis.

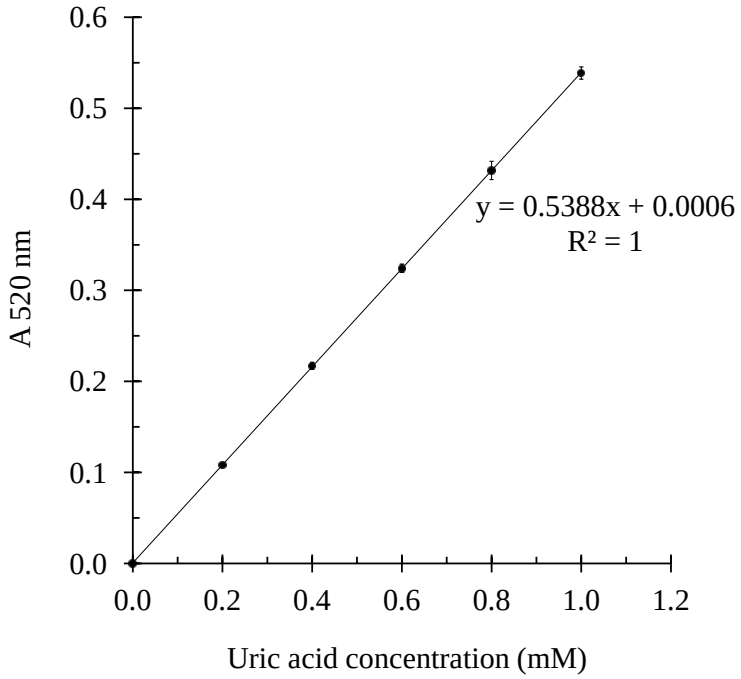


Figure 3.13: Calibration curve used for the calculation of uric acid in fasting plasma and constructed from the average of the 9 calibration curves obtained during the 9 individual assays.

3.5 Discussion and conclusion

Biomarkers can be a valuable tool to assess the health of subjects. These however, need to be good indicators of the health condition or status of interest but also need to be quantified by the appropriate reliable methods. In the present chapter, the improvement and validation of the methods used for the quantification of total aminothiols, glucose, insulin and uric acid in fasting plasma of healthy adult subjects was described.

The method described by Pfeiffer et al. (1999) was used as a reference to develop an improved method for the quantification of total aminothiols. The improved method demonstrates, despite the important sample treatments, that excellent recoveries are achieved and that the SBD-F-aminothiol adducts are stable for at least 48 h when stored at 4 °C. Although some methods allow a faster detection of aminothiols, they also compromise the good chromatographic separation of the compounds. The new chromatographic conditions used for the

quantification of total aminothiols result in an improved and optimal separation of the SBD-F-aminothiol adducts in a 22 min run. Finally, good repeatability and linearity of the calibration curves was obtained for both the calibration curve prepared in milliQ water and the calibration curve prepared in plasma.

The improved method for the quantification of glucose made use of the glucose hexokinase assay (Sigma-Aldrich, 2004). The replacement of cuvettes and spectrophotometer by a UV-compatible 96-microwell plate and microplate reader offered the advantage of reducing the sample and hexokinase reagent volumes. Also, duplicates of 17 samples and a 6-point standard curve can be processed and analysed simultaneously within 3 hours. The use of the hexokinase assay permits the analysis of the sample with little pre-treatment (i.e. centrifugation and dilution) and minimizes the risk of glucose loss by any treatment due to plasma modification, also, methods for sample deproteination may concentrate the plasma sample, so that the measurement of glucose may be overestimated. Finally, deproteination of the samples is time consuming and although glucose is a stable component of plasma, it is desirable to process samples as rapidly as possible. The little sample pre-treatment resulted in a good recovery of the glucose.

For the insulin, the already commercially available Mercodia ELISA assay for human insulin determination was used (Mercodia). Lot-to-lot variations was avoided by purchasing sufficient supplies from the same batch. As guaranteed by the producer, linearity within the range of interest and the reproducibility were high.

Finally, uric acid was quantified by using an enzymatic colorimetric method developed by a colleague (Ms Yuanlu Shi). The strength of the method was verified and confirmed with an excellent linearity within the desired range and reproducibility.

Overall, the methods described in the present chapter have shown to be reliable methods for the measurement of the corresponding biomarkers in fasting plasma of healthy adult subjects. With the exception of the aminothiols, for the lower-end extreme values, these methods would also be suitable for the detection of more extreme values, as the linearity ranges were good, even beyond the desired range.

Chapter 4
Human study and analysis of the food frequency questionnaire

4.1 Abstract

The present observational human study focused on the impact of general (poly)phenol-rich dietary habits, with an emphasis on coffee, tea and cocoa-containing beverages, on the biomarkers of general inflammation and cardiovascular health. The current chapter describes the study design and analyses the consumption habits of the study population regarding these beverages. The influence of age and gender was analysed and the likelihood for a subject with a high consumption of particular (poly)phenol-rich product to consume other (poly)phenol-rich foods was examined. The minimal sample size for a statistical power of 80 % was calculated to be 52 based on the variables for the biomarker of interest requiring the most participants (i.e. homocysteine). Sixty two participants were recruited and completed 1 out of 3 possible visits. Data indicate that the study population had a slightly higher consumption of coffee and tea than the average consumption in the UK. The weekly consumption of coffee and cocoa-beverages was not gender- or age-dependent. Amongst the tea brews, only green tea and herbal infusions were gender- and age-dependent, respectively. The consumption of green tea was also positively associated with the consumption of selected coffee brews. The (poly)phenolic intake from coffee, tea and cocoa preparations was analysed in a semi-quantitative manner by the introduction of indexes representative of the (poly)phenolic content. A positive association between the intake of coffee and tea (poly)phenols was detected. The consumption of fruits was positively associated with that of tea, cocoa-containing beverages, vegetables, herbs and spices, and the intake of supplements, and the consumption of tea was associated with the use of herbs and spices.

4.2 Introduction

In two recent studies from Renouf et al., large inter-individual variations in plasma metabolites were reported following coffee and green tea ingestion (Renouf et al., 2010a, Renouf et al., 2013). It is still unknown why such substantial differences exist between subjects, but genetics, microflora, general nutrition and other life habits can play significant roles. Research studying the effects of coffee consumption and other phenolic-rich foods has led to variable outcomes. This can be explained by different study designs, the amount of administered dose or confounders.

The daily recommendations in the UK for the consumption of fruits and vegetables are currently 5 servings (1 serving defined as 80 g or the equivalent of a fist-size) (http://www.nhs.uk/Livewell/5ADAY/Pages/Portionsizes.aspx). At this level of consumption, meta-analyses have reported a decreased risk in death from all causes, when compared to no consumption of fruits and vegetables (Wang et al., 2014). Also, for subjects increasing their daily consumption from < 3 to > 5 servings, a 17 % decrease in the incidence of coronary heart disease was reported (He et al., 2007). A recent meta-analysis by Li et al. (2014), with 10 prospective cohort studies (n = 434,342 participants, of which 24,013 developed T2DM during the 4.6 to 23-year follow-up) focusing on the effect of fruit and/or vegetable consumption on the incidence of T2DM indicated a lower risk of T2DM with the consumption of fruit or green leafy vegetables. A dose-response analysis further indicated a 6 % and 13 % decrease of T2DM incidence for each additional 1 and 0.2 fruit or green leafy vegetable serving (1 serving defined as 106 g), respectively. Li et al. (2014) could not exclude any residual confounding by unmeasured factors, also incidence of T2DM may have been underestimated as the the information was obtained using a questionnaire. The main possible limitations of the above-mentioned meta-analyses are a difference in the classification of fruits and vegetables (Wang et al., 2014) with the possibility of missclassification (He et al, 2007); the lack of adjustment for some dietary and lifestyle factors (He et al., 2007; Li et al., 2014); a dietary assessment done at baseline only for numerous studies, thus not taking into account possible dietary changes (He et al., 2007); and the older age of the majority of the studies included in the meta-analyses (Wang et al., 2014; He et al., 2007; Li et al., 2014)

No minimal recommendations for the consumption of coffee or tea exist, likely due to the fact that the consumption of these beverages has shown outcomes, which cannot lead to clear conclusions. In a meta-analysis by van Dieren et al. a beneficial effect of tea (mainly black) and coffee consumption on the incidence of T2DM, with the lowest hazard ratios for a consumption of > 5 cups tea and 4.1 - 6.0 cups coffee per day, respectively, was reported (van Dieren et al., 2009). These represent relatively high levels of tea and coffee consumption and not necessarily obtainable in an average population. In this report, however, no association with biomarkers of T2DM was detected. A similar study investigation on the incidence of cardiovascular diseases and associated deaths reported a beneficial effect of tea (mainly black) and coffee consumption on the incidence of coronary heart disease, with the lowest hazard ratios for a consumption of 1 - 6 cups tea and 2.1 - 3.0 cups coffee per day, respectively (de Koning Gans et al., 2010). No associations were identified in this report with the incidence of stroke or death from coronary heart disease, stroke or from all causes. Of note, only a small percentage of the analysed population died from coronary heart disease and therefore the statistical power was reduced for the detection of associations which included coronary heart disease.

The 3 countries with the highest consumption of coffee between May 2013 and June 2014 were Finland (12 kg green coffee equivalent), Austria (8.5 kg) and Sweden (7 kg), as reported by the European Coffee Federation (http://www.ecf-coffee.org/about-coffee/coffee-consumption-in-europe). Taking into account that the coffee beans lose 8.48 - 24.74 % (averaged to 16.61 %) of their weight during roasting (Sualeh et al., 2014), the above mentioned consumptions are equivalent to approximately 10, 7 and 6 kg per capita per year, respectively, and thus, approximately 3, 2 and 1.5 cups per day, assuming a preparation with 10.3 g coffee (averaged from 10 to 10.6 g) (http://www.blackbearcoffee.com/resources/83, Illy and Pizano, 2004). In 2012, the average UK consumption was estimated to be 0.74 cups per day, taking into account the 2.8 kg per capita consumed during that year (http://www.ecf-coffee.org/images/European_Coffee_Report_2013-14.pdf) and assuming that one cup is prepared with 10.3 g coffee. The average consumption of tea in the UK was estimated to be 2 - 3 cups per day, as calculated from the weight consumed weekly in households in 2009 (29 g) and the tea contained in tea bags (1.5

- 2 g) (http://www.statista.com/statistics/284484/weekly-household-consumption-of-tea-in-the-united-kingdom-uk/).

The beneficial effects of coffee, tea, cocoa, fruits and vegetables have often been attributed to the constituent (poly)phenols and therefore, the assessment of the (poly)phenolic intake with each product, and not only the amounts of product consumed, is of major interest if associations with health are sought to be tested. Food frequency questionnaires are extensively used for the self-assessment of food intake. In the present study, a food frequency questionnaire was created based on a questionnaire for estimating caffeine exposure in pregnant women, the caffeine assessment tool (Boylan et al., 2008). Included in the questionnaire was the assessment of exposure to (poly)phenol-rich products including coffee, tea, cocoa, fruit and vegetables; foods consumed during the main daily meals (i.e. breakfast, lunch, dinner) and as snacks; and the consumption of supplements and vitamins. An initial section, the health assessment questionnaire, mainly used for participant selection purposes, contained information about anthropometrics, medication intake, medical history, the bowel movement and in general, blood donation, habits of physical activity, weekly alcohol consumption, smoking as well and bowel movement.

In the present chapter, the calculations of the population size for the present investigations and the study design are described. When doing human studies, the number of participants involved is important in order to ensure sufficient statistical power of the obtained outcome. For the present study, the minimal number of participants required was based on published and personal data obtained during the analysis of the biomarkers of interest in plasma samples. Also, the study population is characterized in terms of the consumption of popular (poly)phenol-rich products, such as coffee, tea, cocoa beverages as well and fruits and vegetables and the (poly)phenolic intakes of coffee, tea and cocoa beverages. Finally, the correlations between the consumption of each beverage, as well as with other sources of (poly)phenols, such as fruits, vegetables, herbs and spices, and dietary supplements, were subsequently tested. The data were analysed as a whole population, by gender and by age group.

4.3 Materials and method

4.3.1 Ethics and eligibility

The study was approved by the joint faculty research ethics committee under the reference number MEEC 10-035. The participants were recruited in Leeds, in an area that included the University of Leeds campus. After written informed consent was obtained, the candidates were provided with a health assessment questionnaire destined to assess their suitability for participating in the study. The questionnaire collected data on gender, age, weight, height, general health status, medication, recent blood donation, smoking and alcohol consumption habits, habits of physical activity and bowel movement.

The subjects were eligible if they were in general good health, between 18 and 70 years of age, had a body mass index between 18.00 and 29.00 kg/m^2, were not diagnosed with any chronic disease, haemophilia or had not previously undergone a gastrointestinal tract operation, were not under any long-term prescribed medication, except for any form of contraceptive medication (i.e. pill, injection, subcutaneous chip), were not pregnant or lactating, were non-smokers or were smoking < 5 cigarettes per day and consumed ≤ 4 units of alcoholic beverages on a daily and regular basis. If the participant had or was going to donate blood in the 4 weeks before or after completion of the questionnaire, the visit was arranged so that there were at least 4 weeks elapsed since the last blood donation or 4 days before the next blood donation. The suitable subjects were provided with a self-assessed food frequency questionnaire to be completed at home and brought back for the first visit. To keep their identity anonymous, the subjects were assigned a code, which was used for processing all the collected data and biological samples.

4.3.2 Sample size

In order to ensure sufficient power, the sample size was computed with the following variables: μ_0, the expected values for the biomarkers of interest at fast, which were based on the data obtained from test measurements performed for the method validation; μ_1, the known values, and σ, the known standard deviation which were based on data reported in the literature; α, the probability of falsely rejecting a

true null hypothesis, which was set to 0.05 (i.e. 5 %), and the desired power, the probability of correctly rejecting a false null hypothesis which was set to 0.8 (i.e. 80 %) (http://www.statisticalsolutions.net/pss_calc.php). The primary outcome variable was total homocysteine, but the sample size was also calculated using the variables from the measurement of total cysteine, total cysteinylglycine, total glutathione, glucose, insulin and uric acid.

4.3.3 Study design

The present study consisted of three visits during which the participants were asked to undergo a 36-hour washout period prior to their visit at the School of Food Science and Nutrition at the University of Leeds, and a 36-hour period with the same diet restrictions, upon attendance to the appointment. The regimen was (poly)phenol-poor and excluded the consumption of alcohol, any form and type of coffee, tea and cocoa-containing products, most fruits and vegetables, wholemeal (wheat) products and any form of fruit- or vegetable-based food supplements. To facilitate the choice of food during the study, participants were supplied with some meal suggestions and a list of allowed foods. The latter included any non-spiced meat and fish, rice, pasta, noodles, eggs, any form of non-spiced peeled potato, white bread, non-wheat cereals, some vegetables in small amounts (i.e. mushrooms, peeled cucumber, peeled courgette, asparagus, lettuce, pumpkin and lentils), one banana and one kiwi per day, milk and any other non-cocoa or -fruit containing dairy product, water, squash drinks and sodas, white chocolate, non-cocoa or -fruit based sweets and cakes, some seasoning in moderation (i.e. salt, pepper, olive oil, aromatic herbs and salad dressing), peeled nuts and vitamins. A notification was sent to each participant as a reminder of the commencement of the restricted diet period. A 72-hour dietary record was used to certify the compliance of the participant with the diet restrictions. In this diary, the participants described the type and quantities of all the consumed foods and beverages, other than water, during the period of dietary restriction.

On the morning of the first study visit, the participants arrived in a fasting state, from 10 pm on the evening before, however, they were allowed to drink water *ad libitum*. During the examination, the identity and the dietary compliance of the

participant were first verified. If the diet had been breached, the participant was asked to repeat the washout period. If the dietary restrictions had been respected, the participant was offered to drink some water and 10 mL of fasting blood was withdrawn by venepuncturing the antecubital vein (see section 3.3.3.1). The blood samples used at a later time point for the quantification of selected biomarkers of health (chapters 3, 5, 6 and 7). Anthropometrics (i.e. weight, height, waist and hip circumference) followed by the measurement of blood pressure were then recorded (chapter 5). To avoid the influence of stress on the blood pressure, the latter was always measured a few minutes after the blood had been collected and when the subject was relaxed. The measurement was repeated 3 times, and when possible, on the opposite arm used for blood collection. The subjects collected a baseline urine sample which was used for compliance purposes, as well as the baseline for the later pharmacokinetics. The fast was finally broken with a (poly)phenol-poor breakfast, which consisted of two slices of white toast bread with butter and cheese, one banana or one kiwi, along with a cup of 4 g "Green Blend" Nescafé instant Coffee (Nestlé) dissolved in approximately 300 mL boiling water. It was ensured that all the coffee had been consumed. The nutrient information of the breakfast products are presented in Table 4.1.

Table 4.1 Nutrient content of the foods provided at breakfast, for the participants.

Food	**Nutrient information**	
Banana (per 100 g)	Energy (89 cal)	Vit C (8.7 mg)
	Total Fat (0.3 g)	Calcium (5 mg)
	Saturated fat (0.1 g)	Iron (0.3 mg)
	Monounsat. fat (0.0 g)	Magnesium (27 mg)
	Polyunsat. fat (0.1 g)	Phosphorus (22 mg)
	Cholesterol (0 mg)	Potassium (358 mg)
	Protein (1.1 g)	Sodium (1 mg)
	Vit A (64.0 IU)	Zinc (0.2 mg)

Banana (continued)	Total Carbohydrate (22.8 g) dietary fiber (2.6 g) starch fiber (5.4 g) Sugars (12.2 g)	Copper (0.1 mg) Manganese (0.3 mg) Water (74.9 g)
Kiwi (per 100 g)	Energy (61 cal) Total Fat 0.5 g Saturated fat (0.0 g) Monounsat. fat (0.0 g) Polyunsat. fat (0.5 g) Cholesterol (0 mg) Total Carbohydrate (14.7 g) dietary fiber (3.0 g) starch fiber (0.0 g) Sugars (9.0 g) Protein (1.1 g)	Vit A (87.0 IU) Vit C (92.7mg) Vit E (1.5 mg) Calcium (34 mg) Iron (0.3 mg) Magnesium (17 mg) Phosphorus (34 mg) Potassium (312 mg) Sodium (3 mg) Zing (0.1 mg) Copper (0.1 mg) Manganese (0.1 mg) Water (83.1 g)
Bread (per 2 slices)	Energy (0.23 cal) Fat (1.8 g) Saturated fat (0.6 g) Carbohydrates (41.6 g)	Fibre (2.4 g) Sugars (2 g) Protein (9.4 g) Sodium (0.94 g)
Butter, Anchor Spreadable (per 10 g)	Energy (70 kcal) Fat (7.7 g) Saturated (3.1 g) Monounsat. (2.9 g)	Polyunsat. (1 g) Carbohydrates (0.03 g) Sugars (0.03 g) Sodium (0.11g)

Coffee, Green blend Nescafé (per 4 g)	Energy (2.36 kcal) Protein (0.35 g) Carbohydrates (0.22 g) Sugars (0.22 g) Fat (8 mg) Unsaturated (4 mg)	Fibre (1.2 g) Sodium (4 mg) Salt equivalent (12 mg) Antioxidants (0.88 g) Polyphenols (0.35 mg)
Mini Babybell (per unit)	Energy (61 kcal) Fat (4.8 g) Saturated (3.2 g) Carbohydrate (traces)	Protein (4.5 g) Sodium (0.36 g) Calcium (140 mg)

After finishing breakfast, the participant was free to leave and collected all the urine produced during the following 36 hours. Five containers for urine collection were provided for all the urine produced from 0 - 4, 4 - 8, 8 - 12, 12 - 24 and 24 - 36 hours after the coffee consumption. At the end of the 36 hours of urine collection, the containers were returned to the School of Food Science and Nutrition at the University of Leeds and the urine samples were centrifuged, aliquoted and stored at -20 °C until analysis. All the urine samples were processed and analysed by former colleague, Mr. Nicolai U. Kraut, and details other than the relevant data for the present thesis will not be discussed in this manuscript (chapter 7). The second and third visits consisted of the same procedures performed during the first visit, however, the food frequency questionnaire was not asked to be filled in again. A period of at least 4 - 8 weeks was left between each visit. This allowed for a change in the biomarkers profile to be detecable, if there was any. Also, a 4 week-range gave some freedom to the participants in case these were not available during a suggested date. During this time, the subjects were allowed to follow their regular diet. The participants were compensated with £10 for each completed visit. A schematic representation of the study design is shown in (Figure 4.1).

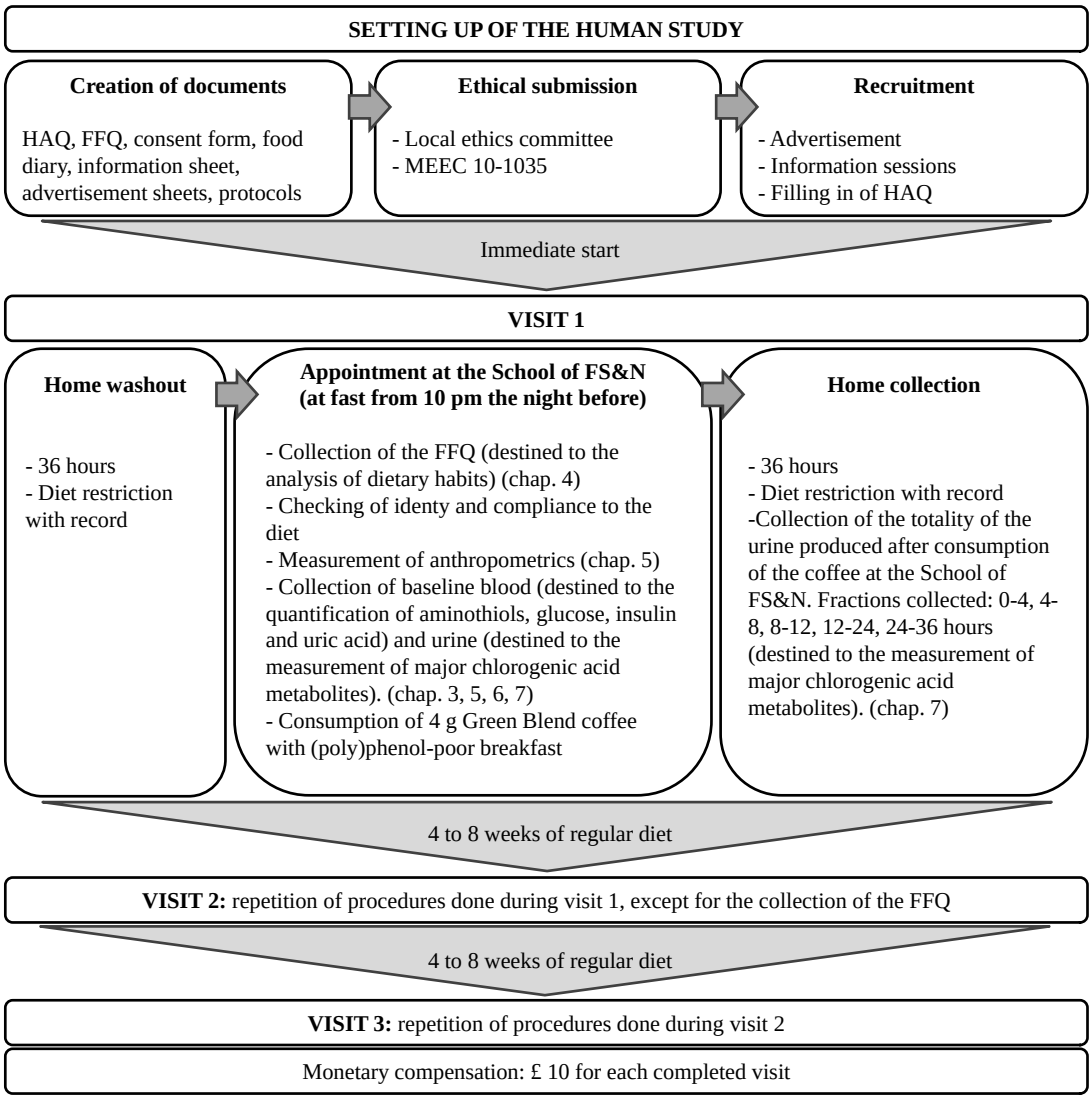


Figure 4.1: Study design. Summarizing chart with the major steps in the study, from the preparation of the documents for the participants to the different procedures in the study. Chap., chapter; HAQ, health assessment questionnaire; FFQ, food frequency questionnaire; FS&N, School of Food Science and Nutrition at the University of Leeds.

4.3.4 Analysis of the food frequency questionnaire

The data collected from the food frequency questionnaire was semi-quantitative and provided information on the consumption of coffee, tea, cocoa and alcohol, as well as the general habits of fruit, vegetables, herbs, spices, supplements and vitamins consumption, and on the frequency and types of foods consumed during the main meals (i.e. breakfast, lunch and dinner) and snacks. In order to

ensure the appropriate comprehension and consequent filling out of the food frequency questionnaire as well as the food diary, both were submitted to persons that were not familiar with the research area. A few improved versions were progressively created. The difficulty of data interpretation for the subsequent use for statistical analysis was equally taken into account.

4.3.4.1 Information on coffee, tea and cocoa preparations habits

Questions were asked on how long participants had been consuming coffee, tea and cocoa, in months or years. For coffee, details of consumption of caffeinated, decaffeinated, strong, medium, weak, instant, filtered, espresso, cappuccino, latte, turkish, arabic or other (with specification) were asked. For tea, details for the consumption of decaffeinated black tea, caffeinated black tea, instant black tea, black tea in form of tea bags, black tea in form of leaves, black tea in form of commercial iced tea, green tea, rooibos, chai, herbal infusion, fruit infusion or other (with specification) were asked. For cocoa, details on the consumption of dark, milk, white, bitter or other type of chocolate beverage was asked. The information was reported as the number of cups consumed in 7 days and was obtained by multiplying by 7 the number of cups consumed in one day. For each of these beverages there were 6 options of answers, ranging from "none" to "more than 8 cups per day". If a subject had a regular consumption of 1 - 2 cups of black tea in a day, the consumption in a week was calculated as the average, 1.5 cups per day, multiplied by 7, that is, 10.5 cups of black tea in a week. For the option "more than 8 cups per day", the value of 9 was multiplied by 7. Additionally, for each of the three types beverages, information on whether sugar, sweetener, milk or anything else was added to it and how much was asked.

4.3.4.2 Information on other dietary habits

For alcoholic beverages, the questionnaire asked information on the amount of units of beer, cider, wine, spirits, alcopops or other were consumed in a week. There were 5 possible answers ranging from "none" to "more than 4 units per days". As for coffee, tea and cocoa, if a subject consumed 3 - 4 units of beer in a day, the total beer consumption in a week was calculated as the average, 3.5 units per day,

multiplied by 7. The total alcohol consumption in a week was obtained from the sum of the weekly consumption of each type of alcoholic drink.

Information on the frequency of consumption of common snack products (i.e. fruit, yoghurt, dried fruits, nuts, crisps, chocolate, cake, crackers) was asked. There were 6 possible answers to select and ranging from "none" to "more than twice a day".

For the consumption of fruit and vegetables, only the most commonly used products in the UK were included. Comprised in the list of common vegetables were salad, cruciferous vegetables, green beans and peas, carrot, sweet corn, tomato and potato in any form. Included in the common fruits consumed were any berries, citrus fruits, kiwi, grape, peaches and nectarines, apple, banana and any dried fruit. The 9 given options of consumption for vegetables and fruits ranged from "none" to "more than 5 times daily" and the unit was a portion, which was defined as the amount of product that was the size of the fist of the participant.

As for the main meals (i.e. breakfast, lunch, dinner), the most common products consumed during these periods were included. For breakfast, cereals, porridge, muesli, white bread, wholemeal bread, yoghurt, fruit, dairy milk, soy milk, tea, coffee, eggs and bacon or sausage were included. For lunch and dinner, salad, soup, sandwich, pizza, pasta, burger and chips, meat or fish, rice or potatoes and curries were within the common list. The 6 options for the type of food consumed at the specific meals ranged from "never" to "daily".

Information on the spices and herbs regularly consumed was also included in the food frequency questionnaire and included pepper, chilli, cumin, saffron, ginger, cardamom, star anise, cinnamon, turmeric, cloves mint, coriander, thyme and rosemary. The final category asked information on the frequency of usage of supplements and vitamins and subjects completed the information for berry-based supplements, seed-based supplements, non-berry of seed-based supplement, minerals and trace elements and vitamins. There were 6 possible answers for the consumption of spices, herbs, supplements and vitamins, ranging from "never" to "daily".

For all the foods and drink categories, an open-ended section was used for reporting frequently consumed items which were not included in the food frequency questionnaire.

4.3.4.3 (Poly)phenolic intake with coffee, tea and cocoa beverages

The estimation of the (poly)phenolic intake from (poly)phenol-containing beverages, such as coffee, tea and cocoa, is a challenging task. Several factors, including the environmental growing conditions, processing and the brewing method, influence the (poly)phenolic composition of these beverages. In the food frequency questionnaire completed by the participants, besides the information asked regarding the overall coffee consumption at different strengths, the possible choices of coffee beverages were instant, filtered, espresso, cappuccino, latte, Turkish and Arabic. The information collected on the consumption of the different brews of coffee was limited to the number of cups consumed per week, but did not provide information on the factors influencing the (poly)phenolic content of the beverage. In order to obtain an accurate individual (poly)phenolic intake due to the consumption of the above-mentioned beverages, the analysis of each consumed beverage should have been conducted, which was realistically not feasible.

Total phenolic content is often estimated by the method of Folin-Ciocalteu. This colorimetric method is based on the reducing properties of the phenol group and is easily performed, however, it is susceptible to interferences from other non-phenolic constituents in the sample, such as ascorbic acid, for which the measurement can be corrected. The most accurate method for total phenolic analysis is chromatography, followed by the sum of all the phenolic compounds analysed. However, many (poly)phenols are difficult to analyse by chromatography and these include theaflavins, thearubigens, some procyanidins, tannins or complex melanoidins. No information exists on the total phenolic composition of coffee, tea and chocolate beverages determined by chromatography and which includes an exhaustive list of the constituent (poly)phenols. A rich database of foods with (poly)phenolic composition determined by different methods exists, but not all the foods of interest are reported (http://phenol-explorer.eu/). In 2010, a list of the 100 products highest in (poly)phenols was generated, and the (poly)phenolic

composition determined by chromatography was compared to the antioxidant content determined using the Folin-Ciocalteu assay (Perez-Jimenez et al., 2010a). The latter was, for the majority, higher when compared to the results obtained by chromatography, however, the (poly)phenolic composition of foods remains incomplete and the phenolics that are missing from the chromatographic analysis would possibly partly explain the difference.

Taking these reflexions into account, the (poly)phenolic intake from coffee, tea and cocoa in the present study was based, when possible, on published data obtained by Folin-Ciocalteu analyses. Briefly, the beverages were attributed an index and ranked within their category (i.e. coffee, tea or cocoa beverages) from lowest to highest according to their total (poly)phenolic contents of per serving.

4.3.4.4 (Poly)phenolic intake from coffee

Two new variables were introduced for the non-quantitative evaluation and comparison of (poly)phenolic intake from coffee between subjects. The first variable, the "coffee level", which did not take into account the consumption of specific brews, was calculated from the total weekly consumption of coffee and its strength, according to what participants had reported. The strength referred to the amount of coffee, rather than the intensity of the roast. An index of strength was attributed to each strength of coffee, namely "1" for weak, "2" for medium, and "3" for strong. To determine the total "coffee level" per week for a participant, the index was then multiplied by the number of cups of the corresponding strength consumed on a weekly basis, and if the participant consumed coffee at variable strengths, the values obtained from the multiplication of the strength index by the number of cups consumed per week were summed. Assuming that a stronger coffee provides more (poly)phenols, a higher "coffee level" index indicates a higher intake of (poly)phenols.

The second variable for the intake of coffee (poly)phenols, the "coffee (poly)phenolic index", was created to estimate the total weekly (poly)phenolic intake from the ingestion of different coffee brews (e.g. filtered coffee or cappuccino). In a research by Sánchez-Gonzalez et al. (2005), the analysis of filtered, espresso and freeze-dried (instant) coffee revealed that, per serving, filtered coffee provided the

most (poly)phenols, closely followed by espresso. In this study, the serving volume of filtered coffee was 111 mL and that of the espresso was 45 mL, however, twice as much ground coffee was used for the preparation of filtered coffee, resulting in a similar ground coffee to water ratio (0.025 g/mL and 0.031 g/mL, respectively). The lowest (poly)phenolic content was measured in a serving of freeze-dried coffee (Sánchez-González et al., 2005). As lattes and cappuccinos are prepared from espresso shots, these brews were attributed the same index as espresso in the present study. The volume of espresso shots most frequently varies between 30 and 60 mL, but no information was asked in the food frequency questionnaire on the strength of specific coffees, therefore no distinction was made for this purpose. Due to the lack of information regarding the content of total (poly)phenols and chlorogenic acids in Turkish and Arabic coffee, and as only 2 participants were regular consumers of Turkish coffee (10.5 cups/week), and an additional 2 were low consumers of Arabic coffee (1.5 cups/week), those brews were regarded as regular coffee and considered to contain the same quantity of total (poly)phenols as a serving of espresso coffee. In conclusion, the following "coffee (poly)phenolic indexes" were attributed to the different brews: "1" for instant coffee, "2" for espresso, latte, cappuccino, Turkish and Arabic coffee, and "3" for filtered coffee.

4.3.4.5 (Poly)phenolic intake from tea and infusions

For tea and cocoa beverages, the created variables were "tea (poly)phenolic index" and "cocoa (poly)phenolic index", respectively. These, as for coffee, were based on information reported in the literature. Along with coffee, tea and cocoa are listed amongst the 50 products with the highest contents of (poly)phenols per serving (Perez-Jimenez et al., 2010a, Perez-Jimenez et al., 2010b). Green tea infusion is frequently reported to contain more (poly)phenols than black tea infusion (Chan and Lim, 2006, Anesini et al., 2008, Almajano et al., 2008, Atoui et al., 2005, Horžić et al., 2009, Fukushima et al., 2009), although there is some disagreement depending on the source (Gorjanovic et al., 2012). Both brews are preparations from the same plant species (*Camellia sinensis*), however, leaves used for green tea are not subject to oxidation, as opposed to those used for black tea. Once the leaves have dried, (poly)phenol oxidase is inhibited, maintaining (poly)phenols in their monomeric

non-oxidised forms (Anesini et al., 2008, Pekal et al., 2011), and resulting in a different (poly)phenolic profile (Almajano et al., 2008). Green tea contains more catechins which are absorbed and black tea (poly)phenols need microflora degradation before absorption. Chai tea is classified as flavoured black tea as it is a preparation from black tea with spices added to it. The latter include cinnamon, cloves, star anise, ginger, cardamom, peppercorn, nutmeg and fennel, which are chiefly products that contain high amounts of (poly)phenols (Perez-Jimenez et al., 2010a). A single publication which includes the simultaneous total phenolic analysis of commercially available and popular fruit infusions, flavoured black teas and black tea was found (Pekal et al., 2011). In a later publication from the same first author, Indian Chai tea was included in the analysis of flavoured black tea infusions (Pekal et al., 2012). Combined, the results of both studies indicate that fruit tea infusions provide the least total phenols, followed by the flavoured black tea infusions, while black tea provided the most total phenols. In 2011, Fu et al. compared the total phenolic composition of 28 herbal infusions and 23 tea infusions from China. Large variations were observed within the herbal infusions, and the highest total phenolic contents were observed within this category, but overall, the average of the herbal infusions (0.41 g gallic acid equivalent/L) was lower than that obtained from the analysis of the tea infusions (0.56 g gallic acid equivalent/L) (Fu et al., 2011). Similar results were previously obtained by other groups (Almajano et al., 2008, Atoui et al., 2005, Chan and Lim, 2006). In a study by Almajano et al. (2008), in which 13 types of teas were tested, the analysis of Rooibos infusion indicated a lower total phenolic composition when compared to that of black and green tea infusions, which is in agreement with a previous publication (Erickson, 2003). In the same research by Almajano et al., the other herbal infusions tested (i.e. peppermint and nettle) had a total phenolic content that was lower than that obtained from the Rooibos infusion (Almajano et al., 2008). The attribution of indexes reflecting the total phenolic composition of the different tea beverages was based on the information above. As no comparative information was found on the total phenolic composition of fruit and herbal infusions, these were joined and given the index of "1". Rooibos tea, as a herbal tea, with a higher total phenolic content, was given the index of "2". Chai tea, as a fragrant black tea, with a lower total phenolic content than black tea, was given the index of "3", black tea was given the index of "4", and green tea, the index of "5".

4.3.4.6 (Poly)phenolic intake from cocoa beverages

Finally, for cocoa, the participants provided information on the number of cups of cocoa beverages consumed in 7 days, with the options of consuming bitter, dark, milk or white chocolate beverages. These are characterised by a different percentage of cocoa and therefore, a different total (poly)phenolic content (Vinson et al., 1999). The latter increases with the cocoa percentage of the chocolate product and a crescent index was attributed to each chocolate beverage accordingly: "0" for white, "1" for milk, "2" for dark and "3" for unsweetened bitter chocolate.

4.3. 5 Statistics

Statistical analyses were performed using the Microsoft Office Excel 2007 and IBM SPSS statistics 20 softwares. All averaged values are expressed as the mean ± standard deviation unless otherwise stated. Normality of the variables was assessed by a Shapiro-Wilk test. Outliers were systematically included in the statistical analysis as they were genuine data points. Most often, non-parametric tests were used, due to the presence of outliers and a non-normal distribution, unless otherwise stated.

4.4 Results

4.4.1 Sample size

Amongst the biomarkers of interest, the sample size obtained when using variables from total homocysteine was the largest, with 52 subjects as shown in Table 4.2. This was also the minimal sample size aimed at for the present study. A total of 62 participants were recruited and successfully completed one visit. Due to dropouts, 49 subjects completed two visits and 40 completed the three possible visits. The main motive behind the drop outs were dietary restrictions, lack of availability and the small monetary compensation. A few subjects with higher regular consumption of

coffee complained about headache and lower level of energy during the diet restriction.

Table 4.2: Variables used for the calculation of the sample size. μ_0, are the expected values, expressed as the mean ± standard deviation (SD). Known values, μ1, and known SD, σ, were based on data reported in publications and are expressed as the mean ± SD (σ). n.d., not determined. † (Pfeiffer et al., 1999), ††(Urgert et al., 2000), ‡ (Pimkova et al., 2014), ‡‡ (Levitzky et al., 2008), ‡ ‡ ‡ (Ruggiero et al., 2006)

Biomarker	μ_0 ± SD	μ_1 (± σ)	N (sample size)
Total cysteine	251 ± 10	289 ± 31 †	6
Total homocysteine	7.4 ± 2	8.5 ± 1.8 †	22
		8.1 ± 1.8 ††	52
Total cysteinylglycine	37 ± 4	32 ± 6 ‡	12
Total glutathione	7.9 ± 0.6	9.07 ± 1.55 ‡	14
Glucose	4.7 ± 0.3	5.3 ±1.3 ‡‡	37
Insulin	7.3 ± 1.9	9.6 ± 3.1	15
Uric acid	0.26 ± 0.06	0.3 ± 0.07 ‡‡‡	25

4.4.2 Analysis of the food frequency questionnaire, emphasis on (poly)phenol-rich beverages

Not all the participants provided complete information on their dietary habits. In these cases, data were excluded pair-wise, explaining the variable number of data available for some analyses.

4.4.2.1 Habits of coffee consumption

Total coffee consumption: As expected, the years of coffee consumption increased with the age of the participant (data not shown). The average weekly consumption of coffee in the UK was estimated to be 5 cups (0.74 cups per day) as

explained in section 4.2. When compared to the level of consumption in the UK, the average level of coffee consumption of the study population was higher. This can easily be explained by the fact that coffee had a central role in the present study and subjects disliking coffee or those that were not regular consumers were less likely to participate in the research. The studied population had an average of total coffee consumption of 13 ± 14 cups per week (Table 4.3). Medium and strong preparations were the most popular, with 8 ± 10 and 5 ± 10 cups consumed per week, respectively. The difference of the preference in the strength of coffee preparation was confirmed by running a non-parametric Friedman's test; X^2 (2) = 31.89, p < 0.0005. Pairwise comparisons were performed with a Bonferroni correction for multiple comparisons and the significant differences are shown in table 4.3. Post hoc analysis revealed statistically significant differences in preference between weak (*Mdn* = 0.00) and medium coffee (*Mdn* = 1.5), p < 0.0005; between weak and strong coffee (*Mdn* = 0.00), p = 0.012; but not between medium and strong coffee, p = 0.182.

The effect of gender on the consumption of coffee was assessed by analysing the significance of the difference between groups, rather than using a method for analysing the associations. A Mann-Whitney U test was therefore run. The distributions of the coffee level between gender were similar, and the median coffee level was not statistically significantly different between females (*Mdn* =10.5) and males (*Mdn* =10.5); U = 318.5, z = - 0.395, p = 0.693. No significant association between the total coffee consumption and the age of the participants was detected, as assessed by running a Spearman's rank-order correlation test; r_s (52) = 0.073, p = 0.599.

Table 4.3: Coffee consumption of the study population. Data are expressed as the average cups consumed in 7 days (means ± standard deviation, SD) for the total coffee consumption and that of the individual strength of coffee. From the 62 recruited participants 54 provided complete information. A Friedman's test was used to assess the significance of the difference in preference for a particular strength of coffee preparation, within the whole population and the differences of preferences between gender were assessed by running a Mann-Whitney U-test due to the presence of outlier(s) and a non-normal distribution within both group.

	Total coffee consumption	Coffee strength		
		Weak	Medium	Strong
	cups/week ± SD			
All population (n=54)	13 ± 14	0 ± 1 †, ††	8 ± 10 †	5 ± 10 ††
Gender				
Female (n=34)	13 ± 13	0 ± 2	9 ± 12	4 ± 7
Male (n=20)	13 ± 16	0 ± 0	6 ± 7	7 ± 14
Age range				
18 - 22 (n=15)	12 ± 12	0 ± 1	7 ± 9	5 ± 8
23 - 29 (n=22)	10 ± 10	0 ± 0	7 ± 10	3 ± 4
30 - 53 (n=17)	18 ± 19	1 ± 3	9 ± 13	8 ± 16

The same symbol indicates a statistically significant difference between the marked groups.

Preferences for selected coffee brews: Only 8 out of the 56 participants that provided information on the matter, consumed decaffeinated coffee, and the population average was 1 ± 4, against 12 ± 13 cups consumed weekly for caffeinated coffee. When the consumption of particular coffee brews was compared, an evident preference for instant coffee was noticed (Table 4.4). The population had an average of 7 ± 12 cups of instant coffee consumed per week. Espresso and filtered coffee were the second most consumed coffee brews, followed by latte and cappuccino, and by Arabic and Turkish coffee brews. The little popularity of the two latter preparations is likely to be due to the ethnicity of the majority of the study population. A Friedman test was run to compare the difference in preference for particular coffee brews and results confirmed that the difference was significant, X^2 (6) = 78.64, $p < 0.0005$. Pairwise comparisons with a Bonferroni correction for multiple comparisons indicated that the difference in preference was significant between filtered and Turkish coffee ($p = 0.034$), instant and Turkish coffee ($p < 0.0005$), Arabic and instant coffee ($p < 0.0005$), espresso and instant coffee ($p < 0.0005$) and latte and instant coffee ($p = 0.037$). No other statistical difference was observed ($p > 0.05$).

Although the weekly consumption was similar, females were in general higher consumers of instant coffee and latte (Table 4.4). These associations were tested by means of a Mann-Whitney U test and the results indicate that only the consumption of latte was significantly different between genders (U = 229.5, z = -2.8, p = 0.05). There was no influence of the age on the preference for any coffee brew, as assessed by a Friedman's test ($p > 0.1$).

Table 4.4: Weekly consumption of individual coffee brews, expressed as mean ± standard deviation (SD). Overall, there were little variations amongst gender and with age, but some differences were revealed to be statistically significant. The significance of the differences in preference for particular brews, within the whole population, were assessed by running a non-parametric Friedman test, and the differences between gender were assessed by running a non-parametric Mann-Whitney U-test due to the presence of outliers and a non-normal distribution within both groups. Esp., espresso; Capp, cappuccino.

	Coffee brews						
	Instant	**Esp.**	**Latte**	**Capp.**	**Filtered**	**Arabic**	**Turkish**
	cups/week ± SD						
All population (n=56)	7 ± 12 $^{**, \dagger, \dagger\dagger, \S}$	2 ± 7††	1 ± 4§	1 ± 3	2 ± 5*	0 ± 0†	0 ± 2$^{*, **}$
Gender							
Female (n=35)	8 ± 10	2 ± 7	2 ± 5	1 ± 3	2 ± 6	0 ± 0	0 ± 2
Male (n=21)	6 ± 14	2 ± 6	1 ± 2	1 ± 3	2 ± 4	0 ± 0	1 ± 2
Age range							
18 - 22 (n=15)	5 ± 8	2 ± 7	3 ± 7	1 ± 3	4 ± 8	0 ± 0	0 ± 0
23 - 29 (n=22)	8 ± 9	1 ± 3	1 ± 2	2 ± 3	1 ± 3	0 ± 0	1 ± 2
30 - 53 (n=19)	8 ± 16	4 ± 9	1 ± 2	1 ± 3	2 ± 4	0 ± 0	1 ± 2

The same symbol indicates a statistically significant difference between the marked groups.

4.4.2.2 Habits of tea consumption

As for the habits of coffee consumption, the time a subject had been a consumer of tea increased with the age of the participant (data not shown). The average consumption of tea was 17 ± 16 cups/week, which corresponds with the average tea consumption in the UK 14 - 21 cups/week (2 - 3 cups/day), as estimated as described in section 4.2. Black tea was most popular (8 ± 13 cups/week), followed by green tea (3 ± 6 cups/week), herbal infusions (2 ± 4 cups/week), fruit infusions (2 ± 3 cups/week), rooibos tea (1 ± 4 cups/week) and chai tea (1 ± 2 cups/week) (Table 4.5).

To assess the influence of age on the level of tea consumption and on the preference for different tea preparations, a Spearman's rank-order correlation was performed. Total tea consumption was not found to be age-dependent, and other than the herbal infusion, which was positively associated with age (r_s (55) = 0.265, p = 0.046), no other tea preparation was found to be associated with age (p > 0.6). The differences in tea consumption between females and males were tested by running a Mann-Whitney U test. The distributions of the different tea preparations for females and males were similar, as assessed by visual inspection. The median consumption of green tea was significantly different between females (*Mdn* = 1.5) and males (*Mdn* = 0.0); U = 252, z = - 2.122, p = 0.034. The median consumption between females and males was not significantly different for the other preparations (p > 0.43). A positive association was detected between the consumption of green tea and herbal infusion (r_s (55) = 0.348, p = 0.008), green tea and fruit infusion (r_s (55) = 0.455, p < 0.0005), and fruit infusion and herbal infusion (r_s (55) = 0.667, p < 0.0005). Finally, a negative association was measured between the consumption of black tea and that of chai tea (r_s (55) = - 0.313, p = 0.018). However, since only two subjects were regular consumers of Chai tea, this association cannot be considered.

Table 4.5: Tea consumption in the study population. Data are expressed as the average cups consumed in 7 days (means ± standard deviation, SD) for the different tea preparations and the total tea consumption. The latter was obtained from the sum of the average consumption of each individual tea and infusion preparation. The difference of preferences between gender was assessed by running a non-parametric Mann-Whitney U-test due to the presence of outliers and a non-normal distribution within both groups. inf., infusion.

	Tea preparations						
	Black tea	**Green tea**	**Rooibos**	**Chai**	**Herbal inf.**	**Fruit inf.**	**Tea and inf.**
	Cups / week ± SD						
All population (n=57)	8 ± 13	3 ± 6	2 ± 4	2 ± 3	1 ± 4	1 ± 2	17 ± 16
Gender							
Female (n=36)	7 ± 12	4 ± 6†	2 ± 3	2 ± 4	1 ± 5	1 ± 2	17 ± 15
Male (n=21)	10 ± 15	3 ± 7†	3 ± 6	1 ± 3	1 ± 3	1 ± 2	18 ± 17
Age range							
18 - 22 (n=16)	9 ± 13	4 ± 9	2 ± 5	2 ± 4	2 ± 7	0 ± 1	19 ± 17
23 - 29 (n=22)	8 ± 11	3 ± 5	3 ± 4	2 ± 4	1 ± 2	1 ± 3	17 ± 16
30 - 53 (n=19)	8 ± 15	3 ± 5	2 ± 4	1 ± 2	1 ± 3	1 ± 2	16 ± 16

The same symbol indicates a statistically significant difference between the marked groups.

4.4.2.3 Habits of cocoa consumption

When compared to the consumption of coffee and tea, the consumption of cocoa beverages was the lowest. Independently of the cocoa contents of the beverage, the study population consumed a total average of 2 ± 4 cups/week, with milk chocolate beverages largely accounting for the cocoa (poly)phenolic intake (Table 4.6). The study population had an average of milk chocolate beverage intake

of 2 ± 4 cups/week, with female (*Mdn* = 1.5) subjects tending to consume more milk chocolate drink than the male (*Mdn* = 0) subjects. This difference between genders was, however, not statistically significant, as assessed by the non-parametric Mann-Whitney U test; $U = 269.5$, $z = -1.838$, $p = 0.066$. A Spearman correlation test did not indicate any association of the consumption of cocoa-containing beverages, with the age, $r_s (56) = 0.006$, $p = 0.965$.

Table 4.6: Cocoa beverage consumption in the study population. Data are expressed as the average ± standard deviation (SD) cups consumed in 7 days for the different chocolate preparations. The difference of preferences between gender was assessed by running a non-parametric Mann-Whitney U-test due to the presence of outliers and a non-normal distribution within both groups.

	Chocolate preparations			
	White	**Milk**	**Dark**	**Bitter unsweetened**
	Cups / week ± SD			
All population (n=58)	0 ± 0	2 ± 4	0 ± 1	0 ± 0
Gender				
Female (n=37)	0 ± 0	3 ± 5	0 ± 1	0 ± 0
Male (n=21)	0 ± 0	1 ± 2	0 ± 1	0 ± 1
Age range				
18 - 22 (n=17)	0 ± 0	2 ± 3	0 ± 0	0 ± 0
23 - 29 (n=22)	0 ± 0	3 ± 6	0 ± 1	0 ± 0
30 - 53 (n=19)	0 ± 0	1 ± 2	0 ± 1	0 ± 0

4.4.3 (Poly)phenolic intake from coffee, tea and cocoa-beverages

The (poly)phenolic indexes attributed to each beverage are summarized in Table 4.7 and were used to calculate the final score representative of the total (poly)phenol consumption from all the beverages of a same category (i.e. coffee, tea

or cocoa). As for the "coffee level" variable, the number of cups of a particular beverage, reported to be consumed by each participant on a weekly basis, was multiplied by the corresponding (poly)phenolic index of Table 4.7. For example, a participant consuming 6 cups of cappuccino per week would have a total score of (poly)phenolic intake from coffee beverages of 12, if no other coffee beverage was consumed. When participants consumed beverages which were attributed a different score, but that were within the same category, the multiplied indexes were summed. Thus if the earlier mentioned subject had also reported to consume 4 cups of filtered coffee per week, the total score of (poly)phenolic intake from coffee beverages would increase to 24. The scores obtained for each participant are representative of their weekly level of total (poly)phenols ingestion from the 3 different sources of beverages.

Table 4.7: Polyphenolic (PP) indexes representative of the level of (poly)phenols ingested with each beverage. A higher index indicates higher amounts of total phenolic content as assessed by the Folin-Ciocalteu method. The 3 categories of beverages are treated independently and the same value of an index does not represent the same total (poly)phenolic content between categories. inf., infusion

Coffee preparation	**Coffee PP index**	**Tea preparation**	**Tea PP index**	**Cocoa preparation**	**Cocoa PP index**
Instant	1	Fruit inf.	1	White	0
Espresso	2	Herbal inf.	1	Milk	1
Latte	2	Rooibos	2	Dark	2
Cappuccino	2	Chai	3	Bitter unsweetened	3
Turkish	2	Black	4		
Arabic	2	Green	5		
Filtered	3				

4.4.3.1 Correlations between (poly)phenolic indexes

When the total level of (poly)phenolic ingestion was analysed between the 3 categories of beverages, there was a significant positive correlation between the total (poly)phenolic intake from coffee and from tea, with a correlation factor r_s (55) = 0.348, p = 0.006, as assessed by a Spearman's correlation test. This indicates that the subjects consuming either a high amount of coffee preparations or coffee preparations higher in total (poly)phenols also tend to consume more tea preparations, or tea preparations that are richer in (poly)phenols, such as green tea. There was no significant correlation identified between the total (poly)phenolic intake from coffee and cocoa beverages (r_s (55) = - 0.008, p = 0.955) or between the total (poly)phenolic intake from tea and cocoa beverages (r_s (55) = 0.059, p = 0.662).

Table 4.8: Introduced variables, which allowed for the comparative analysis of (poly)phenolic intake from different sources. The coffee level took into account the strength and the quantity of coffee consumed in a week. The total (poly)phenolic (PP) indexes of coffee, tea and cocoa beverages took into account the level of (poly)phenols present in a particular brew and the quantity of the corresponding brew consumed in a week. The difference between gender of polyphenolic intake caracterized by the coffee level and total PP index variables, was assessed by running a non-parametric Mann-Whitney U-test, due to the presence of outliers and/or a non-normal distribution within both groups.

	Coffee level (n=54)	Total coffee PP index (n=57)	Total tea PP index (n=57)	Total cocoa PP index (n=57)
	Cups / week ± SD			
All population	31 ± 36	25 ± 33	41 ± 41	3 ± 5
Gender				
Female	30 ± 30	26 ± 35	42 ± 41	4 ± 5
Male	32 ± 45	23 ± 28	40 ± 44	2 ± 3
Age range				
18 - 22	29 ± 30	26 ± 48	44 ± 35	2 ± 3
23 - 29	22 ± 22	20 ± 19	41 ± 35	4 ± 7
30 - 53	44 ± 51	29 ± 30	39 ± 35	2 ± 3

4.4.3.2 Influence of age and gender on (poly)phenolic intake

Coffee level vs. age and gender: The influence of age on the coffee level was analysed by running a non-parametric Spearman's test. No association was detected; r_s (52) = 0.027, p = 0.848. A non-parametric Mann-Whitney U test revealed no gender effect on the coffee level. The distributions of the coffee level for females and males were similar and the difference in the median of the coffee level between females (*Mdn* = 21.00) and males (*Mdn* = 21.00) was not significant; U = 339.5, z = - 0.009, p = 0.993 (Table 4.8).

(Poly)phenolic index vs. age and gender: Analysis of the level of (poly)phenolic ingestion due to the consumption of coffee, tea and cocoa preparations did not reveal any association with age, as assessed by a Spearman's correlation test. The correlation factors, r_s (55), for the coffee, tea and cocoa (poly)phenolic ingestion level with age were 0.136 (p = 0.314), 0.075 (p = 0.579) and 0.020 (p = 0.884), respectively. The outcome did not change when age was analysed as categories. Females had, in general, higher values of (poly)phenolic

indexes (Table 4.8), but the difference was not significant as assessed by a Mann-Whitney U test**.** The distributions of (poly)phenol ingestion from the 3 sources were similar for females and males. For coffee, the median total (poly)phenolic ingestion of the female population was 11.75, and that of the male population was 10.5 with $U = 430$, $z = -0.148$, $p = 0.883$. For tea, the median total (poly)phenolic ingestion of the female population was 28.5 and that of the male population 22.5 with $U = 405.5$, $z = -0.509$, $p = 0.611$. Finally, for cocoa beverages, the difference between the median total (poly)phenolic index for cocoa beverages of the female population was 1.5, and that of the male population, 0.75, was not significant; $U = 293$, $z = -1.604$, $p = 0.109$.

4.4.4 Likelihood of consumption of other (poly)phenols

As a method of testing whether the answers from the food frequency questionnaire were pertinent, the correlation between "coffee level" and "total coffee (poly)phenolic index" was tested. As expected, both variables were strongly correlated (r_s (52) = 0.839, $p < 0.0005$), as assessed by the Spearman's correlation test. This reassuring result indicates that participants did not provide random answers in the food frequency questionnaires.

In the study population, the average weekly consumption of fruit was 23 ± 14 fist-size portions with reported individual consumptions ranging from 1 - 67 portions per week. The average weekly consumption of vegetables (excluding potatoes) was 21 ± 15 fist-size portions with reported individual consumption ranging from 2 - 78 portions per week. Finally, the average combined weekly consumption of fruit and vegetables (excluding potatoes) was 45 ± 25 fist-size portions with reported individual consumption ranging from 10 - 100 portions per week, which is equivalent to a daily consumption of 6.4 portions, higher than the recommended 5 portions per day in the UK (Carter et al., 2010). Of note, 23 subjects had a consumption lower than this recommendation. The consumption of supplements was relatively low in the study population, with an average ± standard deviation weekly consumption of 3.5 ± 5.5 supplements.

A Spearman's correlation test was performed to test any associations between the intake of (poly)phenols from coffee, tea and cocoa beverages, and the intake of

fruit or vegetables, herbs and spices, and supplement consumption. There were strong positive associations between the weekly consumption of coffee (poly)phenols and that of fruit, r_s (55) = 0.569, $p < 0.0005$; vegetables, r_s (53) = 0.437, $p = 0.001$; but no associations with the consumption of herbs and spices, r_s (55) = 0.203, $p = 0.130$ or supplements, r_s (55) = - 0.139, $p = 0.301$. The intake of (poly)phenols from tea preparations was not associated with the consumption of fruit; vegetables, herbs and spices or supplements, $p > 0.091$. There were positive associations between the weekly consumption of tea and that of fruit, r_s (55) = 0.361, $p = 0.004$; and herbs and spices, r_s (55) = 0.323, $p = 0.01$; but no significant association was detected with the consumption of vegetables, r_s (53) = 0.205, $p = 0.133$; or the intake of supplements, r_s (55) = 0.161, $p = 0.211$. There were positive associations between the consumption of (poly)phenol from cocoa beverages and fruit consumption, r_s (55) = 0.285, $p = 0.032$; as well as with the intake of supplements, r_s (55) = 0.427, $p < 0.001$; but no significant association was detected with the consumption of vegetables, r_s (53) = - 0.177, $p = 0.196$; or herbs and spices, r_s (55) = 0.023, $p = 0.867$.

When (poly)phenol sources other than coffee, tea and cocoa beverages were tested for their association, some strong positive correlations were found. These were between the consumption of fruits and vegetables, r_s (53) = 0.476, $p < 0.0005$; fruits and herbs and spices, r_s (55) = 0.525, $p < 0.0005$; and vegetables and the use of herbs and spices, r_s (53) = 0.436, $p = 0.001$. A moderate positive association was detected between the consumption of fruits and the intake of supplements, r_s (55) = 0.297, $p = 0.019$; and no significant association was found between the intake of supplements and the consumption of vegetables, r_s (53) = - 0.017, $p = 0.902$; or the use of herbs and spices (r_s (55) = 0.107, $p = 0.406$).

4.5 Discussion

The details regarding the type of coffee, the frequency and amounts of consumption provided important information and with it, some challenges for the interpretation of the data. Information on the caffeination, strength, brew and any additive added to the coffee, tea and cocoa-containing beverage regularly consumed

was collected from a food frequency questionnaire. For the analysis of the data, the information on the ingredients (i.e. milk, sugar or sweetener) added to a beverage was not taken into account.

In the present observational study, the average total coffee consumption from the study population was above that of the average consumption per capita in the UK with no significant difference between gender or influence of age. The participants were mainly consumers of medium and strong caffeinated preparations, and when individual coffee brews were analysed, instant coffee was clearly the mostly consumed preparation. This could be explained by the practicality and low-cost of instant coffee, when compared to the other brews. Arabic and Turkish coffee preparations were the least consumed and this could be explained by the ethnicity of the study population, which was in majority from Occidental countries. Gender, but not age, had an effect on the choice for a particular coffee brew. The average consumption of tea was higher than that of coffee and was similar to the average consumption in the UK. Apart from the consumption of herbal infusions, which was age-dependent, and green tea, which was gender-dependent, total tea consumption and the consumption of particular tea brews was not influenced by either variable. The consumption of cocoa-containing beverages was the lowest when compared to coffee and tea, but amongst them, milk chocolate beverages were the most consumed. No association was found between age or gender with the consumption of cocoa-containing beverages.

A quantitative estimation of the (poly)phenolic intake from coffee, tea and cocoa sources was not possible and therefore, within a category, products were attributed an index representative of their (poly)phenolic content for the comparative statistical analysis of (poly)phenolic intake. The (poly)phenolic index was not comparable between categories, as the indexes did not necessarily correspond to the same level of (poly)phenols. The coffee level and coffee (poly)phenolic index were not influenced by gender, nor by age. The female and male populations were estimated to have a similar intake of (poly)phenols from coffee, tea and cocoa. There was a significant association between the (poly)phenolic index from coffee and from cocoa tea. Some significant associations were detected between the consumption of (poly)phenols from other sources. These were namely positive between the intake of coffee (poly)phenols and fruit and vegetables; there was a positive association

between tea consumption and fruit, and herbs and spices; a positive association between the intake of (poly)phenols from cocoa beverages and the consumption of fruit, and the intake of supplements; and a strong positive association between fruits and vegetables; fruits and herbs and spices; vegetables and herbs and spices; and fruits and the intake of supplements.

Unfortunately not all the study population (n = 62) provided complete information on their general dietary habits and consistently completed the food frequency questionnaire, resulting in a variable number available for statistical analysis.

Chapter 5
Study population characteristics and consistency at baseline

5.1 Abstract

The target study group in the present study was a healthy adult population. Anthropometrics, blood pressure and selected biomarkers of health in fasting plasma were recorded in all the participants (n = 62) in order to characterize the study population and verify the health status of the participants, as well as to assess the consistency of their metabolism. The influence of gender and age on the target variables was investigated. To assess the general health status of the study population, 7 biomarkers of health in fasting plasma, anthropometric variables and general daily habit were analysed for each subject at baseline. The averages ± standard deviation for the investigated biomarkers were 257 ± 25 μM for total cysteine, 9.5 ± 3.5 μM for homocysteine, 30.5 ± 5.6 μM for total cysteinylglycine, 7.7 ± 1.5 μM for total glutathione, 4.7 ± 0.3 mM for glucose, 55.5 ± 16.6 pM for insulin and 0.26 ± 0.06 mM for uric acid. These were consistent with reported values in the literature, for healthy subjects, thus confirming that the study population was in general good health and that the corresponding data were appropriate to undergo further statistical analysis. The study population was relatively young with an average ± standard deviation of 28 ± 8 years old. On average, when compared to the female participants, the male participants were taller, heavier, had a larger waist circumference and waist-to-hip ratio, were more active and had higher levels of plasma total cysteine, homocysteine, cysteinylglycine and uric acid. Age only was associated with a few variables, namely the waist circumference, the diastolic blood pressure, the total cysteinylglycine and the glucose concentration. Overall, the variables of interest were consistent throughout the completed visits. Total cysteinylglycine and glutathione concentrations varied between visits and the outcome for insulin was inconsistent, depending on the choice of the statistical test performed. However, the coefficients of variation were considered to be low for all the biomarkers analysed, therefore suggesting that overall, the analysed biomarkers have a stable metabolism over a period of at least 8 weeks.

5.2 Introduction

Characterizing a recruited study population is important in order to ensure that the population will be adequate to test a hypothesis. Biomarkers of health are primary indicators of the health status of a person. They have long been used as indicators of the general health status or the progression of particular diseases (Alderman et al., 1999, Alper et al., 2005, Alvarez et al., 2009, Andersson et al., 1999, Burack et al., 1985, Vasan, 2006). In numerous human studies investigating the effects of coffee and other (poly)phenol-rich foods on health, various biomarkers of health have been measured in biological samples (Agardh et al., 2004, Ahmed et al., 2009). As the consumption of coffee and other (poly)phenol-rich products has been linked to a reduced risk of inflammation and cardiovascular health (Andersen et al., 2006, Bohn et al., 2012, Bonita et al., 2007, Cano-Marquina et al., 2013), a few reliable biomarkers in fasting plasma that are indicators of these two health statuses were selected for analysis in the population of the present study.

In the present study, a healthy adult population was targeted in order to investigate the impact of a habitual consumption of coffee and other (poly)phenol-rich products, as well as non-dietary habits on the general health status in healthy subjects. The weight and height were recorded to measure the body mass index (BMI) of each participant. The BMI is a useful measure for characterizing the dimension of a person, however it may be misinterpreted. Typical BMI values for both genders and at any age are < 18.50 kg/m^2 for underweight, from 18.50 - 24.99 kg/m^2 for healthy, ≥ 25.00 kg/m^2 for overweight and ≥ 30.00 kg/m^2 for obese subjects (WHO, 2014). For the present study, participants were eligible if they had a BMI of 18.00 - 29.00 kg/m^2. Subjects practicing sport at higher level have a more significant muscular mass, resulting in a higher BMI value and a misclassification of the subject. Therefore, the waist-to-hip ratio variable was recorded as a complementary variable to the BMI. The waist-to-hip ratio is often used as a measure of visceral obesity and has been significantly correlated with myocardial infarction risk (Yusuf et al., 2005). Individuals with a waist-to-hip ratio ≥ 0.85 for females and ≥ 0.90 for males are considered to suffer from visceral obesity and be at a substantially increased risk of metabolic syndrome and consequent complications including Type 2 diabetes Mellitus (Alberti and Zimmet, 1998).

As circulating biomarkers of inflammation and cardiovascular health, the aminothiols cysteine, cysteinylglycine, homocysteine and glutathione, as well as glucose, insulin and uric acid were chosen (chapter 3).

In the present chapter, the study population was characterized in terms of certain non-dietary lifestyle habits, anthropometrics, blood pressure and selected biomarkers of health. The consistency of the measured biomarkers was then evaluated in order to assess the consistency of the metabolism of the study participants.

5.3 Materials and methods

5.3.1 Assessment of selected non-dietary lifestyle habits

Information on smoking habits, frequency of bowel movements and exercising habits were obtained from a health assessment questionnaire filled in once by each participant and used to assess their suitability in taking part in the present study (chapter 3). The number of cigarettes smoked per week was obtained by multiplying the number of cigarettes reported to be smoked in one day by 7. For the frequency of bowel movement, the participants had the options of reporting a movement "once every three days or less", "once every two days", "once per day" or "more than once per day". The corresponding weekly bowel movements were 2.5 (rounded up from 2.33), 3.5, 7 and 14 weekly bowel movements, respectively. It is clear that half bowel movements are not possible and therefore these values were considered as bowel movement scores and used to order the variable on an ordinal scale for the statistical analyses. For the habits of physical activity, the participants were first asked to provide the information on whether they generally never exercised, whether they exercised "once", "twice", "three to four times" per week or "once a day or more". Physical activity was then categorised into light, moderate, strenuous, muscle gain exercise or other, with each category containing a few types of activity. Participants were asked to assess how many hours of each category they were exercising per week. For the interpretation of data, each exercise category was attributed a score that was representative of its intensity. The hours reported by the participants were then multiplied by the score of the corresponding activity. These

scores were "1" for physical activities of light intensity (e.g. walking, yoga), "2" for physical activities of moderate intensity (e.g. fast walking, easy swimming, easy cycling) and "3" for strenuous physical activities (e.g. running, vigorous swimming, high impact aerobics) and muscle gain. If a subject reported multiple activities of different scores, the multiplied scores were summed up. Therefore, if a subject reported on a weekly basis to practice 3 hours of a moderate intensity physical activity and 5 hours of a light intensity physical activity, the final physical activity score was 11. The same score could be obtained by a subject practicing 7 hours of a light physical activity and 2 hours of a moderate physical activity weekly. For the bowel movement, the number of movements were reported as the frequency in 7 days.

5.3.2 Anthropometrics

The anthropometrics of each participant were recorded once during each visit. Before the measurements, the participants were asked to remove their shoes and any other objects and accessories susceptible to influence the measurements (i.e. belt, watch, wallet, coins, keys, heavy upper body outer clothing). The weight was measured on a calibrated portable electronic scale from Seca (SECA 877) to the nearest 0.1 kg. The height was measured with a Leicester manual portable stadiometer from Seca (SECA 214) that was placed against a wall. To have an accurate measure of the distance between the vertex and the bottom of their feet, the participants were asked to keep an upright posture, with the feet close together, the back against the stadiometer and the head in the Frankfurt plane. The reading was taken to the nearest 0.1 cm, however, the averages were rounded to the nearest cm. Both the weight and height were used to calculate the BMI by dividing the weight (kg) by the squared height (m^2). Hip and waist circumferences were measured with a flexible, but resistant to stretch, measuring tape. For both measurements, the participants were asked to keep their arms down and maintain a relaxed but straight posture. For the lower body part, the participants kept their clothing on, but for the upper body part, any thicker clothes were asked to be removed so that there was no more than the thickness of a t-shirt on top of the skin. The hip circumference was the distance recorded around the widest part of the buttocks and the waist circumference

was the abdomen girth measured at its narrowest point, between the lowest ribs and the iliac bone crest (WHO, 2008).

5.3.3 Blood pressure

When the participant was in a relaxed state, the blood pressure was measured using a portable cuffless upper arm blood pressure monitor with elbow support (Panasonic). The measurement was performed in triplicate, and when possible, on the opposite arm from which the blood had been withdrawn and with the hand in a supine position. If the participant was wearing clothes that were tightening their arm, they were asked to remove the arm from the sleeve.

5.3.4 Blood sampling and processing

The participants were seated for the blood collection. Ten milliliters of blood was withdrawn by venepuncture of the antecubital vein in the opposite arm to the one used for writing. The blood was collected in previously ice-cooled EDTA-containing tubes from BD Vacutainer®. In order to slow down the cellular metabolism, the tubes with the collected blood were placed on ice immediately after venepuncture. The samples were then rendered acellular within 30 min through a 10 min centrifugation at 3,000 g and 4 °C, as frequently done (Ubbink et al., 1992). The plasma was carefully separated from the cellular fraction, aliquots were prepared and the samples were stored at - 80 °C until analysis. For the analysis of glucose, 500 µL of freshly drawn EDTA-whole blood was mixed with two antiglycolytic agents, glyceraldehyde and sodium fluoride at a final concentration of 11 and 119 mM, respectively (chapter 3).

5.3.5 Measurement of the biomarkers in plasma

All the selected biomarkers of inflammation and cardiovascular health were measured in fasting plasma from the blood collected on each visit. For all the biomarkers analysed, the fasting plasma was thawed, centrifuged for 10 min, at 3,000 g and 4 °C and processed and analysed as described in chapter 3. For all biomarkers, spiked and non-spiked plasma samples were compared for the

calculation of recoveries. These were prepared with a pool of a combination of plasma from all samples that were included in a set of analysis. The plasma samples were mixed in equal volumes.

5.3.5.1 Total aminothiols

Total cysteine, homocysteine, cysteinylglycine and glutathione were measured in plasma of all the completed visits. Two calibration curves containing the four aminothiols were prepared. One was a milliQ water-based matrix and the other a plasma-based matrix. Both had the same final concentrations ranges for the selected aminothiols, from 0 - 200 μM for cysteine, 0 - 50 μM for homocysteine, 0 - 100 μM for cysteinylglycine and 0 - 50 μM for glutathione. Cystamine dihydrochloride was used as the internal standard and kept at a constant final concentration of 6 μM. Calibration curves were constructed from the normalized areas under the curves plotted against the prepared concentrations of aminothiols on a double-logarithmic scale. To calculate the total aminothiol concentrations in plasma, only the matrix-matched calibration curves were used.

The procedure detailed in section 3.3.3.2 was followed for the preparation of reagents, standards and samples as well as for the sample analysis.

5.3.5.2 Glucose

The glucose measurement in fasting plasma was performed using the hexokinase assay reagent from Sigma following the improved modified method described in chapter 3 (section 3.3.3.3) (Sigma-Aldrich, 2004).

For each participant, two technical replicates were prepared on two different days. The average fasting glucose for each completed visit is therefore based on the analysis of 4 sets of data.

5.3.5.3 Insulin

The quantification of insulin in the fasting plasma was by done by ELISA, using an assay in the form of a kit, from Mercodia (Mercodia). The protocol

described in detail in section 3.3.3.4 was used for the sample preparation and subsequent analysis.

The calibration curve was constructed on a double-logarithmic scale. For each participant, two technical replicates were prepared on two different days. The average fasting insulin for each completed visit is therefore based on the analysis of 4 sets of data.

5.3.5.4 Uric acid

Uric acid was quantified by an enzymatic colorimetric method run in a 96-microwell plate and adapted from a previously published procedure by Fossati et al. (Fossati et al., 1980). For each experiment, the reagents and solutions were prepared as described in section 3.3.3.5. The sample preparation and subsequent analysis were carried out as described in section 3.3.3.5.

Each plasma sample was analysed twice in duplicate so that averages were calculated from 4 technical replicates.

5.3.6 Statistics

Along with the information obtained on selected lifestyle habits, the averaged anthropometrics, blood pressure measurements and concentrations of the selected biomarkers for each participant (n = 62) and all corresponding completed visits (i.e. 1 to 3) were used to characterize the study population at baseline. Any link between the variables measured and the gender and age were tested with an appropriate statistical test for the analysis of differences between groups and inter-variable associations, respectively. To evaluate the consistency of the measured anthropometrics, blood pressure and biomarkers of health, only data from the participants who completed three visits (n = 40) were included. The average measurements obtained for each visit were analysed and compared between visits by conducting the appropriate statistical tests for repeated measures.

Normality of distribution was assessed by a Shapiro-Wilk test and the presence of outliers by visual inspection of box plots. Unless otherwise stated, genuine outliers were included in the statistical analysis and non-parametric tests were used only when significant outliers were present and the variables did not

follow a normal distribution. All averaged values are expressed as the mean ± standard deviation unless otherwise stated. Statistical analyses were performed using the Microsoft Office Excel 2007 and IBM SPSS statistics 20 softwares.

5.4 Results

5.4.1 Baseline characteristics of the study population (n = 62)

The study population consisted of 40 females and 22 males (chapter 4) ranging from 18 - 53 years old. The average age was 28 ± 8 years old and was similar between female (28 ± 8) and male (29 ± 9) participants (Table 5.1). The young study population is easily explained by the fact that the present study was advertised and participants were recruited in an area which included the University of Leeds campus.

5.4.1.1 Non-dietary lifestyle habits

The study population was relatively active, with an average physical activity score of 12.3 ± 12.6. In general, males (15.5 ± 9.9) were more active than females (10.5 ± 13.7). Three outliers were detected and the distribution was similar but non-normal for both genders. A non-parametric Mann-Whitney U-test was run to determine the impact of gender on physical activity. The analysis revealed significantly different medians for the female (*Mdn* = 8.0) and male (*Mdn* = 18.0) study population, $U = 538$, $z = 2.422$, $p = 0.015$. A non-parametric Spearman's correlation test indicated no association with age, r_s (56) = - 0.022, $p = 0.868$.

The average bowel movement score reported by the study population was 7.9 ± 2.9 and was very similar for the female (7.8 ± 3.3) and male (7.9 ± 2.5) subjects. A Mann-Whitney U-test confirmed that the difference between females (*Mdn* = 7.00) and males (*Mdn* = 7.00) was not statistically significant and no association with age was detected either when a Spearman's correlation test was conducted, r_s (60) = 0.110, $p = 0.404$.

As only 6.65 % and 4.83 % were current and former smokers, respectively, no statistical analysis was performed with this lifestyle habit variable. The majority of the female participants (75 %) reported being under contraceptive medication (Table 5.1).

Table 5.1 : Age and non-dietary habits characteristics of the study population (n = 62) at baseline. The variables are expressed as means ± standard deviations (SD). The differences between gender were assesse by running a non-parametric Mann-Whitney U-test due to the presence of outliers and a non-normal distribution within both group. Statistically significant differences between gender are marked with an asterisc in the "mean ± SD" column for the total population. [a)] Four female participants did not provide information on their habits of physical activity. [b)] One female participant did not provide information on the bowel movement and the frequency of another female subject was once per week and assumed to be wrongly reported and therefore excluded from the analysis.n.a. (not applicable).

	Total population (n = 62)		**Female population (n = 40)**		**Male population (n = 22)**	
	Mean ± SD	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Age (years)	28 ± 8	18 - 53	28 ± 8	20 - 53	29 ± 9	18 - 49
Exercise score (per week)[a)]	12.3 ± 12.6 *	0.5 - 85.0	10.5 ± 13.7	0.5 - 85.0	15.5 ± 9.9	3.0 - 40.5
Bowel movement score (per week)[b)]	7.9 ± 2.9	3.5 - 14.0	7.8 ± 3.3	3.5 - 14.0	7.9 ± 2.5	7 - 14
Smokers (%)	6.65	n.a.	2.5	n.a.	13.63	n.a.
Former smokers (%)	5	n.a.	3	n.a.	9	n.a.
Contraceptive medication (%)	n.a.	n.a.	30	n.a.	n.a.	n.a.

* $p < 0.05$

5.4.1.2 Anthropometrics and blood pressure

The detailed anthropometric characteristics of the present study population at baseline are summarized in Table 5.2. The average weight of the study population was 66.7 ± 11.2 kg, females (62.7 ± 9.7 kg) weighing on average less than the males (73.9 ± 9.9 kg). A non-parametric Mann-Whitney was used to assess the influence of gender on the weight of a participant. One genuine outlier was present and the variable was not normally distributed but was similar for females and males. The difference between the median weight for females (*Mdn* = 61.4 kg) and males (*Mdn* = 73.2 kg) was statistically significant, $U = 697.5$, $z = 3.789$, $p < 0.0005$. There was however, no association between the weight and the age, as assessed by a non-parametric Spearman's correlation test, $r_s\,(60) = 0.172$, $p = 0.1781$.

The average height of the study population was 1.69 ± 0.08 m and the female population (1.65 ± 0.06 m) was on average shorter than the male population (1.77 ± 0.05 m). No outliers were detected in the height variable and the latter followed a normal distribution within both gender groups, so that a parametric independent T-test was used to analyse the difference between genders. The variances within both groups were equal, as assessed by a Levene's test for equality of variances ($p = 0.469$). The difference of the means was - 0.12 (95 % CI, - 0.15 to - 0.09), with the female population being statistically significantly shorter than the male population, $t\,(60) = -7.710$, $p < 0.0005$. As for the weight, a Spearman's correlation test showed no correlation between the height and the age, $r_s\,(60) = -0.0013$, $p = 0.921$.

The average BMI of the study population was 23.3 ± 3.1 km/m^2, with very similar values for the female (23.1 ± 3.2 kg/m^2) and male participants (23.7 ± 2.8 kg/m^2). Conforming to the World Health Organization standards, from the 62 study participants, 2 were classified as underweight (BMI ≤ 18.50), 41 were within the normal range (BMI 18.50 to 24.99) and 19 were classified as overweight (BMI ≥ 25.0). No outlier was present within both gender groups but the variable which had a similar distribution for females and males did not follow a normal distribution, so that a non-parametric Mann-Whitney U test was run to study the influence of gender on the BMI. Results indicated that the median for the female (*Mdn* = 22.5 kg/m^2) and male (*Mdn* = 23.3 kg/m^2) subjects was not statistically significantly different, $U = 505$, $z = 0.957$, $p = 0.339$. A correlation analysis with a Spearman's correlation test

did not reveal any association between the BMI and the age, r_s (60) = 0.242, p = 0.058.

The study population had an average waist circumference of 75.3 ± 8.1 cm. The average obtained for females (72.3 ± 6.0 cm) was below the average of the total population and that obtained for males (80.6 ± 8.8 cm) was above. One outlier was present and the variable did not follow a normal distribution but was similar for both genders. A non-parametric Mann-Whitney U test was conducted to assess the influence of gender on the waist circumference and revealed that the median for females (*Mdn* = 71.5 cm) was statistically significantly lower than that for males (*Mdn* = 79.3 cm), U = 711, z = 3.990, p < 0.0005. A positive correlation was detected between the waist circumference and the age, as assessed by a non-parametric Spearman's correlation test, r_s (60) = 0.283, p = 0.026.

Inversely to the waist circumference, the female population had a higher hip circumference (99.4 ± 7.7 cm) than the male population (98.1 ± 5.9 cm) and than the average obtained for the study population (98.9 ± 7.1 cm). The significance of the difference was tested by an independent T-test. A Levene's test revealed equal variances in both gender groups (p = 0.292) and the difference of the means between genders, 1.3 (95 % CI, -2.5 to 5.1), was not significant, t (60) = 0.671, p = 0.505. A non-parametric Spearman's correlation test did not detect any association between the hip circumference and age, r_s (60) = 0.105, p = 0.418.

The average waist-to-hip ratio within the study population was 0.76 ± 0.06 cm and females (0.73 ± 0.03 cm) had a slightly lower average than males (0.82 ± 0.06 cm). No female but two male subjects had a waist-to-hip ratio higher than the cutoff point adopted by the World Health Organization to classify someone as visceral obese (ratio ≥ 0.85 for females and ≥ 0.90 for males). For both gender groups, no outliers were present and the distribution was similar but non-normal. A Mann-Whitney U-test was therefore conducted to determine if there was a difference in waist-to-hip ratio between genders and revealed that the median for the female (*Mdn* = 0.73 cm) and male subjects (*Mdn* = 0.80 cm) was statistically significantly different, U = 839, z = 5.875, p < 0.0005. When a non-parametric Spearman's correlation test was run to assess the association with age, however, no significant correlation was detected, r_s (60) = 0.194, p = 0.130.

The blood pressure could not be recorded for one female participant due to the small diameter of the arms. The average blood pressures were therefore based on data of 61 participants for the total population, and on data of 39 participants for the female population. The average systolic and diastolic blood pressures within the total population were 114 ± 9 and 71 ± 8 mmHg, respectively, with similar pressures observed in female (Syst.: 113 ± 10 mmHg, Diast.: 71 ± 9 mmHg) and male (Syst.: 116 ± 9 mmHg, Diast.: 70 ± 7 mmHg) subjects. These values are classified as healthy (http://www.heart.org/) and are lower than the reported average in the UK. Five and three outliers were present in the systolic and diastolic blood pressures, respectively. The distribution of the systolic and diastolic blood pressures was similar for both genders but was non-normal. A Mann-Whitney U-test was run to assess the effect of gender on both blood pressures and results indicate that the median blood pressures for females (Syst.: *Mdn* = 113 mmHg, Diast.: *Mdn* = 70 mmHg) and for males (Syst.: *Mdn* = 117 mmHg, Diast.: *Mdn* = 71 mmHg) were not statistically significantly different (Syst.: $p = 0.166$, Diast.: $p = 0.741$). The influence of age was different for both blood pressures. When a Spearman's correlation test was run, no influence of age on the systolic blood pressure was detected, $r_s\,(59) = -0.105$, $p = 0.419$, but a strong positive association with the diastolic blood was detected, $r_s\,(59) = 0.315$, $p = 0.013$.

Table 5.2 : Anthropometric characteristics of the study population (n = 62) measured in fasting plasma at baseline. The variables are expressed as means ± standard deviations (SD). Except for the hip, the differences between gender were assessed by conduction of a Mann-Whitney U-test due to the presence of outliers and/or a non-normal distribution between both groups. Statistically significant differences are marked with the corresponding number of asteriscs in the "mean ± SD" column for the total population. a) The average of the total population and female population did not include the measurement of one female subject for which the arms were too thin to obtain a blood pressure record. BP: blood pressure.

	Total population (n = 62)		**Female population (n = 40)**		**Male population (n = 22)**	
	Mean ± SD	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Weight (kg)	66.7 ± 11.2 ***	47.1 - 95.6	62.7 ± 9.7	47.1 - 82.9	73.9 ± 9.9	56.6 - 95.6
Height (m)	1.69 ± 0.08 ***	1.51 - 1.89	1.65 ± 0.06	1.51 - 1.77	1.77 ± 0.05	1.65 - 1.89
BMI (kg/m^2)	23.3 ± 3.1	17.7 - 29.6	23.1 ± 3.2	17.7 - 29.4	23.7 ± 2.8	17.7 - 29.6
Waist (cm)	75.3 ± 8.1 ***	63.5 - 104.5	72.3 ± 6.0	63.5 - 85.5	80.6 ± 8.8	66.0 - 104.5
Hip (cm)	98.9 ± 7.1	86.5 - 117.0	99.4 ± 7.7	86.5 - 117.0	98.1 ± 5.9	87.0 - 111.5
Waist to Hip ratio	0.76 ± 0.06 ***	0.67 - 0.95	0.73 ± 0.03	0.67 - 0.83	0.82 ± 0.06	0.75 - 0.95
Systolic BP (mmHg)[a)]	114 ± 9	89 - 134	113 ± 10	91 - 134	116 ± 9	89 - 129
Diastolic BP (mmHg)[a)]	71 ± 8	54 - 107	71 ± 9	56 - 107	70 ± 7	53 - 83

* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

5.4.1.3 Biomarkers of inflammation and cardiovascular health

One of the study subjects did not come in a fasting state on the first visit. This participant reported to have eaten a banana prior to the examination. The first data set for the biomarkers of this participant were therefore not included in the calculation to obtain the study population average. All the selected biomarkers of inflammation and cardiovascular health measured in fasted plasma were within the expected healthy ranges. Detailed concentrations are presented in Table 5.3.

The average concentration of total cysteine in the present study population was 257 ± 27 μM, the female participants (250 ± 26 μM) having a lower average when compared to the male population (270 ± 23 μM). These values are similar to previously reported concentrations in healthy subjects (Guttormsen et al., 1994, Andersson et al., 1999). No outliers were present and the distribution was similar and normal for both genders. An independent t-test was run to assess the influence of gender on the total fasting plasma cysteine. The variances in both groups were equal, as assessed by an initial Levene's test (p = 0.650) and the difference of the means was - 20.86 (95 %, - 34.28 to - 7.44) and statistically significant, t (60) = - 3.111, p = 0.003 (Figure 5.1, A). When total cysteine was analysed by age, the distribution was non-normal and a non-parametric Spearman's correlation test revealed no significant association between both variables, r_s (60) = 0.099, p = 0.448.

The average fasting total homocysteine concentration was 9.6 ± 4.0 μM for the study population, which is similar to fasting values reported by Christensen et al. in a healthy adult population (Christensen et al., 2001). In the present study, the female population had a lower average (8.5 ± 2.4 μM) than the male population (11.7 ± 5.5 μM). Three outliers were present and the distribution of homocysteine within both genders was similar and non-normal so that the influence of gender on this biomarker was assessed by a Mann-Whitney U-test. The results indicated that the median obtained for the females (*Mdn* = 7.94 μM) was statistically significantly different than that obtained for the males (*Mdn* = 10.77 μM), U = 622, z = 3.06, p = 0.002 (Figure 5.1, B). When a non-parametric Spearman's correlation test was conducted to assess the association with age, no significant association between both variable was detected, r_s (60) = - 0.208, p = 0.108.

An average of 31.3 ± 6.8 μM was obtained for total cysteinylglycine, for the study population, which is close to previously reported concentrations in healthy adults (Guttormsen et al., 1994). The female study population (30.1 ± 6.6 μM) had a lower average concentration than the male study population (33.7 ± 6.8 μM). The significance of the difference was tested by an independent t-test. An initial Levene's test indicated the variances within both groups to be equal (p = 0.782) and the difference of the means between genders was - 3.59 (95 % CI, - 7.18 to - 0.004) and significant, t (60) = - 1.999, p = 0.05 (Figure 5.1, C). When total cysteinylglycine was analysed by age, no bivariate normality was observed and a non-parametric Spearman's correlation test revealed a negative association between total cysteinylglycine and age, r_s (60) = - 0.348, p = 0.006 (Figure 5.1, E).

The fasting plasma concentration of total glutathione in the study population was 8.4 ± 2.4 μM, with a neglectable difference between the female (8.4 ± 2.2 μM) and the male population (8.5 ± 2.9 μM). The extracellular concentration of glutathione can range from 2 to 20 μM (Wu et al., 2004). In the study by Guttormsen et al. earlier referenced, an average of 5.69 ± 1.33 μM was reported for healthy adults (Guttormsen et al., 1994). The concentrations obtained in the present study thus fit in this range. A Levene's test indicated equality of variances in both gender groups (p = 0.205). The mean difference between genders was - 0.03 (95 % CI, - 1.36 to 1.30) and an independent sample t-test confirmed the non-significance of the difference, t (60) = - 0.44, p = 0.965. No significant association between total glutathione and the age was detected when a Spearman's correlation test, r_s (60) = - 0.223, p = 0.09.

The average fasting glucose concentration in the study population was 4.7 ± 0.4 mM, with similar values for females (4.7 ± 0.4 mM) and males (4.9 ± 0.5 mM). These values are considered healthy, as defined by the American Diabetes Association (healthy glycaemia at fast: < 5.6 mM), often taken as a reference and whose cutoff the World Health Organization has also adopted (ADA, 2010, WHO, 2006). A Levene's test indicated equality of variances in both gender groups (p = 0.589) and the mean difference between both genders was - 0.19 (95 % CI, - 0.43 to 0.03). This difference was not significant, as assessed by an independent sample t-test, t (60) = - 1.703, p = 0.094. When glucose was analysed by age, no bivariate normality was observed and a non-parametric Spearman's correlation test was

conducted to assess the association with age. Glucose was not significantly associated with the age, r_s (60) = 0.142, p = 0.276.

The average fasting insulin concentration in the study population was 55.2 ± 19.3 pM, with very similar concentrations obtained for females (55.6 ± 17.8 pM) and males (54.5 ± 22.3 pM). As for the other biomarkers measured, these values are considered to be healthy, as reported by Mercodia that measured a range of 12 - 150 pM insulin in 137 apparently healthy adults (Mercodia). Three outliers were present and the distribution was similar within both genders but did not follow normality. A non-parametric Mann-Whitney U-test was therefore run and indicated no statistically significant difference between the median for females (*Mdn* = 52.65) and males (*Mdn* = 49.90), U = 369, z = - 0.767, p = 0.443. A non-parametric Spearman's correlation test was conducted to assess the association with age and no significant association with age was identified for insulin, r_s (60) = - 0.085, p = 0.514.

Using the fasting glucose and insulin variables, the pancreatic β-cell function, the insulin sensitivity and the insulin resistance were estimated using the HOMA calculator available online, from the University of Oxford (https://www.dtu.ox.ac.uk/homacalculator/download.php). The average β-cell function was 108 ± 22 % for the total study population, 112 ± 21 % for the female population and 101 ± 22 % for the male population. The average insulin sensitivity was 108 ± 30 % for the total study population, 106 ± 27 % for the female population and 111 ± 35 % for the male population. The insulin resistance was normal in the study population, with values of 1.03 ± 0.37 for the total study population, 1.05 ± 0.34 % for the female population and 1.01 ± 0.43 % for the male population. For these three variables the difference between genders was non-significant (p > 0.05) and only the β-cell function was negatively associated with the age r_s (60) = - 0.258, p = 0.045 (Figure 5.1, F).

As well as for the above mentioned biomarkers of health, the average fasting uric acid for the total study population (0.26 ± 0.06 mM) was within the expected normal range (Sautin and Johnson, 2008). The average for the female population (0.23 ± 0.04 mM) was lower than for the male population (0.31 ± 0.06 mM). A Levene's test revealed equality of variances between gender (p = 0.063). The difference of the means was - 0.08 (95 % CI, - 0.11 to - 0.06) and as assessed by the independent

sample t-test the difference was significant, t (60) = - 6.362, $p < 0.0005$ (Figure 5.1, D). No bivariate normality was observed when uric acid was analysed by age and a non-parametric Spearman's correlation test was conducted to assess the association with age. No significant association between uric acid and the age was detected, r_s (60) = - 0.014, $p = 0.917$.

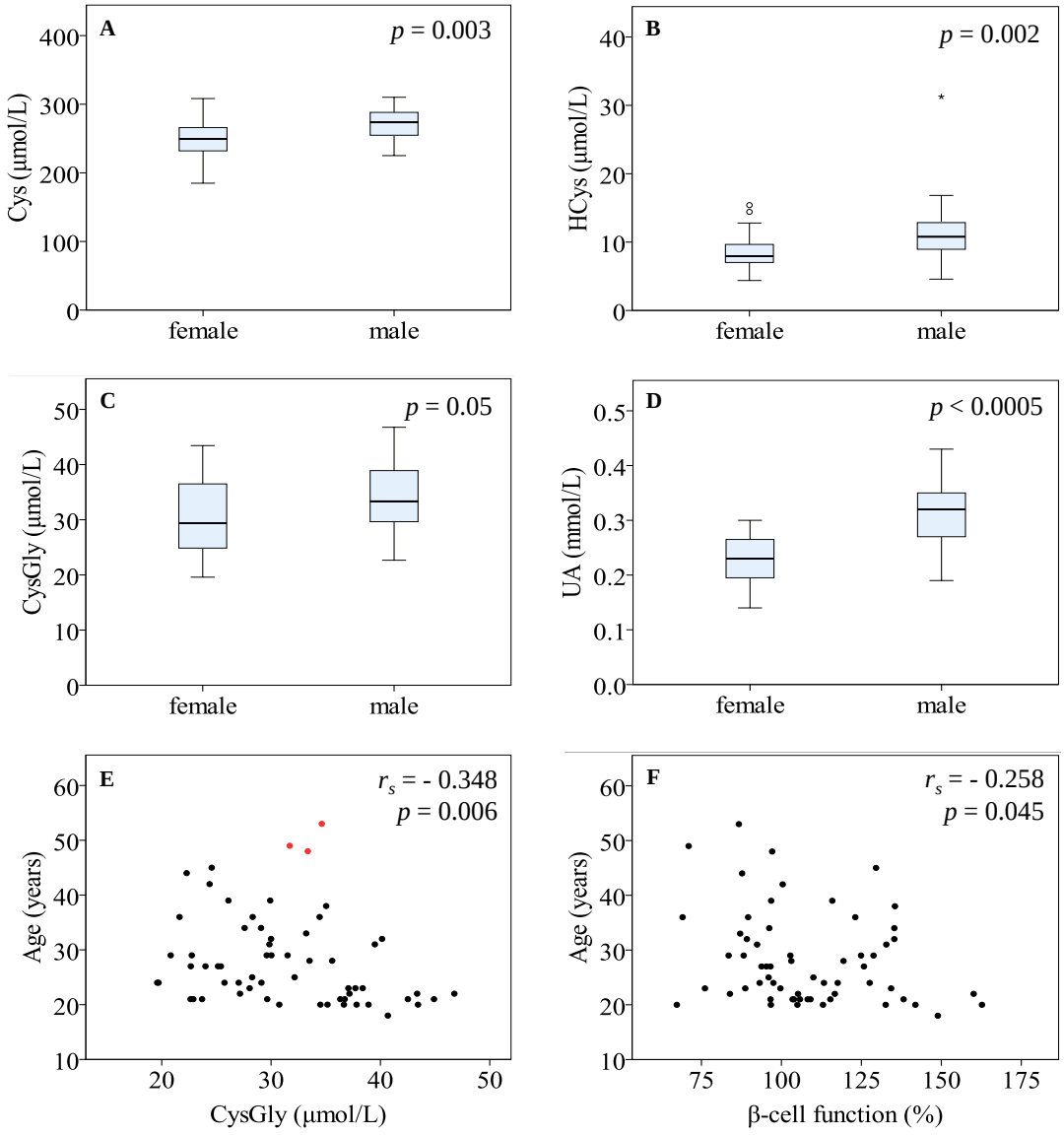


Figure 5.1: Biomarkers for which a statistically significant difference between gender or an association with age was identified at baseline. tCys, total cysteine; tCysGly, total cysteinylglycine; UA, uric acid; tHCys, total homocysteine; **O**: non-extreme outlier (the data point is > 1.5 box-length away from the corresponding plotted box edge), *: extreme outlier (the data point is > 3 box-lengths away from the corresponding plotted box edge)

Table 5.3 : Selected inflammation and cardiovascular biomarkers of the study population (n = 62) measured in fasting plasma at baseline. The variables are expressed as means ± standard deviations (SD). All biomarkers measured were within ranges that have previously been reported in healthy subjects. For the biomarker-gender pairs with no outlier, a normal distribution and equal variance between groups (i.e. tCys, tCysGly, tGSH, Glc, UA). The differences between gender for the remaining biomarkers were assessed by conduction of a Mann-Whitney U-test, due to the presence of outliers and/or a non-normal distribution between groups. **S**ignificant differences between gender are marked with asteriscs. tCys, total cysteine; tHCys, total homocysteine; tCysGly, total cysteinylglycine; tGSH, total glutathione; Glc, glucose; Ins, insulin; UA, uric acid.

	Total population (n = 62)		**Female population (n = 40)**		**Male population (n = 22)**	
	Mean ± SD	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Total thiols (µmol/L)						
tCys	257 ± 27 **	185 - 310	250 ± 26	185 - 308	270 ± 23	225 - 310
tHCys	9.6 ± 4.0	4.4 - 31.3	8.5 ± 2.4	4.4 - 15.4	11.7 ± 5.5	4.6 - 31.3
tCysGly	31.3 ± 6.8 *	19.6 - 46.8	30.1 ± 6.6	19.6 - 43.4	33.7 ± 6.8	22.7 - 46.8
tGSH	8.4 ± 2.4	4.9 - 17.25	8.4 ± 2.2	5.17 - 15.3	8.5 ± 2.9	4.9 - 17.3
Glc (mmol/L)	4.7 ± 0.4	3.9 - 6.4	4.7 ± 0.4	3.9 - 5.6	4.9 ± 0.5	4.2 - 6.4
Ins (pmol/L)	55.2 ± 19.3	30.6 - 134.7	55.6 ± 17.8	31.0 - 134.7	54.5 ± 22.3	30.6 - 129.5
UA (mmol/L)	0.26 ± 0.06 ***	0.14 - 0.43	0.23 ± 0.04	0.14 - 0.30	0.31 ± 0.06	0.19 - 0.43
HOMA, B (%)	108 ± 22	67 - 163	112 ± 21	76 - 163	101 ± 22	67 - 149
HOMA, S (%)	108 ± 30	39 - 178	106 ± 27	40 -178	111 ± 35	40 - 176
Insulin resistance, IR	1.03 ± 0.37	0.57 - 2.52	1.05 ± 0.34	0.64 - 2.49	1.01 ± 0.43	0.57 - 2.52

* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

5.4.2 Consistency of the baseline variables

5.4.2.1 Anthropometrics and blood pressure

The consistency of the anthropometric (i.e. weight, height, BMI, waist and hip circumference, waist-to-hip ratio) and blood pressure measurements taken between the three visits was tested by conducting a one way ANOVA with repeated measures. Outliers were present for the waist circumferences (n = 1), the waist-to-hip ratio (n = 3) and the systolic pressure (n = 5), but due to the likelihood of their genuine nature, these were not excluded. A Shapiro-Wilk test indicated that data were not normally distributed at each visit for the waist circumference (visit 1: p = 0.012, visit 3: p = 0.043), the waist-to-hip ratio (visit 1: p = 0.007) and the diastolic blood pressure (visit 2: p = 0.007). Due to the considerable robustness of the ANOVA with repeated measures test to deviations of normality, this test was still used for the analysis of consistency.

The variances of the differences between the possible pair combinations between the three visits were not equal for the weight (X^2 (2) = 10.097, p = 0.006), BMI (X^2 (2) = 7.654, p = 0.022), waist circumference (X^2 (2) = 17.93, p < 0.0005) and hip circumference (X^2 (2) = 8.477, p = 0.014), as assessed by a Mauchly's test of sphericity. The Mauchly's test is not very robust to deviations of normality and it is mostly conventional to perform a Greenhouse-Geisser correction regardless of whether the sphericity has or has not been violated (Moulton, 2010). Therefore, a Greenhouse-Geisser correction, in which the degrees of freedom are altered, was performed. The Greenhouse-Geisser epsilon values obtained for the weight (ε = 0.811), height (ε = 0.924), BMI (ε = 0.846), waist circumference (ε = 0.727), hip circumference (ε = 0.833), waist-to-hip ratio (ε = 0.919), systolic blood pressure (ε = 0.988) and diastolic blood pressure (ε = 0.882) were used to correct the one-way ANOVA with repeated measures. The resulting adjusted data did not indicate any significant difference of measurement between the three visits (Table 5.4), for weight, F (1.622, 63.243) = 0.664, p = 0.488, partial η^2 = 0.017; height, F (1.847, 72.034) = 0.949, p = 0.386, partial η^2 = 0.024; BMI, F (1.691, 65.966) = 0.927, p = 0.387, partial η^2 = 0.023; waist circumference, F (1.453, 56.680) = 0.257, p = 0.703, partial η^2 = 0.007; hip circumference, F (1.667, 65.003) = 0.044, p = 0.934, partial η^2 = 0.001; waist-to-hip ratio F (1.838, 71.678) = 0.241, p = 0.768, partial η^2 = 0.006;

systolic blood pressure, F (1.976, 77.074) = 0.312, p = 0.731, partial η^2 = 0.008; and diastolic blood pressure, F (1.765, 68.818) = 0.519, p = 0.575, partial η^2 = 0.013.

The outcome did not change when, for comparative purposes, the alternative Friedman's test was run to test the consistency of the variables which had outliers and/or, which did not follow a normal distribution. These variables were the waist circumference, waist-to-hip ratio, systolic blood pressure and diastolic blood pressure. The measurements of the waist circumference (*Mdn* visit 1 = 73.25, *Mdn* visit 2 = 75.2, *Mdn* visit 3 = 73.75), waist-to-hip ratio (*Mdn* visit 1 = 0.755, *Mdn* visit 2 = 0.760, *Mdn* visit 3 = 0.760), systolic blood pressure (*Mdn* visit 1 = 116.0, *Mdn* visit 2 = 116.0, *Mdn* visit 3 = 114.50) and diastolic blood pressure (*Mdn* visit 1 = 71.0, *Mdn* visit 2 = 73.5, *Mdn* visit 3 = 70.0) throughout the three visits of the human study were statistically not significantly different, with Chi square (degrees of freedom) values of X^2 (2) = 2.471, p = 0.291, X^2 (2) = 0.870, p = 0.647, X^2 (2) = 0.039, p = 0.980 and X^2 (2) = 2.554, p = 0.279, respectively.

Table 5.4 : Average anthropometrics and blood pressure obtained with the participants that completed the three possible visits (n = 40). Data are presented for each visit as the average ± standard deviation (SD). All the anthropometric and blood pressure measurements were consistent between visits ($p > 0.05$) as assessed by an parametreic ANOVA with repeated measures test. WHR, waist-to-hip ratio; n.d.: not determined.

	Average ± SD			**ANOVA RM, *p*-value**
	Visit 1	**Visit 2**	**Visit 3**	
Weight (kg)	65.9 ± 10.1	66.1 ± 9.7	66.3 ± 9.8	0.488
Height (m)	1.69 ± 0.09	1.69 ± 0.09	1.69 ± 0.09	0.386
BMI (kg/m^2)	23.13 ± 2.96	23.19 ± 2.90	23.28 ± 3.08	0.387
Waist (cm)	75.2 ± 8.4	75.5 ± 7.3	75.2 ± 7.7	0.703
Hip (cm)	98.1 ± 6.9	98.2 ± 6.6	98.1 ± 6.8	0.934
WHR	0.77 ± 0.07	0.77 ± 0.06	0.77 ± 0.06	0.768
Systolic BP (mmHg)	116 ± 8	114 ± 7	115 ± 9	0.731
Diastolic BP (mmHg)	71 ± 6	72 ± 6	71 ± 6	0.575

5.4.2.2 Biomarkers of inflammation and cardiovascular health

Although 40 subjects completed three visits, the analysis of consistency for the biomarkers of inflammation and cardiovascular health was performed on the data of 38 participants. One of the two excluded data belonged to a subject which, due to fear of needles, had no blood withdrawn on the first visit. The second data set excluded belonged to a subject that had consumed a banana in the morning, prior to the first visit and for which glucose and insulin measurements would be influenced. Although the consumption of a banana is unlikely to significantly influence the measurements for the other biomarkers, the complete data set was excluded as a precaution.

As for the anthropometrics and blood pressure measurements, some outliers were detected by visual inspection of boxplots in the data set for total homocysteine (n = 1), total cysteinylglycine (n = 3), total glutathione (n = 3), glucose (n = 3), insulin (n = 2) and uric acid (n = 1) and all were included in the statistical analyses. Also, a Shapiro-Wilk test indicated that total homocysteine (visit 1, 2 and 3: $p < 0.0005$), total glutathione (visit 1: $p = 0.007$), insulin (visit 2: $p = 0.038$, visit 3: $p = 0.001$) and uric acid (visit 3: $p = 0.004$) did not follow a normal distribution in all the visits.

As assessed by a Mauchly's test of sphericity, the variances of the differences between the possible pair combinations between the three visits for total glutathione only were not equal, X^2 (2) = 18.411, $p < 0.0005$. For the same reason as previously mentioned, a Greenhouse-Geisser correction was performed for all the biomarkers of health variables and not only for glutathione. The Greenhouse-Geisser epsilon values obtained for the total cysteine ($\varepsilon = 0.958$), total homocysteine ($\varepsilon = 0.961$), total cysteinylglycine ($\varepsilon = 0.913$), total glutathione ($\varepsilon = 0.714$), glucose ($\varepsilon = 0.922$), insulin ($\varepsilon = 0.876$) and uric acid ($\varepsilon = 0.996$) were used to correct the one-way ANOVA with repeated measures. After correction, the means of the dependent variables were similar between visits for total cysteine, F (1.917, 70.919) = 0.039, $p = 0.962$, partial $\eta^2 = 0.001$; total homocysteine, F (1.923, 71.135) = 1.247, $p = 0.293$, partial $\eta^2 = 0.033$; glucose, F (1.844, 68.225) = 0.970, $p = 0.378$, partial $\eta^2 = 0.026$ and uric acid F (1.991, 73.678) = 0.204, $p = 0.815$, partial $\eta^2 = 0.005$. A statistically significant difference between visits was however detected for total cysteinylglycine, F (1.827, 67.591) = 7.849, $p = 0.001$, partial $\eta^2 = 0.175$; total glutathione, F (1.428, 52.844) = 11.515, $p < 0.0005$, partial $\eta^2 = 0.237$ and insulin, F (1.753, 64.861) = 3.333, $p = 0.048$, partial $\eta^2 = 0.083$. For those three variables, further investigation was undertaken to identify the cause of the statistical significance. Post hoc analysis with a Bonferroni adjustment revealed that the total cysteinylglycine concentration was statistically significantly higher in visit 1, when compared to visit 2, with a mean difference of 3.249 μM (95 % CI, 1.118 to 5.380), $p = 0.001$. For total glutathione, the concentration obtained in visit 1 was statistically significantly higher when compared to visit 2 and visit 3, with mean differences of 1.500 μM (95 % CI, 0.611 to 2.390), $p < 0.0005$ and 1.537 μM (95 % CI, 0.3871 to 2.687), $p < 0.006$, respectively. Finally, the average concentration obtained for the insulin during visit

1 was statistically significantly lower when compared to visit 3, with a mean difference of - 5.932 μM (95 % CI, -10.971 to -0.892), $p = 0.016$.

When the alternative non-parametric Friedman's test was run to analyse the consistency of the variables which had outliers and/or were not normally distributed, the results indicated that the concentration of total cysteinylglycine obtained throughout the three visits were statistically significantly different, X^2 (2) = 11.692, $p = 0.003$. Pair-wise comparisons with a Bonferroni correction for multiple comparisons indicated that the concentrations were statistically significantly different between visit 1 (*Mdn* = 32.150) and visit 2 (*Mdn* = 28.465), $p = 0.002$. The concentrations of total glutathione were also statistically significantly different between the three visits, X^2 (2) = 20.667, $p < 0.0005$, and pair-wise comparisons with a Bonferroni correction for multiple comparisons indicated that the concentrations were statistically significantly different between visit 1 (*Mdn* = 8.75) and visit 2 (*Mdn* = 7.34), $p < 0.0005$ and between visit 1 (*Mdn* = 8.75) and visit 3 (*Mdn* = 7.41), $p = 0.001$. On the contrary, when the consistency of the insulin concentrations was tested with the Friedman's test, no statistically significant difference was detected, X^2 (2) = 5.842, $p = 0.54$.

To further examine the intra-individual variation, the coefficient of variation for each biomarker was calculated for each participant. The average for each biomarker was then calculated for the total population and is presented in Table 5.5. The relatively low values support the idea that the metabolism of the selected biomarkers of health is stable over a period of at least 8 weeks.

Table 5.5 : Average concentrations and coefficients of variation of the biomarkers of inflammation and cardiovascular health obtained for the participants that completed the three possible visits. The comparisons included data from 38 participants. Data are presented for each visit as the average ± standard deviation (SD). A parametric ANOVA with repeated measures test indicated that not all biomarkers were consistent for the studied period, but the low coefficients of variation suggest otherwise. tCys, total cysteine; tHCys, total homocysteine; tCysGly, total cysteinylglycine; tGSH, total glutathione; Glc, glucose; Ins, insulin; UA, uric acid.

	Average ± SD			**Average CV (%) ± SD**	**ANOVA RM, *p*-value**
	Visit 1	**Visit 2**	**Visit 3**		
tCys (µmol/L)	260 ± 27	261 ± 25	260 ± 26	3.9 ± 1.9	0.962
tHCys (µmol/L)	9.9 ± 4.7	9.3 ± 3.9	9.5 ± 4.0	15.1 ± 8.1	0.293
tCysGly (µmol/L)	32.8 ± 6.4	29.5 ± 5.3	30.4 ± 6.6	11.3 ± 6.3	0.001
tGSH (µmol/L)	8.9 ± 2.6	7.4 ± 1.4	7.4 ± 1.8	18.4 ± 10.6	< 0.0005
Glc (mmol/L)	4.7 ± 0.3	4.8 ± 0.4	4.8 ± 0.4	4.6 ± 2.4	0.378
Ins (pmol/L)	52.1 ± 14.1	55.0 ± 16.4	58.0 ± 18.4	15.7 ± 7.3	0.048
UA (mmol/L)	0.26 ± 0.06	0.26 ± 0.05	0.26 ± 0.08	8.6 ± 4.5	0.815

5.5 Discussion and conclusion

As for the analysis of the food frequency questionnaire discussed in chapter 4, the totality of the study participants (n = 62) did not always provide complete information on certain life habits, such as bowel movements or physical activity.

Also, some data could not be measured or considered for some participants. This resulted in an unequal number of data available depending on the statistical analyses performed. Ideally, the weight measurement would have been recorded on the participants wearing their underwear only, however, all the participants underwent the measurement under the same conditions and the excess weight estimated to be < 200 g would be very similar amongst the participants, thus still allowing the comparative measurements.

The age range of the study population was broad (18 - 53 years), but the majority of the population was young, resulting in an average of 28 ± 8 years. This relatively young average could be explained by the fact that the recruiting area included the University of Leeds campus. The male population was on average taller and heavier than the female population, but both genders had similar BMI averages. Age did not influence any of these three variables. The female population had an average waist circumference that was lower than that of the male population and the waist circumference increased with age. No influence of gender or age on the hip circumference was detected. The waist-to-hip ratio was lower in the female subjects, but age did not have any influence on the variable. Two male subjects were considered to possess visceral obesity but the corresponding data was still considered for the analyses. The study population had in general a healthy blood pressure which was gender-independent, but diastolic blood pressure was positively associated with age. Although few subjects were in the older age rage, the blood pressure is known to increase with age. The excess consumption of certain dietary components such as sodium, alcohol, fat, the reduction of physical activity with the age and the long-term effect of stress, are examples of factors contributing to structural modifications in the arteries leading to an increasing stiffness and subsequent resistance, a major cause of increase of blood pressure (Pinto, 2007). Males were in general more active than females but the physical activity was not influenced by age. The bowel movement was not influenced by either the gender or

age. A low percentage in the population smoked or had smoked in the past and therefore no statistical analyses were performed on the variable.

Overall, the recruited study population was confirmed to be in general good health with regards to inflammation and cardiovascular health, as assessed by the selected biomarkers measured at fast in plasma and when compared to values previously reported for healthy adult subjects (Wu et al., 2004, Guttormsen et al., 1994, Christensen et al., 2001, Mercodia, Sautin and Johnson, 2008). The observed concentrations in the present study are all consistent with those reported in a recent study carried out by Pimková at al. (2014). In this study, Pimková et al. compared aminothiol concentrations in healthy subjects and Myelodysplastic Syndrome patients. Healthy subjects had lower levels of cysteine, homocysteine and cysteinylglycine (total, reduced and oxidized), while higher levels of glutathione (total and oxidized) were reported in healthy subjects, when compared to the patient group. Of note, Pimková et al. did not specify whether the blood was collected at fast or not. The lower cysteine concentrations obtained in the female population of the present studyare consistent with a previous report by El-Khairy et al. (2001) but not by Bates et al. (2002). In the study by El-Khairy et al. (2001), the healthy subjects were < 60 years, but no details on the age range is provided. Similarly, the lower homocysteine levels measured in the female population are consistent with a report by Powers et al. (2002), in which105 healthy adult females and males of different age categories (pre- and postmenopausal females, but with no specified age for either gender) were subject to analysis., Bates et al. did not report any difference between genders in subjects < 15 years old, in subjects aged 15 to 18 years old (Bates et al., 2002, Powers et al., 2002). Both biomarkers were independent from age, in the present research. In the same study by Bates et al. and Powers et al., older subjects had higher levels of homocysteine. This different outcome could, however, be explained by the fact that the recruited population in the present study was mainly composed of young adults with only one subject > 50 years old. The female population also had a lower concentration of cysteinylglycine than the males in the study, and the biomarker was negatively associated with age. These observations are consistent with the report of Bates et al. (2002). Glutathione concentrations were not gender- or age-dependent. The higher levels of cysteine, homocysteine and cysteinylglycine in males could suggest that the estrogens have an effect on the metabolism of aminothiols. This would be supported by several researches. In a

study with post-menopausal women, a 6-month treatment with estrogen decreased the homocysteine levels by 21.7 %, when compared to the values prior to the treatment. The female group receiving a placebo for the same period increased the homocysteine values by 16.5 %, when compared to the values prior to the treatment (Lakryc et al., 2015). Since the subjects in the present study were in general good health an unbalance in the transsulfuration pathway was not expected. It would therefore be expected that a healthy subject with higher homocysteine levels would also have a more active transsulfuration pathway, starting with a higher rate of catalysis of homocysteine into cystathione and subsequent hydrolysis into cysteine. Glucose was positively associated with age, but not gender. Insulin was also gender-independent but as there was a positive association between glucose and age, the same was expected between glucose and insulin, which was not the case. This could suggest that there is a tendency towards reduced response of the pancreatic β-cells towards glucose with increasing age. Uric acid was gender- but not age-dependent. As for the aminothiols, the gender difference could be explained by the sex hormones. In an *in vivo* experiment using a murine model, Takiue et al. (2011) investigated the effect of a hormone replacement treatment with oestradiol and progesterone on the regulation of the renal uric acid transporters expression in female mice that were or not subject to bilateral ovariectomy. In both groups, the mice were injected subcutaneously with either estradiol dipropionate (2.5 or 0.025 mg/kg), progesterone (2.5 mg/kg), estradiol (0.025 mg/kg) with progesterone (2.5 mg/kg), or the vehicle medium, i.e. sunflower oil. The mRNA of four uric acid trasporters was quantified, namely, Urat1, Glut9, Smct1 and Abcg2 and no change was significant between the different mice and treatments. When the protein levels were quantified, when compared to the ovariectomized mice with no hormone treatment, the hormone treatment with estradiol only significantly lowered the protein levels of Urat1, Glut9 and Abcg2, and th treatment with progesterone only decreased the Smct1 levels. Both hormones thus are able to reduce the reabsorption of uric acid, by reducing post-transciptional expression of the tested transporters.

When the consistency of the population characteristics was tested, the analyses suggested that the anthropometric variables and blood pressure were consistent over the three visits. This is also what was expected as the study population was an adult population, therefore with a stable height; the study participants were not following any prescribed diet or following a weight-loss diet,

therefore having a stable weight, BMI, waist circumference, hip circumference and consequently a stable waist to hip ratio; and the study population was in general good health and later analyses confirmed this judgement, therefore large fluctuations in blood pressure in a relaxed state were not expected. For the biomarkers measured, cysteinylglycine and glutathione were systematically non-consistent between visits, as assessed by an ANOVA test with repeated measures. However, the averaged coefficients of variation in the population were suggestive of a low intra-individual variation. As for insulin, the difference between visits was statistically significant when an ANOVA test with repeated measures was conducted, but not significant when a non-parametric Friedman's test was run instead.

Particular attention needs to be paid when it comes to statistical analyses. Although both parametric and non-parametric tests can be conducted with a same data set in an attempt to answer a certain question, the choice for a particular statistical test requires careful thought as the outcomes can vary. In this current case, the choice of taking into account the results from the non-parametric Friedman's test instead of those from the ANOVA with repeated measures, for those data which have outliers and/or do not follow a normal distribution, is likely the most appropriate. In conclusion, the recruited population was, first self-reported and subsequently confirmed by measurements, in general good health and with a consistent metabolism for the selected biomarkers of health.

Chapter 6
Impact of habitual consumption of (poly)phenol-rich products, general daily habits and anthropometrics on health biomarkers and associations amongst health biomarkers

6.1 Abstract

The associations between the data collected by procedures discussed in earlier chapters are discussed in the present chapter. Firstly, associations between variables were assessed, followed by the correction for age, gender and waist-to-hip ratio. Adjusted statistical analyses revealed numerous significant associations, although fewer when compared to the unadjusted analyses. Variables of coffee consumption were positively associated with the four aminothiols studied, the homocysteine-to-cysteine ratio and uric acid, but had no association with glucose or insulin. No association with the time a subject had reported to be a consumer of tea was detected upon adjustment for the age, gender and the waist-to-hip ratio. The (poly)phenolic intake from cocoa beverages was negatively associated with uric acid. For other (poly)phenol-containing dietary products studied, only the consumption of herbs and spices was positively associated with glucose and the consumption of vegetables was positively associated with uric acid. Data suggested that a habitual consumption of coffee and associated components, specifically including (poly)phenols but not excluding other compounds, are more susceptible to have a higher impact on homocysteine than on cysteinylglycine, cysteine and glutathione, as assessed by the number of significant associations. Also a habitual consumption of tea did not have any effect on the selected biomarkers in the adjusted analyses. Finally, the observations of the present study cannot be extrapolated to a population that does not match the selection criteria of the present study.

6.2 Introduction

Biomarkers arise from normal metabolic processes and constitute a useful tool for the prediction, diagnosis and monitoring of health conditions, as well as for monitoring the impact of interventions (BDWG, 2001). In the present observational study, biomarkers of inflammation and cardiovascular health were monitored in the fasting plasma of a healthy adult population. Total aminothiols are frequently quantified as a measure of inflammation and cardiovascular health, and recently, a few substrate-to-product ratios within the transsulfuration pathway have been described (Mangoni et al., 2013). Higher concentrations of aminothiols may result from an increased synthesis and/or a decrease of degradation, while lower concentrations may result from a decreased synthesis and/or an increase of degradation. Substrate-to-product ratios between thiols have therefore been considered to be representative of the enzymatic activity in the transsulfuration pathway (Figure 3.1). As part of their research, Mangoni et al. measured the homocysteine-to-cysteine ratio as a marker of the cystathionine β-synthase and cystathionine γ-lyase activity, as well as the glutathione-to-cysteinylglycine ratio as a marker of the γ-glutamyltranspeptidase activity (Mangoni et al., 2013). Cystathionine β-synthase catalyses the synthesis of cystathionine from the condensation of homocysteine and serine, the initial reaction in the transsulfuration pathway (Figure 3.1). Cystathionine β-synthase-deficient humans and rats suffer from hyperhomocysteinemia and show earlier development of atherosclerosis, vascular disease and further non-cardiovascular conditions (Beard and Bearden, 2011, Ishii et al., 2010). Cystathionine γ-lyase catalyses the hydrolysis of cystathionine into α-ketobutyrate and cysteine. As a result of a lack of activity or absence of the cystathionine γ-lyase, human patients exhibit increased levels of cystathionine in plasma and urine and lower levels of cysteine. The supplementation of the diet with cysteine has however, shown promising results with cystathionine γ-lyase-deficient mice (Ishii et al., 2010, Paul et al., 2014), while a low cysteine diet led to lethal myopathy in the same mice (Ishii et al., 2010). The γ-glutamyltranspeptidase catalyses the degradation of glutathione into cysteinylglycine and has been linked to a higher risk of developing adverse cardiovascular events, although the referenced research was conducted with T2DM patients (Sluik et al., 2012). Mangoni et al. found lower serum cysteine, higher homocysteine and lower glutathione concentrations to be independently associated with cardiovascular risk

scores which were obtained from an equation taking into account the age, gender, systolic blood pressure, total and HDL cholesterol, smoking, diabetes, and electrocardiographic parameters of the left ventricular hypertrophy of subjects (Anderson et al., 1991, Mangoni et al., 2013).

Circulating levels of glucose and one of its major regulating hormones, insulin, are finely balanced in order to maintain homeostasis and small variations of glucose lead to prompt pancreatic response for the secretion of insulin or glucagon (chapter 3). Imbalances in the glucose and insulin system may lead to diabetes and, subsequently, to cardiovascular events. As a result of genetic factors, lifestyle and environment, and despite the numerous prevention methods available, the incidence and prevalence of diabetes is increasing. Recently, 8.3 and 8.5 % of the global and European adult population, respectively, was estimated to suffer from diabetes. These numbers are predicted to reach 8.8 and 10.3 %, respectively, by 2035. Higher fasting glucose, as well as impaired glucose tolerance, which is characterized by abnormally elevated concentrations of glucose following a meal consumption, are considered to belong to a pre-diabetic state. Phenotypic features of diabetic patients, which include, but not exclusively, hyperglycaemia, hypertension and high levels of triglycerides and LDL cholesterol, put these subjects in a more vulnerable position in regards to developing cardiovascular and other health complications (IDF, 2013, Laakso and Kuusisto, 2014). For these reasons, the quantification of glucose, insulin and the insulin-to-glucose ratio are useful for identifying subjects at a higher risk of developing diabetes and subsequent complications that may lead to cardiovascular events.

The final biomarker investigated in the present study was uric acid. Although uric acid can eliminate singlet oxygen and free radicals, higher levels of circulating uric acid (hyperuricaemia) have been positively linked with a higher risk of developing gout (Choi et al., 2005, Roddy and Doherty, 2010, Wortmann, 2002). This condition, which is characterized by a long-term deposition of uric acid in the joints in the form of monosodium urate salt, has in turn been positively associated with insulin resistance, increased adiposity, hypertension, the metabolic syndrome and a higher risk for developing cardiovascular events (Choi et al., 2005, Richette and Bardin, 2010, Richette and Bardin, 2012, Roddy and Doherty, 2010, Terkeltaub, 2010, Wortmann, 2002).

Although the prevalence of hyperuricaemia and gout are known to be higher within the male population, women, most frequently menopausal and postmenopausal, are also affected (Choi and Curhan, 2010, Choi et al., 2005, Doherty, 2009, Richette and Bardin, 2010, Wortmann, 2002). The balance of both genetics and environmental factors influences the concentration of uric acid in the body, and hyperuricaemia often results from the combination of an insufficient urinary excretion, to a lesser extent from an excessive production, and from an excessive purine ingestion through diet (Choi et al., 2005, Richette and Bardin, 2012, Roddy and Doherty, 2010, Terkeltaub, 2010, Wortmann, 2002).

How the uric acid level is affected by diet does not only depend on the administered dose, but also on the quality of the constituent purine precursors (Choi et al., 2005, Richette and Bardin, 2010, Roddy and Doherty, 2010). Thus, an RNA- and purine-rich diet from animal sources are higher contributors to the stocks of uric acid than DNA- and purine-rich vegetables, respectively. The latter have not been linked to any increase in uric acid and risk of developing gout. Furthermore, in a prospective study by Choi et al. with a healthy adult male population, alcohol consumption, more particularly beer followed by spirits, increased the risk of developing gout (Choi et al., 2004). Fructose was also shown to increase the circulating levels of uric acid (Choi et al., 2005). In the analysis of a large scale health and nutrition survey (NHANES-III), Choi and Curhan reported a negative association between the consumption of coffee, but not tea or caffeine, and the circulating levels of uric acid, after adjusting for age and gender (Choi and Curhan, 2007). In a later cohort study, the same authors confirmed similar outcomes in a female adult population by reporting a negative association between long-term coffee consumption and the risk of developing gout. No association between the intake of caffeine in non-coffee consumers or the consumption of tea and the risk of developing gout was detected (Choi and Curhan, 2010).

Further factors associated with hyperuricaemia include an increased body mass index, waist-to-hip ratio, hypertension and insulin resistance. The mechanism by which hyperuricemia may be linked to insulin resistance may be an increased reabsorption of sodium through the proximal tubules of the nephron and a subsequent reduction of uric acid excretion in the urine (Wortmann, 2002). With the consumption of coffee having been positively associated with a lower concentration of insulin and increased insulin sensitivity, the negative correlation reported between

the consumption of coffee and the levels of circulating uric acid may be explained by the increased urinary excretion of uric acid resulting from the decreased levels of insulin (Choi and Curhan, 2010, Wortmann, 2002).

Hyperuricaemia is essential to develop into gout, but does not necessarily develop into the condition (Roddy and Doherty, 2010). The risk of hyperuricemia developing into gout is determined by the combination of multiple factors, which include the local physicochemical properties (Choi et al., 2005, Richette and Bardin, 2010). Before developing into a chronic disease (gouty arthritis), gout is preceded by an asymptomatic hyperuricaemia stage and subsequent periods of acute gout attacks with asymptomatic intervals (Richette and Bardin, 2012).

Previous researches thus indicate that the quantification of biomarkers but also the correlation amongst themselves and with other variables, such as lifestyle and dietary habits, are key procedures to understand mutual effects and consequences on health. Significant correlations may be masked by confounders and the opposite, significant correlations may become non-significant when other confounders are taken into account. Therefore, it is important to conduct analyses adjusting for confounders. Numerous factors can be confounders but when performing a multiple regression analysis, it is not possible to take all of these into account at the risk of reducing the reliability of the results. The number of confounders introduced in the analysis is generally recommended to be 1 for every 10 - 15 case number (Mangoni et al., 2013).

In the present chapter, the associations between selected biomarkers of inflammation and cardiovascular health themselves, and their association with non-dietary and (poly)phenol-rich dietary lifestyle habits are investigated and discussed. Due to the vast amount of data, when adjusted analyses were conducted, only correlations that were significant upon adjustment for confounders are graphically presented.

6.3 Materials and methods

6.3.1 Data collection and investigated relationships

The data analysed in the present chapter were obtained from the earlier described health assessment and food frequency questionnaires filled in by each participant and from the analysed plasma samples. The data collection and sample processing was discussed in earlier chapters (chapters 3 to 5). As a brief recall, besides the information on the age and gender, information on the anthropometrics, general daily habits, general (poly)phenol-rich dietary habits and concentration of selected inflammation and cardiovascular health biomarkers were collected for each participant. The weight, height, BMI, waist, hip and waist-to-hip ratio were recorded as the anthropometric measurements. Information on general daily habits included habits of physical activity, bowel movement frequency, smoking and alcohol consumption. Information on general (poly)phenol-rich dietary habits, included details on the consumption habits of coffee, tea, cocoa beverages, fruits, vegetables, herbs and spices, and supplements and vitamins together. Finally, the total aminothiols cysteine, homocysteine, cysteinylglycine and glutathione, as well as glucose, insulin and uric acid, were quantified in fasting plasma as the biomarkers of inflammation and cardiovascular health.

As the biomarkers of inflammation and cardiovascular health play a central role in the present study, the associations between the latter and relevant anthropometrics, general daily habits, general dietary habits and amongst themselves were investigated. In the present chapter a few new variables were introduced. As mentioned earlier, substrate-to-product ratios between aminothiols have been considered to be useful markers of enzymatic activity and for this purpose, the homocysteine-to-cysteine was measured to assess the activity of the cystathionine β-synthase and cystathionine γ-lyase, while the glutathione-to-cysteinylglycine ratio was used to assess the activity of the γ-glutamyltranspeptidase. A further ratio, cysteinylglycine-to-cysteine, which was not found in literature, was introduced in order to assess the enzymatic activity of the dipeptidase responsible for the cleavage of cysteinylglycine into cysteine and glycine.

Due to the low percentage of reported smokers and former smokers, associations which included these variables were not part of the investigation. For the bowel movement, the data of one participant was excluded as it was assumed to

result from an error. Further investigation aimed at assessing the associations between the anthropometric variables and general daily and dietary habits. The associations between the general daily habits and with general dietary habits were assessed. The associations between (poly)phenol-rich dietary habits amongst themselves have already been discussed in chapter 4. Since the focus of the present study was not the anthropometrics or the general daily habits themselves, no relationship analysis amongst the variables within each of these categories was performed. Also, associations which included the height were not studied.

6.3.2 Statistics

Statistical analyses of the present chapter were performed using the Microsoft Office Excel 2007 and IBM SPSS statistics 20 software packages. The first visit data set (baseline) of the complete population (n = 62) was taken into account. For the assessment of relationships, including the biomarkers of interest, the data sets of two subjects were excluded. These belonged to one subject that did not fast during the first visit and to another for which, due to fear of needles, no blood was withdrawn on the first visit. For this reason and due to the incomplete information provided by some subjects, the analyses rely on a maximal number of 60 data sets and vary depending on the variables analysed. The data are expressed as mean ± standard deviation unless otherwise stated. When the analysis was limited to the relationship between two variables, this was performed by conducting simple correlation tests. When possible, a parametric Pearson's test was run. The presence of outliers was tested by visual inspection of scatter plots and boxplots. If outliers were detected and considered to be genuine unusual data points, these were included in the analyses unless otherwise stated. When outliers were detected and/or bivariate normality was not obtained, an alternative non-parametric Spearman's test was conducted. The differences between genders were assessed by running a parametric independent t-test or a non-parametric Mann-Whitney U-test. The associations between the anthropometrics, general daily habits and general (poly)phenol-rich dietary habits with age and gender have already been investigated and discussed in chapters 4 and 5, however the influence of both age and gender are assessed here on the newly introduced substrate-to-product ratios of the transsulfuration pathway and insulin-to-glucose ratio.

All the relationships which included the biomarkers of health variables (i.e. biomarkers vs. biomarkers, biomarkers vs. anthropometrics, biomarkers vs. general daily habits and biomarkers vs. general (poly)phenol-rich dietary habits) were subsequently analysed in a model of multiple linear regression for the adjustment for age, gender and waist-to-hip ratio. When required, some of the continuous variables were subject to transformation in order to achieve homoscedasticity, a normal distribution of the residuals and to deal with influential outliers. These are specified in the relevant sections. For some variables, such as the consumption of cocoa beverages or the consumption of supplements, numerous subjects had a value of "0". Applying a transformation such as a log10 to these variables would result in a reduction of the data available. In order to overcome this issue, the value of "0.01" was added to each data point of the variables prior to transformation and is described in the relevant sections.

6.4 Results

6.4.1 Effect of gender, age on selected biomarkers of inflammation and cardiovascular health

6.4.1.1 Influence of gender on selected biomarkers of health

No outlier was detected upon inspection of box plots and the distribution was normal in both groups for the cysteinylglycine-to-cysteine ratio (females: $p = 0.066$, males: $p = 0.999$). For this pair, the association with gender was tested by conducting a parametric independent samples t-test. The results of a Levene's test indicated that equality of variances between genders was met for the cysteinylglycine-to-cysteine ratio ($p = 0.387$). The difference of the means between the female (0.122 ± 0.029) and male (0.127± 0.026) populations was - 0.005 (95 % CI, - 0.021 to - 0.010) and was statistically not significant, t (58) = - 0.672, p = 0.504.

A non-parametric Mann-Whitney U-test was conducted to assess the difference between gender for the glutathione-to-cysteinylglycine, the homocysteine-to-cysteine and the insulin-to-glucose ratios. For these variables, outliers were present and normality was not met. The distribution patterns of the

glutathione-to-cysteinylglycine ratio was similar for both genders, as assessed by visual inspection. The median glutathione-to-cysteinylglycine ratio was statistically significantly different between females (*Mdn* = 0.29) and males (*Mdn* = 0.24), *U* = 249, *z* = - 2.368, *p* = 0.018 (Figure 6.1, A). The distribution of the homocysteine-to-cysteine ratio was similar for both genders, as assessed by visual inspection. The median homocysteine-to-cysteine concentration was statistically significantly different between females (*Mdn* = 0.032) and males (*Mdn* = 0.034), *U* = 525, *z* = 1.960, *p* = 0.050 (Figure 6.1, B). The distributions of insulin-to-glucose were similar for both genders, as assessed by visual inspection. The median insulin concentration was statistically not significantly different between females (*Mdn* = 1.85) and males (*Mdn* = 1.70), *U* = 297.5, *z* = - 1.613, *p* = 0.107.

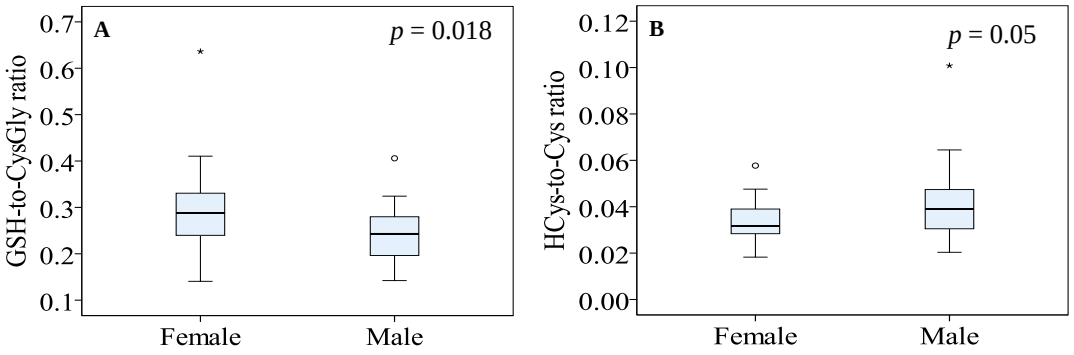


Figure 6.1: Significant differences between genders, in biomarkers of health measured in plasma at baseline. GSH-to-CysGly, glutathione-to-cysteinylglycine; HCys-to-Cys, homocysteine-to-cysteine. **O**: non-extreme outlier (the data point is > 1.5 box-length away from the corresponding plotted box edge), *: extreme outlier (the data point is > 3 box-lengths away from the corresponding plotted box edge).

6.4.1.2 Influence of age on selected biomarkers of health

When the aminothiol substrate-to-product and insulin-to glucose ratios were analysed by the age, only cysteinylglycine-to-cysteine significantly correlated, r_s (58) = - 0.398, *p* = 0.002 (Figure 6.2). This correlation is likely to be due to the cysteinylglycine, as cysteine had no significantly association with age, as seen in chapter 5.

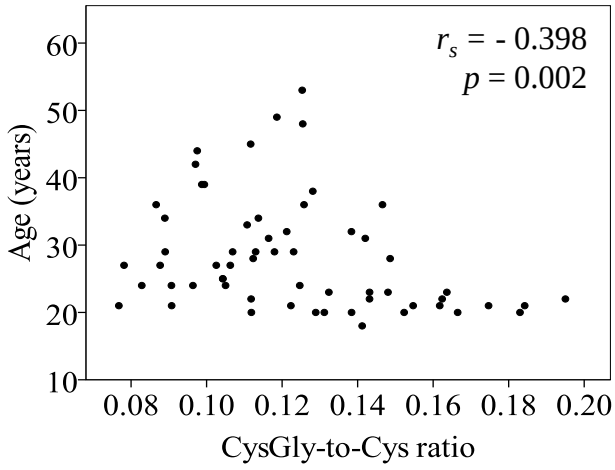


Figure 6.2: Scatter plot representation of the only variable that revealed a significant association with the age at baseline. The significance of the association between cysteinylglycine and age observed in chapter 5 is a likely explanation for the significant correlation detected between the cysteinylglycine-to-cysteine ratio and age. CysGly-to-Cys, cysteinylglycine-to-cysteine.

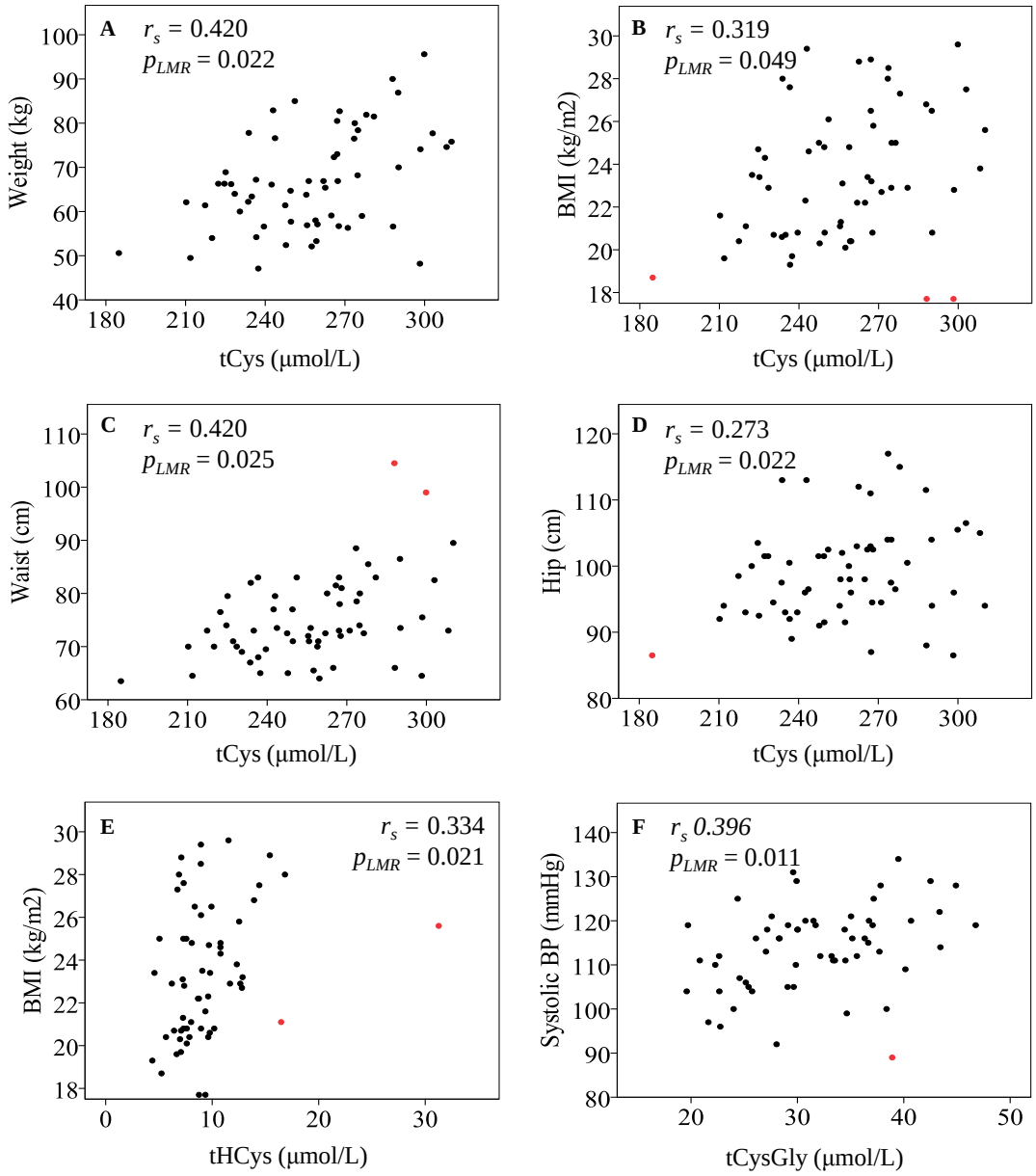
6.4.1.3 Influence of anthropometrics on selected biomarkers of health

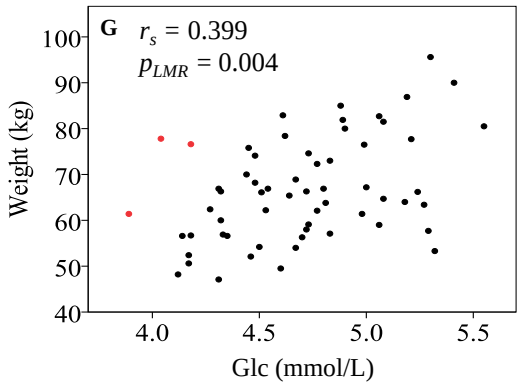
When the associations between biomarkers of health and anthropometrics were tested, positive correlations were detected by running a Spearman's test, between the total concentration of cysteine and the weight, r_s (58) = 0.420, p = 0.001; BMI, r_s (58) = 0.319, p = 0.013; waist circumference, r_s (58) = 0.420, p = 0.001; hip circumference, r_s (58) = 0.273, p = 0.035; and waist-to-hip ratio, r_s (58) = 0.319, p = 0.013. Numerous positive correlations were also detected between the concentration of total homocysteine and anthropometrics. A Spearman's test revealed significant positive associations between total homocysteine and the weight, r_s (58) = 0.398, p = 0.002; BMI, r_s (58) = 0.334, p = 0.009; waist r_s (58) = 0.380, p = 0.003; waist-to-hip ratio, r_s (58) = 0.321, p = 0.012; and systolic blood pressure, r_s (57) = 0.363, p = 0.005. Cysteinylglycine was only significantly positively associated with the systolic blood pressure, as assessed by a Spearman's test, r_s (57) = 0.396, p = 0.002. A Spearman's test detected negative associations between the concentration of total glutathione and the weight, r_s (58) = - 0.260, p = 0.044; BMI, r_s (58) = - 0.278, p = 0.031; and waist circumference, r_s (58) = - 0.257, p = 0.047. A negative association was also detected between the glutathione-to-cysteinylglycine ratio and the weight, r_s (58) = - 0.272, p = 0.035. The only

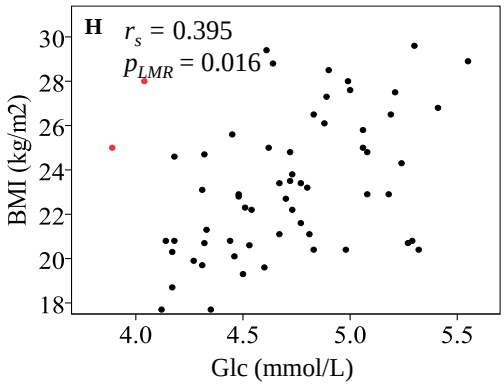
significant association with the cysteinylglycine-to-cysteine ratio was a positive correlation with the systolic blood pressure, as assessed by a Spearman's correlation test, r_s (58) = 0.297, p = 0.023. When the associations between the homocysteine-to-cysteine ratio and the anthropometrics were investigated, positive correlations were detected by conducting a Spearman's test, with the weight, r_s (58) = 0.294, p = 0.023; BMI, r_s (58) = 0.279, p = 0.031; waist circumference, r_s (58) = 0.282, p = 0.029; and systolic blood pressure, r_s (58) = 0.366, p = 0.004. Positive associations only were detected by a Spearman's test between the concentration of glucose and the weight, r_s (58) = 0.399, p = 0.002; BMI, r_s (58) = 0.395, p = 0.002; waist, r_s (58) = 0.419, p = 0.001; and hip circumference, r_s (58) = 0.393, p = 0.002. When the associations between the concentration of insulin at fast and the anthropometrics were tested by conducting a Spearman's test, a positive correlation was detected with the BMI, r_s (58) = 0.266, p = 0.040. Finally, numerous significant associations were observed between the concentration of uric acid in plasma at fast and the anthropometrics. All of the latter were assessed by a Spearman's correlation test and were between uric acid and the weight, r_s (58) = 0.457, $p < 0.0005$; BMI, r_s (58) = 0.274, p = 0.034; waist, r_s (58) = 0.432, p = 0.001; and waist-to-hip ratio, r_s (58) = 0.497, $p < 0.0005$.

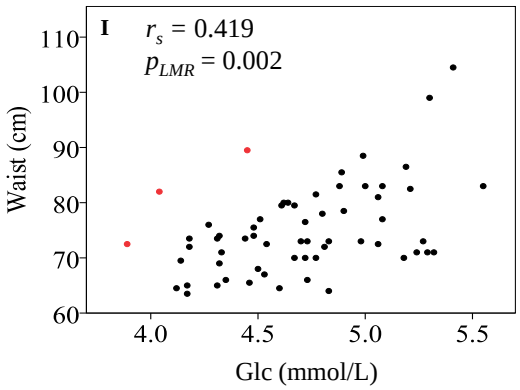
Upon adjustment for the age, gender and waist-to-hip ratio, fewer associations were detected. The concentration of total cysteine was positively associated with the weight, B = 0.79, t (58) = 2.36, p = 0.022; BMI, B = 0.21, t (58) = 2.01, p = 0.049; waist, B = 1.319, t (58) = 2.30, p = 0.025 and hip circumferences, B = 1.03, t (58) = 2.35, p = 0.022 (Figure 6.3, A-D). The cysteine and BMI variables were subject to a log10 transformation prior to the analysis between each other. The concentration of total homocysteine was only positively associated with the BMI, B = 0.73, t (58) = 2.38, p = 0.021 (Figure 6.3, E). Both variables were also log10-transformed for the regression analysis. The association between the concentration of cysteinylglycine and the systolic blood pressure remained unchanged, B = 0.22, t (57) = 2.64, p = 0.011(Figure 6.3, F). All the associations between the concentration of glucose and the anthropometric variables were unchanged and glucose was positively associated with the weight, B = 0.02, t (58) = 3.02, p = 0.004; BMI, B = 0.04, t (58) = 2.50, p = 0.016; waist circumference, B = 0.03, t (58) = 3.17, p = 0.002 and hip circumference, B = 0.02, t (58) = 3.11, p = 0.003 (Figure 6.3, G-J). Insulin remained positively associated with the BMI, B = 0.62, t (58) = 2.34, p =

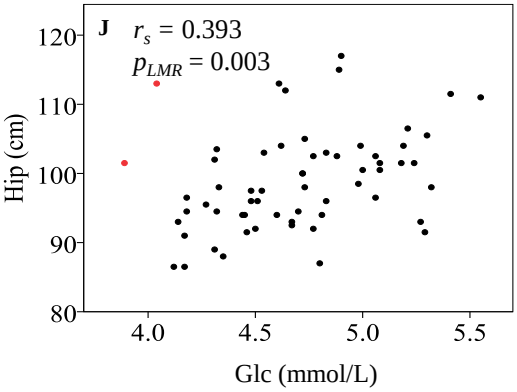
0.023 (Figure 6.3, K). Both variables were subject to a log10-transformed for the regression analysis. The insulin-to-glucose ratio was newly positively associated, with the BMI, B = 0.52, t (58) = 2.05, p = 0.045 (Figure 6.3, L). The insulin-to-glucose ratio and the BMI were log10-transformed prior to the regression analysis. Finally, the only significant association detected with uric acid was with the weight, B = 0.11, t (58) = 2.10, p = 0.041 (Figure 6.3, M). For this final regression, the uric acid variable was squared and the weight subject to a log10 transformation.

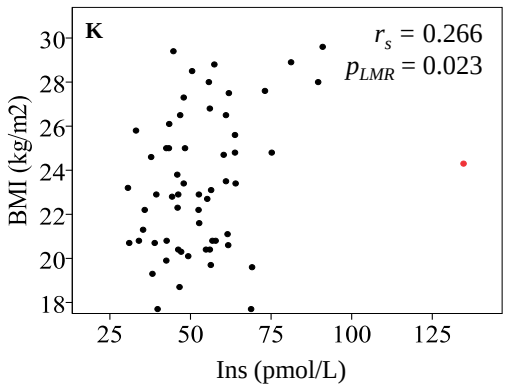


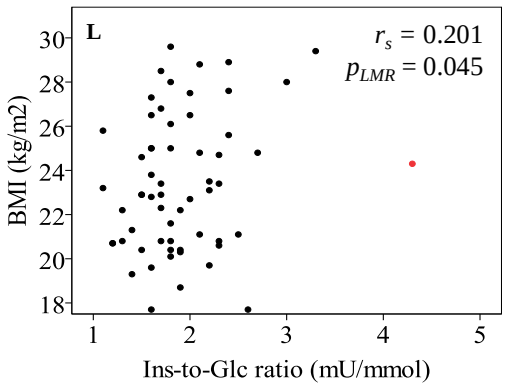

G
r_s = 0.399
p_{LMR} = 0.004
Weight (kg)
Glc (mmol/L)


H
r_s = 0.395
p_{LMR} = 0.016
BMI (kg/m2)
Glc (mmol/L)


I
r_s = 0.419
p_{LMR} = 0.002
Waist (cm)
Glc (mmol/L)


J
r_s = 0.393
p_{LMR} = 0.003
Hip (cm)
Glc (mmol/L)


K
r_s = 0.266
p_{LMR} = 0.023
BMI (kg/m2)
Ins (pmol/L)


L
r_s = 0.201
p_{LMR} = 0.045
BMI (kg/m2)
Ins-to-Glc ratio (mU/mmol)

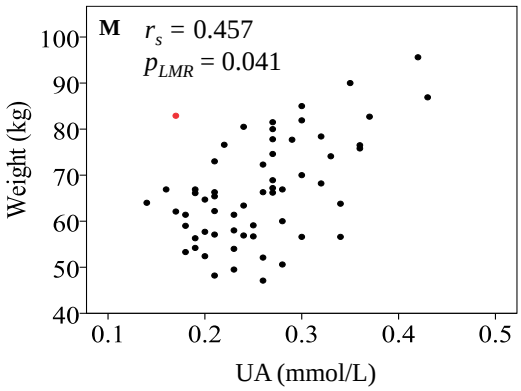

M
r_s = 0.457
p_{LMR} = 0.041
Weight (kg)
UA (mmol/L)

Figure 6.3: Scatter plot representation of the significant relationships at baseline observed between biomarkers of health in fasting plasma and anthropometric variables upon adjustment for age, gender and waist-to-hip ratio. The red data points indicate distinct and ambiguous outliers. p_{LMR}, *p*-value obtained upon multiple regression, tCys, total cysteine; tHCys, total homocysteine; tCysGly, total cysteinylglycine; Glc, glucose; Ins, insulin; Ins-to-Glc, insulin-to-glucose; UA, uric acid; BMI, body mass index; Systolic BP, systolic blood pressure.

6.4.2 Impact of general daily habits on selected biomarkers of health

All the associations between the biomarkers of health and variables of general daily lifestyle habits were tested by conducting a Spearman's correlation test due to the presence of outliers and/or the absence of bivariate normality, and when the bowel movement was part of the analysed pair, due to the ordinal nature of the variable.

Few statistically significant associations were detected within this category. The bowel movement was not associated with any of the biomarkers of health ($p > 0.05$). Glucose was negatively associated with the physical activity score, as assessed by a Spearman's correlation test, r_s (55) = - 0.284, p = 0.032. Positive associations were detected between the consumption of alcohol and cysteinylglycine, r_s (58) = 0.524, $p < 0.0005$; the cysteinylglycine-to-cysteine ratio, r_s (58) = 0.371, p = 0.004; and uric acid, r_s (58) = 0.336, p = 0.009. Finally, a negative association was detected between the consumption of alcohol and the glutathione-to-cysteinylglycine ratio, r_s (58) = - 0.402, p = 0.001.

Upon adjustment for the age, gender and waist-to-hip ratio, no new association was identified and the only unchanged statistically significant associations were between the consumption of alcohol and the fasting concentration of cysteinylglycine, B = 0.453, t (58) = 2.176, p = 0.034; the cysteinylglycine-to-cysteine ratio, B = 0.002, t (58) = 2.110, p = 0.039 and the glutathione-to-cysteinylglycine ratio, B = - 0.007, t (58) = -2.531, p = 0.014 (Figure 6.4 A-C).

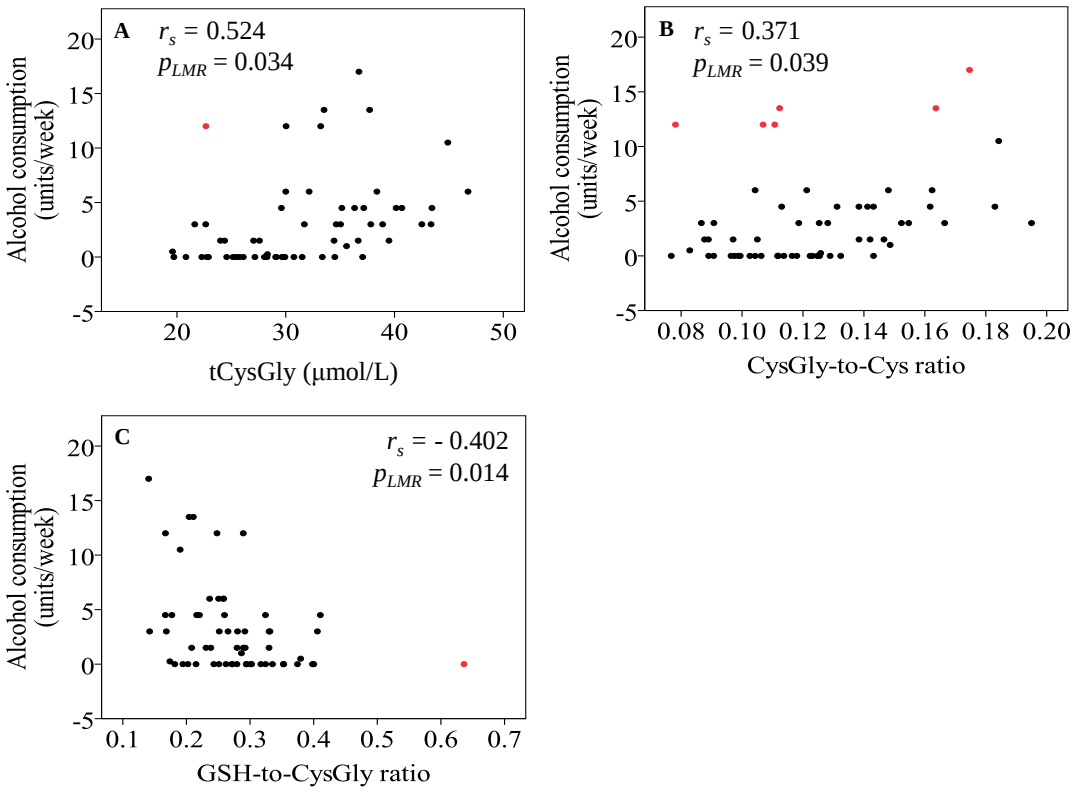


Figure 6.4: Scatter plot representation of the significant relationships observed at baseline between biomarkers in fasting plasma and general daily lifestyle habits after adjustment for age, gender and waist-to-hip ratio. The red data points indicate distinct and ambiguous outliers. p_{LMR}, *p*-value obtained upon multiple regression, tCysGly, total cysteinylglycine; CysGly-to-Cys, cysteinylglycine-to-cysteine ratio; GSH-to-CysGly ratio, glutathione-to-cysteinylglycine ratio.

6.4.3 Impact of habitual consumption of coffee and other (poly)phenol-rich products on selected biomarkers of health

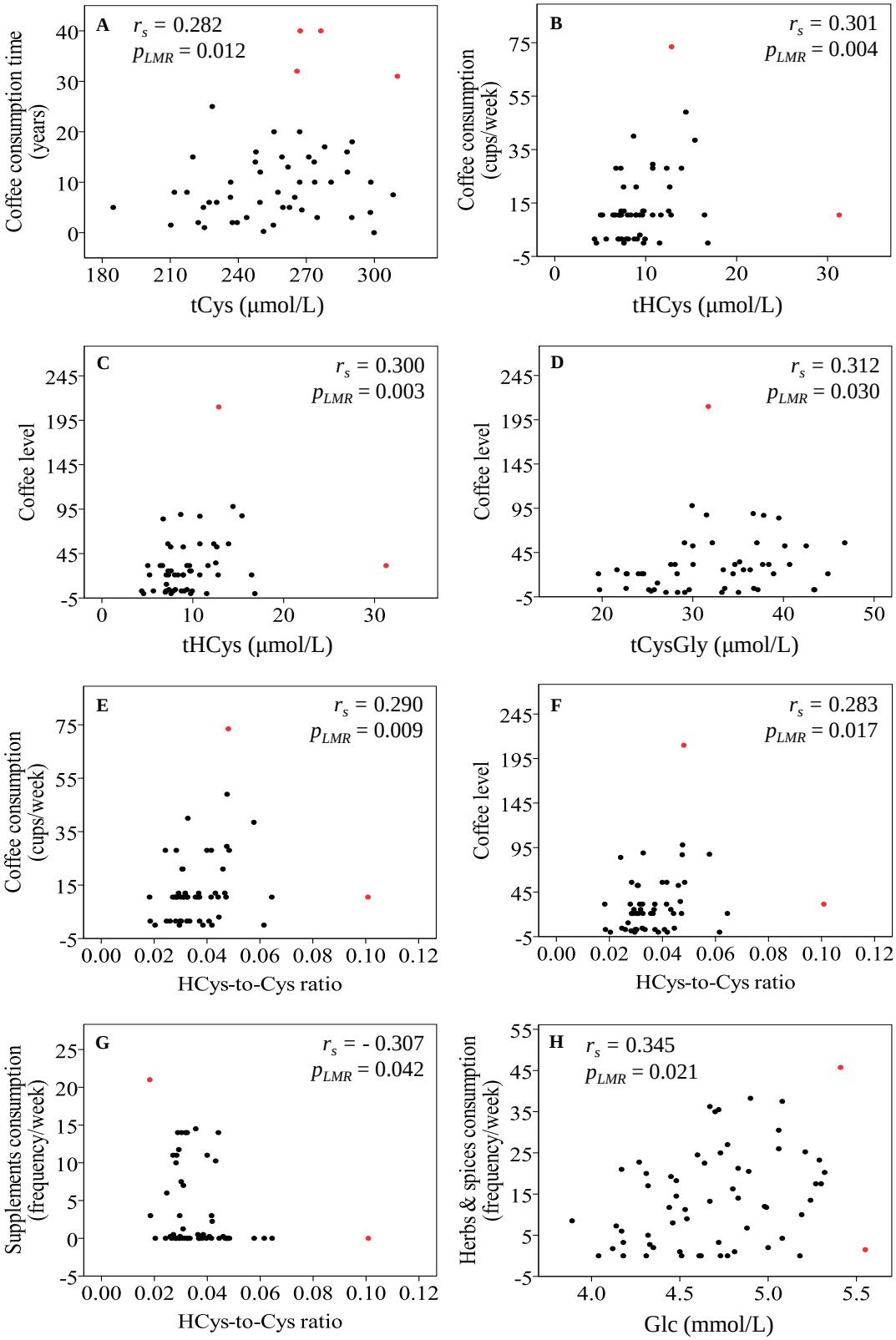
The relationships between all the pairs were assessed by running a non-parametric Spearman's correlation test due to the presence of outliers and/or the absence of bivariate normality. The analyses revealed a statistically significant association between the following pairs: the concentration of total cysteine and the time a subject had reported to be a consumer of coffee, r_s (48) = 0.282, p = 0.047; the concentration of total cysteine and the coffee level, r_s (50) = 0.326, p = 0.018; the concentration of total homocysteine and the weekly coffee consumption, r_s (52) = 0.301, p = 0.030; the concentration of total homocysteine and the coffee level, r_s (50) = 0.300, p = 0.030; the concentration of total homocysteine and the

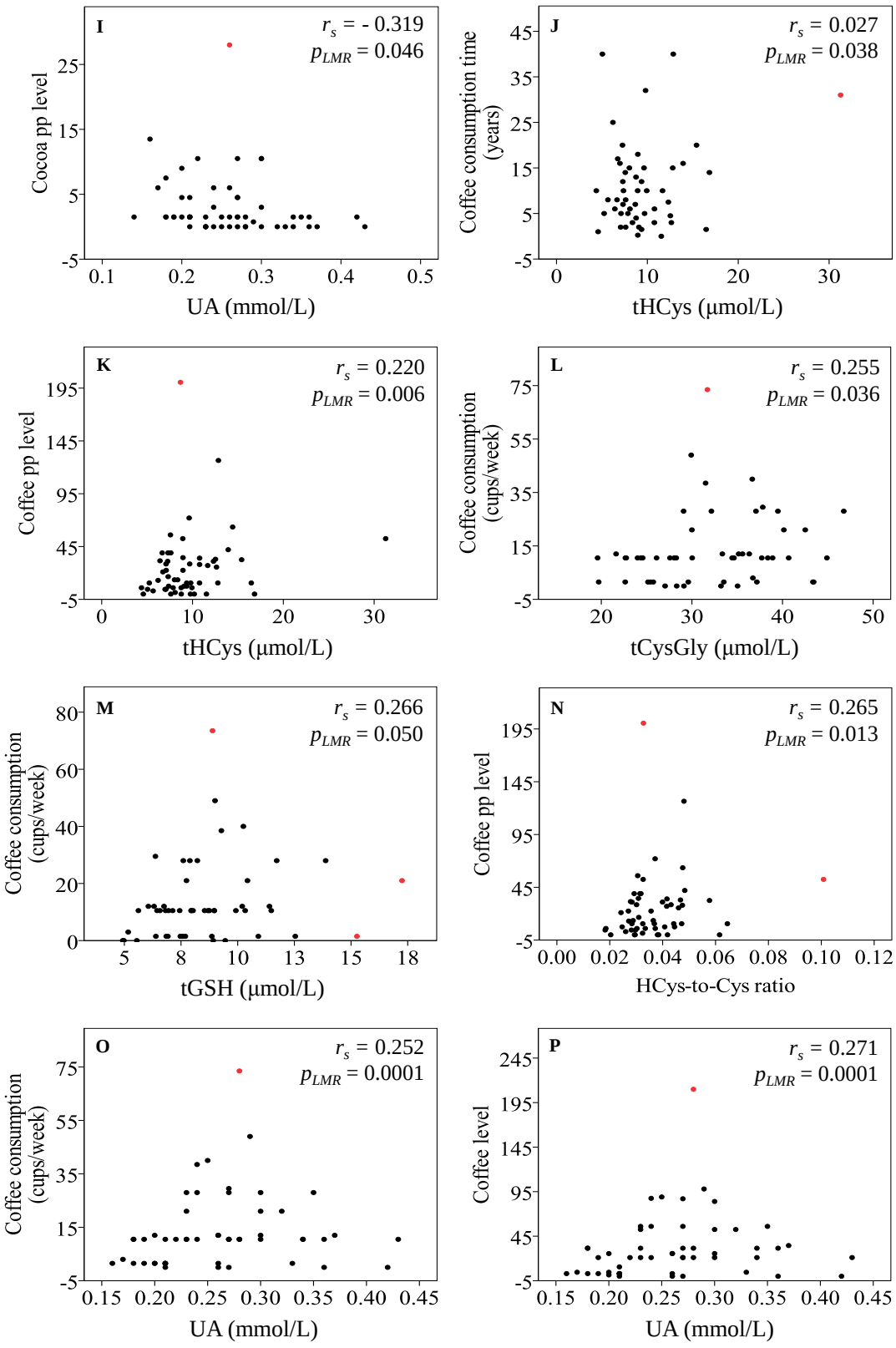
consumption of supplements, r_s (55) = - 0.275, p = 0.034; the concentration of total cysteinylglycine and the coffee level, r_s (50) = 0.312, p = 0.025; the concentration of total cysteinylglycine and the time a subject had reported to be a consumer of tea, r_s (50) = - 0.364, p = 0.009; the concentration of total glutathione and the coffee level, r_s (50) = 0.307, p = 0.027; the concentration of total cysteinylglycine and the consumption of filtered coffee (r_s (51) = 0.304, p = 0.027). This association suggests a beneficial effect from consuming coffee lacking the majority of diterpenes. Although coffee diterpenes are known to possess cholesterol-rising effects, in these have also shown anti-inflammatory effects in several *in vitro* experiments and, therefore, such an association was not expexted. Also associated were the cysteinylglycine-to-cysteine ratio and the time a subject had reported to be a consumer of tea, r_s (50) = - 0.299, p = 0.035; the homocysteine-to-cysteine ratio and the weekly coffee consumption, r_s (52) = 0.290, p = 0.037; the homocysteine-to-cysteine ratio and the coffee level, r_s (50) = 0.283, p = 0.042; the homocysteine-to-cysteine ratio and the time a subject had reported to be a consumer of tea, r_s (50) = - 0.292, p = 0.040; the homocysteine-to-cysteine ratio and the consumption of supplements, r_s (55) = - 0.307, p = 0.017; the concentration of glucose and the consumption of herbs and spices, r_s (55) = 0.345, p = 0.007; the concentration of glucose and the consumption of arabic coffee, r_s (52) = 0.277, p = 0.042. Arabic coffee is prepared with a mixture of spices (e.g. cardamom, saffron, cloves, cinnanon) which could contribute to this association, however only two subjects were regular consumers of this brew and this relation should not be considered. The concentration of insulin was negatively associated with the consumption of espresso (r_s (51) = - 0.309, p = 0.025) and dark cocoa beverages (r_s (53) = - 0.308, p = 0.022), however, as for the relation between glucose and arabic coffee, few subjects (n = 11 for each beverages) were regular consumers and considering these relationships as definite would be borderline.The concentration of uric acid was negatively associated with the consumption of cappuccino (r_s (51) = - 0.341, p = 0.012) and the intake of (poly)phenols from cocoa beverages (r_s (53) = - 0.319, p = 0.018).

As for the previous relationships analysed, a fewer number of statistically significant associations were detected after adjustment for the age, gender and waist-to-hip ratio. The concentration of total cysteine remained positively associated with only the time a subject had reported to be a consumer of coffee, B = 1.270, t (48) = 2.632, p = 0.012 (Figure 6.5, A). Total homocysteine was positively associated with

both the weekly consumption of coffee, B = 0.117, *t* (52) = 3.010, *p* = 0.004, and the coffee level variable, B = 0.102, *t* (50) = 2.789, *p* = 0.008 (Figure 6.5, B and C). The concentration of total cysteinylglycine was positively associated with the coffee level variable, B = 0.054, *t* (50) = 2.243, *p* = 0.030 (Figure 6.5, D). The homocysteine-to-cysteine ratio maintained its positive association with both the weekly consumption of coffee and the coffee level, with B = 0.097, *t* (52) = 2.752, *p* = 0.009 and B = 0.083, *t* (50) = 2.483, *p* = 0.017, respectively (Figure 6.5, E and F). For the latter analyses, a log10 transformation was applied to the homocysteine-to-cysteine ratio, the coffee level and the weekly consumption of coffee variables. The homocysteine-to-cysteine ratio was also negatively associated with the consumption of supplements, B = - 0.036, *t* (55) = - 2.079, *p* = 0.042 with a log10 transformation having been applied to the homocysteine-to-cysteine ratio and to the consumption of supplement variable + 0.01 (Figure 6.5, G). The concentration of glucose was positively associated with the consumption of herbs and spices, B = 0.010, *t* (55) = 2.371, *p* = 0.021 and that of uric acid was negatively associated with the intake of (poly)phenols from cocoa beverages, B = - 0.28, *t* (53) = - 2.049, *p* = 0.046 (Figure 6.5, H and I).

Numerous new significant associations were detected upon multiple regression. The concentration of total homocysteine was associated with the time a subject had reported to be a consumer of coffee and the intake of (poly)phenols through coffee preparations, with B = 0.100, *t* (49) = 2.142, *p* = 0.038 (Figure 6.5, J) and B = 0.117, *t* (55) = 2.876, *p* = 0.006 (Figure 6.5 K), respectively. For these two analyses, the three variables underwent a log10 transformation. The concentrations of total cysteinylglycine and glutathione were positively associated with the weekly consumption of coffee, with B = 0.133, *t* (52) = 2.158, *p* = 0.036 (Figure 6.5, L) and B = 0.049, *t* (52) = 2.008, *p* = 0.050 (Figure 6.5, M), respectively. The homocysteine-to-cysteine ratio was positively associated with the intake of (poly)phenols from coffee preparations, B = 0.096, *t* (53) = 2.578, *p* = 0.013 (Figure 6.5 N). Finally, the concentration of uric acid was positively associated with the weekly consumption of coffee, B = 0.081, *t* (52) = 3.678, *p* = 0.0001 (Figure 6.5, O); the coffee level variable, B = 0.076, *t* (50) = 3.708, *p* = 0.0001 (Figure 6.5, P); the intake of (poly)phenols from coffee preparations, B = 0.074, *t* (53) = 2.974, *p* = 0.005 (Figure 6.5, Q) and the consumption of vegetables, B = 0.046, *t* (52) = 2.364, *p* = 0.022 (Figure 6.5, R).


A
r_s = 0.282
p_{LMR} = 0.012
Coffee consumption time (years)
tCys (µmol/L)
B
r_s = 0.301
p_{LMR} = 0.004
Coffee consumption (cups/week)
tHCys (µmol/L)
C
r_s = 0.300
p_{LMR} = 0.003
Coffee level
tHCys (µmol/L)
D
r_s = 0.312
p_{LMR} = 0.030
Coffee level
tCysGly (µmol/L)
E
r_s = 0.290
p_{LMR} = 0.009
Coffee consumption (cups/week)
HCys-to-Cys ratio
F
r_s = 0.283
p_{LMR} = 0.017
Coffee level
HCys-to-Cys ratio
G
r_s = - 0.307
p_{LMR} = 0.042
Supplements consumption (frequency/week)
HCys-to-Cys ratio
H
r_s = 0.345
p_{LMR} = 0.021
Herbs & spices consumption (frequency/week)
Glc (mmol/L)


I
r_s = - 0.319
p_{LMR} = 0.046
Cocoa pp level
UA (mmol/L)
J
r_s = 0.027
p_{LMR} = 0.038
Coffee consumption time (years)
tHCys (µmol/L)
K
r_s = 0.220
p_{LMR} = 0.006
Coffee pp level
tHCys (µmol/L)
L
r_s = 0.255
p_{LMR} = 0.036
Coffee consumption (cups/week)
tCysGly (µmol/L)
M
r_s = 0.266
p_{LMR} = 0.050
Coffee consumption (cups/week)
tGSH (µmol/L)
N
r_s = 0.265
p_{LMR} = 0.013
Coffee pp level
HCys-to-Cys ratio
O
r_s = 0.252
p_{LMR} = 0.0001
Coffee consumption (cups/week)
UA (mmol/L)
P
r_s = 0.271
p_{LMR} = 0.0001
Coffee level
UA (mmol/L)

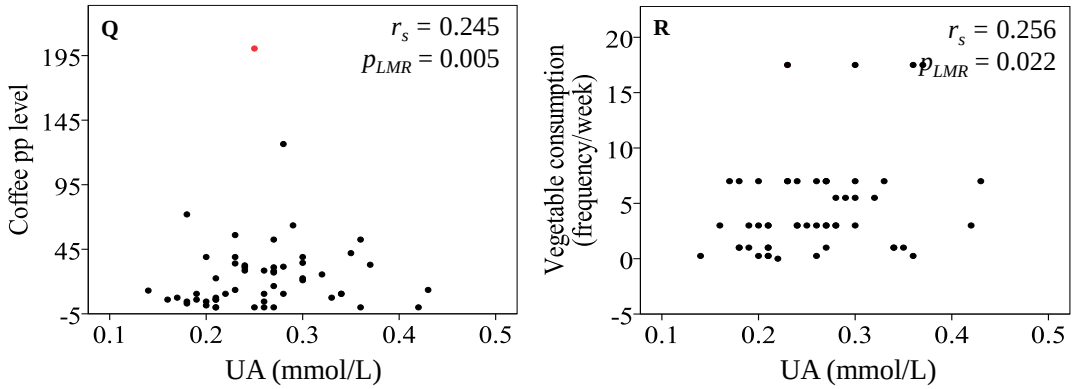


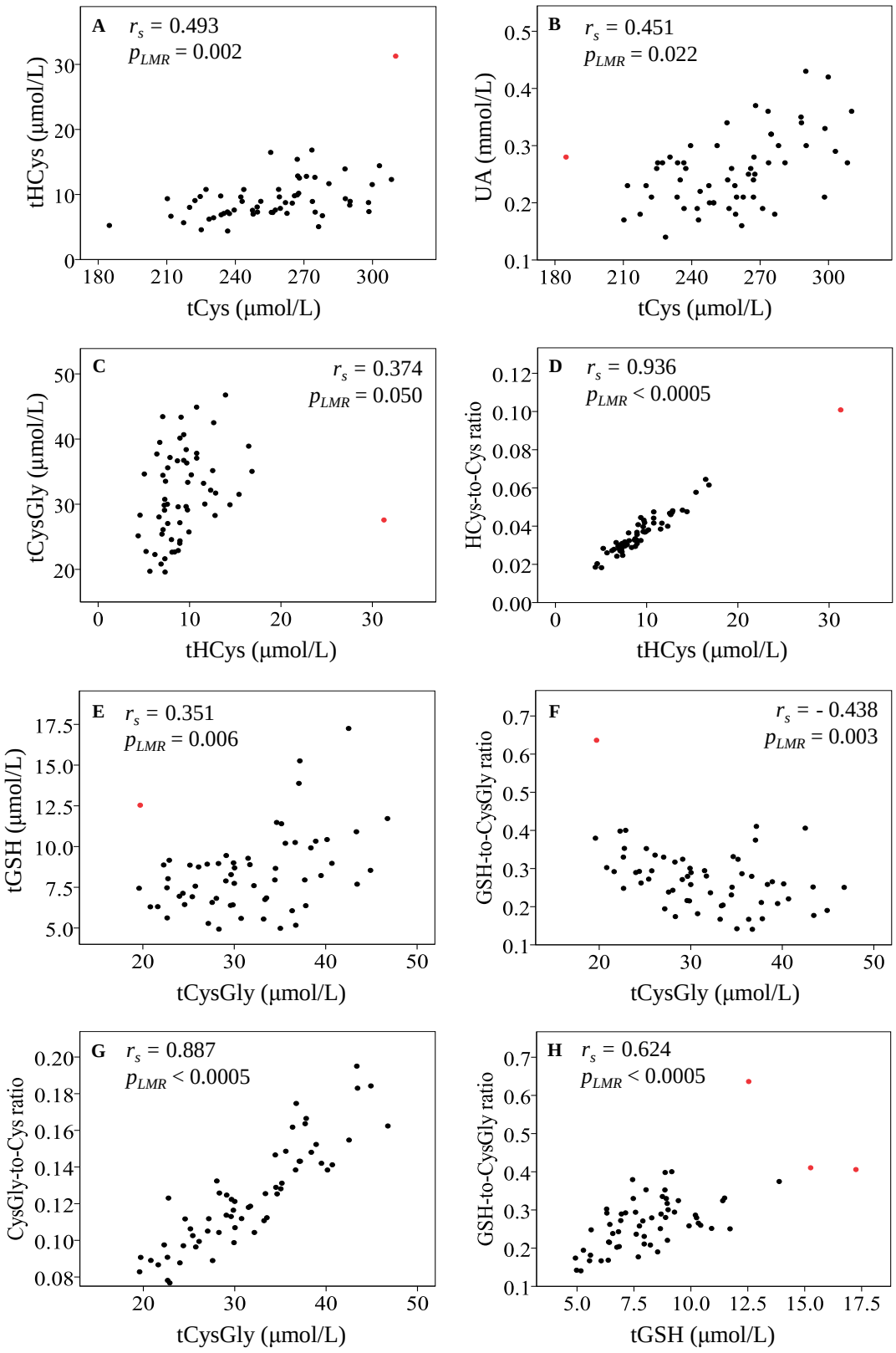
Figure 6.5: Scatter plot representation of the statistically significant correlations observed at baseline between biomarkers in fasting plasma and general (poly)phenol-rich dietary habits upon adjustment for age, gender and waist-to-hip ratio. The red data points indicate distinct and ambiguous outliers. p_{LMR}, *p*-value obtained upon multiple regression, tCys, total cysteine; tHCys, total homocysteine; tCysGly, total cysteinylglycine; L-GSH, total glutathione; HCys-to-Cys ratio, homocysteine-to-cysteine ratio; Glc, glucose; UA, uric acid.

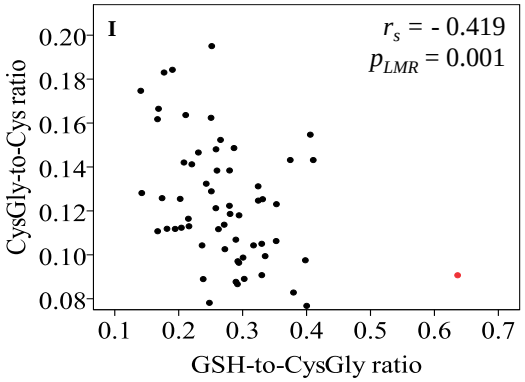
6.4.4 Associations amongst biomarkers of health

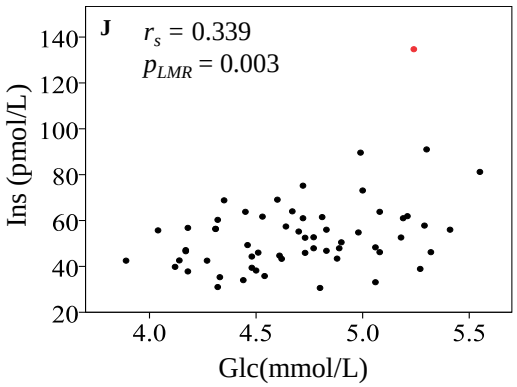
The association between cysteine vs. cysteinylglycine, cysteinylglycine vs. cysteinylglycine-to-cysteine, cysteinylglycine vs. glucose, cysteinylglycine vs. uric acid and glucose vs. uric acid were analysed by running a Pearson's correlation test. Due to the presence of outliers and/or both variables in the analysed pairs not being normally distributed, all the other associations between biomarkers amongst themselves were tested by conducting a non-parametric Spearman's correlation test. The significant associations revealed by carrying out of a Pearson's or Spearman's test were between the following pairs: cysteine vs. homocysteine, r_s (58) = 0.473, $p < 0.0005$; cysteine vs. uric acid, r_s (58) = 0.451, $p < 0.0005$; homocysteine vs. cysteinylglycine, r_s (58) = 0.374, $p = 0.003$; homocysteine vs. homocysteine-to-cysteine ratio, r_s (58) = 0.936, $p < 0.0005$; homocysteine vs. glucose, r_s (58) = 0.273, $p = 0.035$; homocysteine vs. uric acid, r_s (58) = 0.330, $p = 0.010$; cysteinylglycine vs. glutathione, r_s (58) = 0.351, $p = 0.006$; cysteinylglycine vs. glutathione-to-cysteinylglycine ratio, r_s (58) = - 0.438, $p < 0.0005$; cysteinylglycine vs. cysteinylglycine-to-cysteine ratio, r_p (58) = 0.887, $p < 0.0005$; cysteinylglycine vs. homocysteine-to-cysteine ratio, r_s (58) = 0.369, $p = 0.004$; cysteinylglycine vs. uric

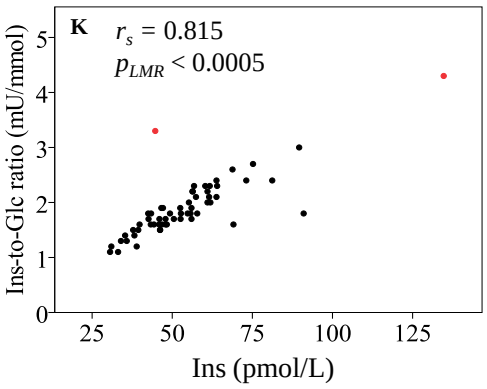
acid, r_s (58) = 0.281, p = 0.029; glutathione vs. glutathione-to-cysteinylglycine ratio, r_s (58) = 0.624, p < 0.0005; glutathione vs. cysteinylglycine-to-cysteine ratio, r_s (58) = 0.271, p = 0.036; glutathione-to-cysteinylglycine ratio vs. cysteinylglycine-to-cysteine ratio, r_s (58) = - 0.419, p = 0.001; cysteinylglycine-to-cysteine ratio vs. homocysteine-to-cysteine ratio, r_s (58) = 0.271, p = 0.036; glucose vs. insulin, r_s (58) = 0.339, p = 0.008; and insulin vs. insulin-to-glucose ratio, r_s (58) = 0.815, p < 0.0005.

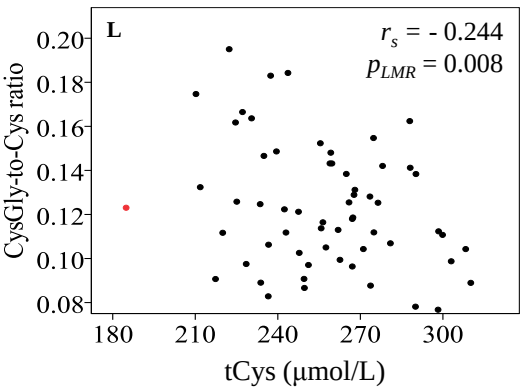
Upon analysis by multiple regression with adjustment for the age, gender and waist-to-hip ratio, the concentration of total cysteine remained significantly associated with total homocysteine (B = 2.842, t (58) = 3.181, p = 0.002) and uric acid (B = 161.964, t (58) = 2.353, p = 0.022) (Figure 6.6, A and B). Total homocysteine was significantly associated with total cysteinylglycine (B = 0.387, t (58) = 2.002, p = 0.050) and the homocysteine-to-cysteine ratio (B = 1.046, t (58) = 21.451, p < 0.0005) (Figure 6.6, C and D). The three variables were subject to a log10 transformation. Total cysteinylglycine remained significantly associated with total glutathione (B = 0.931, t (58) = 2.841, p = 0.006), the glutathione-to-cysteinylglycine ratio (B = - 30.118, t (58) = -3.137, p = 0.003) and with the cysteinylglycine-to-cysteine ratio (B = 209.040, t (58) = 14.479, p < 0.0005) (Figure 6.6, E to G); total glutathione was significantly associated with the glutathione-to-cysteinylglycine ratio (B = 20.360, t (58) = 6.962, p < 0.0005) (Figure 6.6, H); and the glutathione-to-cysteinylglycine ratio was significantly associated with the cysteinylglycine-to-cysteine ratio (B = - 0.578, t (58) = - 3.660, p = 0.001) (Figure 6.6, I). Both variables were subject to a log10 transformation. Glucose was significantly associated with insulin (B = 0.009, t (58) = 3.150, p = 0.003) and insulin with the insulin-to-glucose ratio (B = 0.880, t (58) = 10.544, p < 0.0005) (Figure 6.6, J and K). Four new associations were revealed. Total cysteine was negatively associated with the cysteinylglycine-to-cysteine ratio (B = - 329.439, t (58) = - 2.776, p = 0.008) (Figure 6.6, L) and the glutathione-to-cysteinylglycine ratio with the insulin-to-glucose ratio (B = - 0.300, t (58) = - 2.071, p = 0.043) (Figure 6.6, M), while the homocysteine-to-cysteine ratio was positively associated with insulin (B = 0.271, t (58) = 2.010, p = 0.049) (Figure 6.6, N) and the insulin-to-glucose ratio (B = 0.341, t (58) = 2.447, p = 0.018) (Figure 6.6, O).

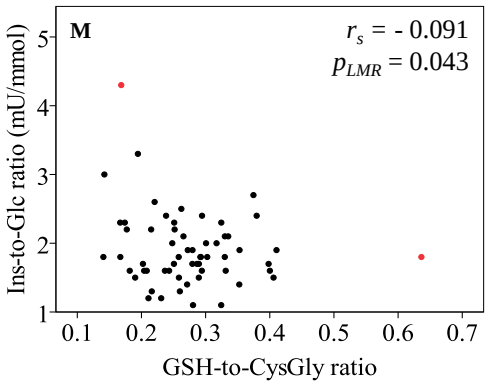

A
r_s = 0.493
p_{LMR} = 0.002
tHCys (µmol/L)
tCys (µmol/L)
B
r_s = 0.451
p_{LMR} = 0.022
UA (mmol/L)
tCys (µmol/L)
C
r_s = 0.374
p_{LMR} = 0.050
tCysGly (µmol/L)
tHCys (µmol/L)
D
r_s = 0.936
p_{LMR} < 0.0005
HCys-to-Cys ratio
tHCys (µmol/L)
E
r_s = 0.351
p_{LMR} = 0.006
tGSH (µmol/L)
tCysGly (µmol/L)
F
r_s = - 0.438
p_{LMR} = 0.003
GSH-to-CysGly ratio
tCysGly (µmol/L)
G
r_s = 0.887
p_{LMR} < 0.0005
CysGly-to-Cys ratio
tCysGly (µmol/L)
H
r_s = 0.624
p_{LMR} < 0.0005
GSH-to-CysGly ratio
tGSH (µmol/L)

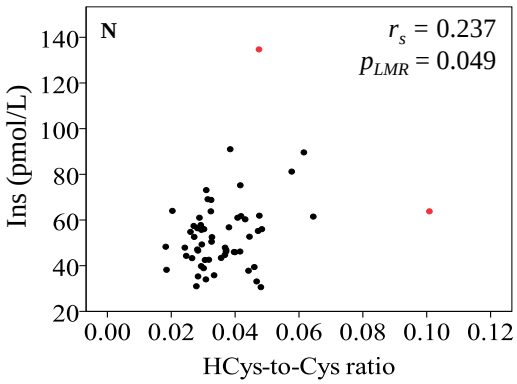

I
r_s = - 0.419
p_{LMR} = 0.001
CysGly-to-Cys ratio
GSH-to-CysGly ratio


J
r_s = 0.339
p_{LMR} = 0.003
Ins (pmol/L)
Glc(mmol/L)


K
r_s = 0.815
p_{LMR} < 0.0005
Ins-to-Glc ratio (mU/mmol)
Ins (pmol/L)


L
r_s = - 0.244
p_{LMR} = 0.008
CysGly-to-Cys ratio
tCys (µmol/L)


M
r_s = - 0.091
p_{LMR} = 0.043
Ins-to-Glc ratio (mU/mmol)
GSH-to-CysGly ratio


N
r_s = 0.237
p_{LMR} = 0.049
Ins (pmol/L)
HCys-to-Cys ratio

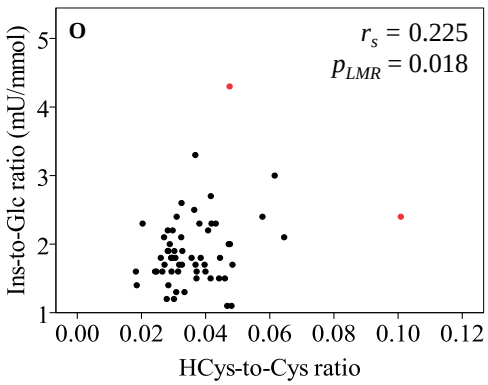

O
r_s = 0.225
p_{LMR} = 0.018
Ins-to-Glc ratio (mU/mmol)
HCys-to-Cys ratio

Figure 6.6: Scatter plot representation of the statistically significant correlations observed at baseline between biomarkers in fasting plasma themselves upon adjustment for age, gender and waist-to-hip ratio. The red data points indicate distinct and ambiguous outliers. p_{LMR}, *p*-value obtained upon multiple regression, tCys, total cysteine; tHCys, total homocysteine; tCysGly, total cysteinylglycine; tGSH, total glutathione; GSH-to-CysGly ratio, glutathione-to-cysteinylglycine ratio; CysGly-to-Cys, cysteinylglycine-to-cysteine ratio; HCys-to-Cys ratio, homocysteine-to-cysteine ratio; Glc, glucose; Ins, insulin; Ins-to-Glc, insulin-to-glucose; UA, uric acid.

6.4.5 Impact of habitual consumption of (poly)phenol-rich foods on the anthropometrics

No bivariate normality was observed in all pairs analysed between the anthropometric variables and the dietary lifestyle habits. The majority of the pairs also had outliers present, as detected by visual inspection of scatter plots. For these reasons, a Spearman's correlation test was conducted to assess the relationship between (poly)-phenol-rich dietary habits and the anthropometrics. Only one significant correlation was detected. This was a negative correlation between the years of tea consumption and the systolic blood pressure, r_s (50) = - 0.328, p = 0.019 Figure 6.7.

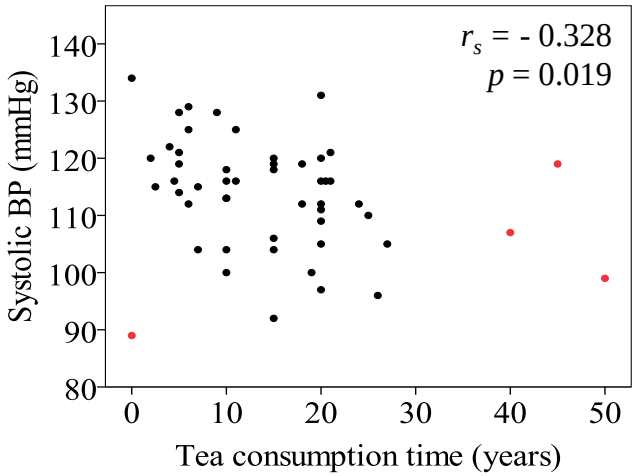


Figure 6.7: Scatter plot illustrating the only statistically significant correlation observed at baseline between the anthropometric and the general dietary habit variables, between the time a subject had declared to be a consumer of tea and the systolic blood pressure (BP). The red data points indicate distinct and ambiguous outliers.

6.4.6 Association between the habitual consumption of (poly)phenol-rich products and general daily habits

No bivariate normality was observed between the possible pairs of general daily habits and the general dietary habits variables. Numerous outliers were also detected by visual inspection of scatter plots. A Spearman's correlation test was conducted to analyse the relationships between the variables in this category. There were five statistically significant associations detected. The bowel movement was positively correlated with the consumption of herbs and spices, r_s (55) = 0.324, p = 0.012 (Figure 6.8A). Alcohol was positively correlated with the consumption of fruits, r_s (55) = 0.308, p = 0.015 (Figure 6.8B); fruits and vegetables, r_s (55) = 0.289, p = 0.023 (Figure 6.8C); and herbs and spices, r_s (55) = 0.333, p = 0.008 (Figure 6.8D); and negatively correlated with the time a subject had been a tea consumer, r_s (50) = - 0.283, p = 0.042 (Figure 6.8E). The positive association identified between the consumption of alcohol and that of fruits and vegetables could be explained by the positive statistically significant correlation between the consumption of alcohol and that of fruits, since no association was detected between the consumption of alcohol and the consumption of vegetables.

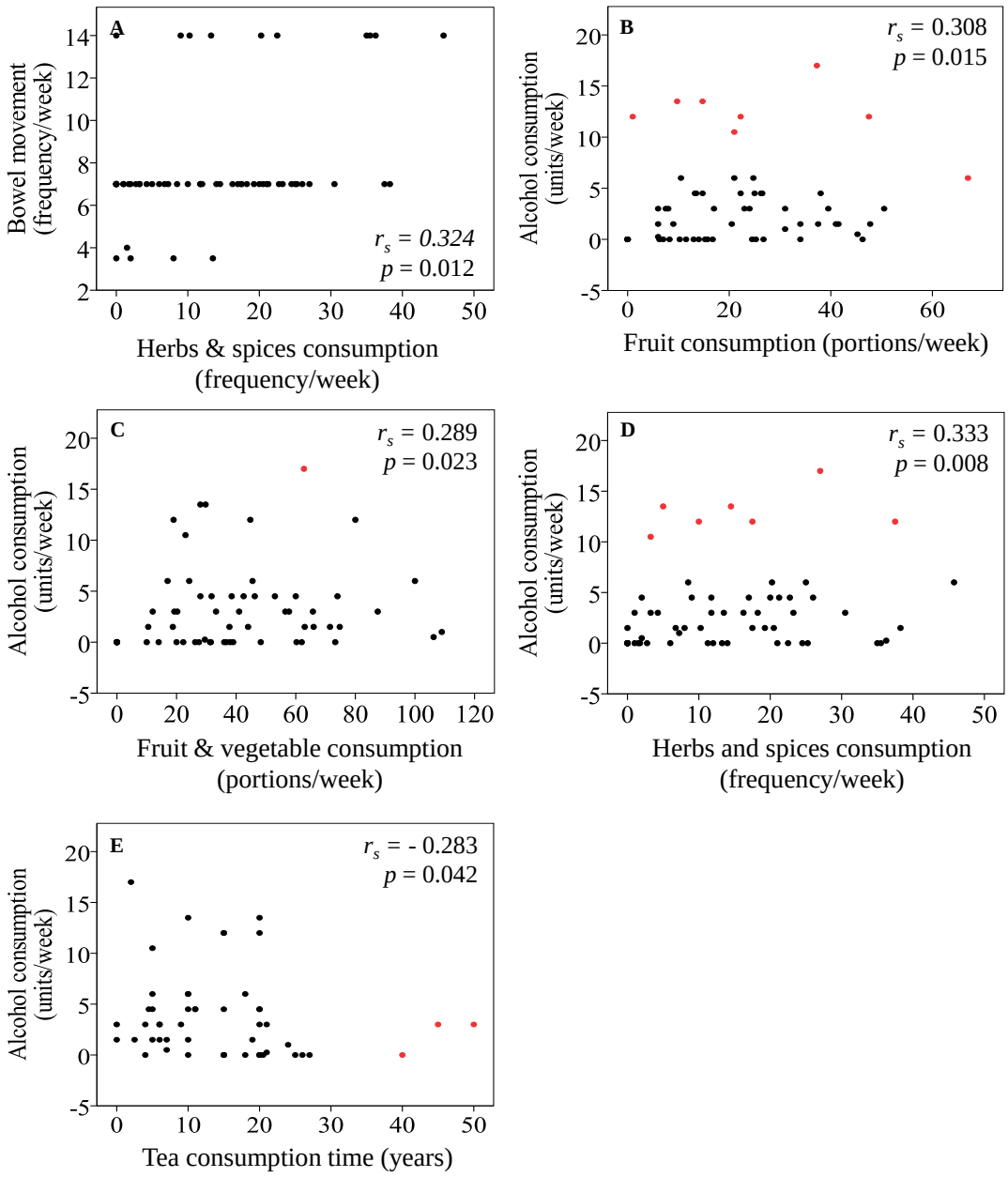


Figure 6.8: Statistically significant associations between the (poly)phenol-rich dietary habits and daily habits variables. Only few significant associations were revealed and most of them included the variable of alcohol consumption. The red data points indicate distinct and ambiguous outliers.

6.5 Discussion and Conclusion

In the present chapter, the impact of a habitual consumption of coffee and other (poly)phenol-rich products on the anthropometrics and the biomarkers of health of interest, the influence of anthropometrics on the same biomarkers, as well

as the associations between biomarkers themselves were investigated in the study population. In a first stage, the relationship between two variables was assessed by conducting simple parametric and non-parametric tests of correlation. As a correlation between two variables may be masked or significant when analysed by simple correlation tests, multiple regression was used in a second stage in order to adjust for the simultaneous effect of selected risk factors for cardiovascular disease, namely the age, gender and waist-to-hip ratio. The waist-to-hip ratio was favoured over the BMI as it is a better indicator for abdominal adiposity. Unfortunately, due to the relatively small sample size when compared to epidemiological studies, the correction was limited to three confounders. Adjustment for further variables, such as the physical activity, the alcohol consumption and physiological measures may have been included; however, this would have resulted in a decreased statistical power. Multiple regression analysis was performed when in the pair analysed, one of the variables was a biomarker of health, this is, for the biomarkers vs. biomarkers, biomarkers vs. anthropometrics, biomarkers vs. general daily habits and biomarkers vs. (poly)phenol-rich dietary habits pairs. Some variables were subject to transformation in order to meet the conditions for running the analysis. Overall, fewer significant associations were identified upon multiple regression analysis, when compared to the bivariate analysis.

The lower homocysteine-to-cysteine and higher glutathione-to-cysteinylglycine ratios observed in the female population could suggest that females have a higher activity of cystathionine β-synthase and/or cystathionine γ-lyase as well as a higher activity of γ-glutamyltranspeptidase, when compared to men. A possible regulator of these enzymes would be estrogen, which would be in line with a report by Dimitrova et al. (2002). When Mangoni et al. (2013) reported the substrate-to-product ratios between homocysteine and cysteine and between glutathione and cysteinylglycine, data were not separated between gender, and no comparision can be made with the results obtained in the present research. From the substrate-to-product ratios tested, only the cysteinylglycine-to-cysteine ratio decreased with the age, suggesting a lower enzymatic activity. Although a decreased hepatic activity with the age could be the cause of this observation, this was the only substrate-to-product ratio decreasing with age and few subjects were in the higher age range. To test this hypothesis, further subjects in the older age range would need to be tested.

Numerous significant associations with the fasting concentration of total circulating aminothiols were detected. Some of the associations identified with total cysteine (i.e. weight, BMI, waist and hip circumferences, time a subject had reported to be a consumer of coffee) could suggest the following. Subjects likely to have a higher body fat content have a higher concentration of total cysteine and a link between the metabolism of lipids and cysteine may explain the association, which is consistent with previous reports by Elshorbagy et al. (Elshorbagy et al., 2008, Elshorbagy et al., 2009). The link between the metabolism of amino acids and that of lipids is the acetyl coenzyme A. The synthesis of the acetyl coenzyme A requires cysteine and once synthesized, it can be converted to fatty acids. Therefore, higher amino acids level would lead to more elevated fatty acids production. The time a subject had reported to be a consumer of coffee, but not any other variable of coffee or other (poly)phenol-rich product consumption was associated with the concentration of cysteine, suggesting little to no influence of (poly)phenols on this association. The positive association between cysteine and homocysteine observed in the present study is consistent with previous researches. Moat et al. detected a positive relationship between cysteine and homocysteine for subjects with concentrations of total homocysteine < 20 μM (Moat et al., 2001). Of note, the healthy subjects had an average age of 42 years old, which is 1.5 times that obtained in the present study (28 ± 8 years old). In a later study by Özkan et al., a similar outcome was reported for healthy subjects, however, the comparison with these reported data needs to be regarded carefully, as the contribution of other variables was not considered (Özkan et al., 2002). Finally, cysteine was positively a uric acid, and was negatively associated with the cysteinylglycine-to-cysteine ratio. Cysteine is a precursor of glutathione, taurine and by extension, of uric acid. Due to the lack of association between cysteine and glutathione, the data obtained suggest that in case of higher levels of cysteine, rather than the production of glutathione being stimulated, there is a shift towards the production of uric acid, putting subjects at a higher risk of developing hyperuricaemia.

The concentration of total homocysteine was positively associated with the BMI, which was also observed by Jacques et al. (2001) and Panagiotakos et al. (2005). In the present study, the time a subject had reported to be a consumer of coffee, the weekly coffee consumption, the coffee level and the intake of (poly)phenols through coffee preparations were associated with higher

concentrations of homocysteine. These associations suggest a homocysteine-rising effect from coffee components. Since the adjusted regression analyses did not reveal any significant asociations between individual coffee brews (e.g. espresso, latte, turkish) it was not possible to identify a particular influent beverage or specific component on the aminothiols. The concentration of circulating homocysteine has been reported to be influenced by the intake of food (Grubben et al., 2000, Jacques et al., 2001, Özkan et al., 2002). However, this observation cannot be generalized for all foods. Grubben et al. reported that the ingestion of higher amounts of dietary protein methionine, a precursor to homocysteine, did not influence the fasting concentration of homocysteine (Grubben et al., 2000). Upon analysis of samples from the Hordaland study, Nygard et al. identified a positive association between homocysteine and the consumption of caffeinated coffee brews (Nygard et al., 1997). Of note, the The Hordaland study population consisted of healthy adults aged 40 - 67 years old, which is higher than the average in the present study. The same was observed by other groups (Grubben et al., 2000, Urgert et al., 2000). Both referenced studies had a crossover design and in both studies, the amount of coffee administrated was high, 1 L, a volume which is likely to be consumed by only a minority of the coffee-consuming population. In the study by Grubben et al. (2000), unfiltered coffee was given to the study participants for two week and in the study by Urgert et al. (2000), filtered coffe was given for a period of four weeks. In the present study, the time a subject had reported to be a consumer of coffee, the amount of coffee consumed weekly, the coffee level and the (poly)phenolic intake from coffee preparations, but not any other reported (poly)phenol-rich product, were found to be associated with the concentration of total homocysteine after multiple regression, suggesting that caffeine and coffee (poly)phenols could possibly contribute to the observed associations. As (poly)phenols possess antioxidant properties, it would be coherent to detect no association or a negative association, however, a higher level of (poly)phenolic content, as assessed by a method described in chapter 4, does not take into account the content of other compounds with the potential to influence the concentration of homocysteine (Daly et al., 2009, Obeid and Herrmann, 2009). The data obtained in the present study also suggest that the habitual consumption of coffee and associated compounds, specifically including (poly)phenols but not excluding other compounds, are more susceptible to have a

higher impact on homocysteine than on cysteine. Homocysteine was associated with total cysteinylglycine and the homocysteine-to-cysteine ratio, which was expected.

The concentration of total cysteinylglycine was positively associated with the systolic blood pressure, the consumption of alcohol, the weekly consumption of coffee, the coffee level, the concentration of total glutathione and the cysteinylglycine-to-cysteine ratio, and was negatively associated with the glutathione-to-cysteinylglycine ratio. Özkan et al. reported no difference in the concentration of cysteinylglycine in healthy subjects which had never consumed alcohol and which consumed alcohol (Özkan et al., 2002). The results obtained in the present study cannot be compared to this outcome, as only two categories (i.e. coffee consumers vs non-consumers) were compared, contrarily to the present study where the consumption of alcohol was assessed on a continuous scale.

The concentration of total glutathione was positively associated with the weekly consumption of coffee and the glutathione-to-cysteinylglycine ratio only. Since a higher intake of coffee and associated components was associated with increased concentrations of homocysteine, cysteinylglycine and glutathione, potential mechanisms by which coffee components could contribute to an elevation of these aminothiols could be an inhibition of the conversion of homocysteine to methionine by the betaine homocysteine methyltransferase, and/or to cystathionine by the vitamin B6-dependant cystathionine β-synthase, an inhibition of the dipeptidase responsible for the hydrolyses cysteinylglycine into cysteine and glycine, and an increased activity of the glutathione synthetase, which catalyses the sysnthesis of glutathione from γ-glutamylcysteine.

The glutathione-to-cysteinylglycine ratio was negatively associated with the consumption of alcohol, the cysteinylglycine-to-cysteine ratio and the insulin-to-glucose ratio. The cysteinylglycine-to-cysteine ratio was positively associated with the consumption of alcohol. The homocysteine-to-cysteine ratio was positively associated with the weekly coffee consumption, the coffee level, the intake of (poly)phenols from coffee preparations, insulin and the insulin-to-glucose ratio, and was negatively associated with the consumption of supplements. Although substrate-to-product ratios in the transsulfuration pathway were used previously as an indicator of enzymatic activity and some were subject of analyses in the present study, the earlier described homocysteine-to-cysteine ratio (Mangoni et al., 2013)

and the newly introduced cysteinylglycine-to-cysteine ratio do not seem to be appropriate indicators of the enzymatic activity responsible for the corresponding reactions. In the transsulfuration pathway, cysteine can be obtained from the hydrolysis of cystathionine and the breakdown of cysteinylglycine in reactions catalyzed by the cystathionine γ-lyase and a peptidase, respectively (Himmelfarb et al., 2002). Measuring the above-mentioned ratios would therefore not distinguish between the cysteine obtained by either reaction.

Glucose was positively associated with the weight, the BMI, both the waist and the hip circumferences, the consumption of herbs and spices and insulin. The latter was positively associated with the BMI and the insulin-to-glucose ratio. The insulin-to-glucose ratio was positively associated with the BMI. The weight, BMI, waist and hip circumferences are all direct or indirect measures of body fat and therefore the associations observed with the ciculating concentrations of glucose and insulin are consistent with the link between three metabolic pathways. In a normal situation, upon elevation of glycemia, the secretion of insulin by the pancreatic β-cells increases. Consequently, the endogenous glucose production by gluconeogenesis and glycogenolysis is inhibited and its uptake, utilization and storage in muscle and fat is stimulated (Saltiel and Kahn, 2001). During adipocyte lipolysis, triglycerides are hydrolysed into glycerol and fatty acids. While glycerol is converted to glyceraldehyde phosphate, an intermediate in both gluconeogenesis and glycolysis, fatty acids are subject to β-oxidation and enter the Krebs cycle in form of acetyl coenzyme A to contribute to the production of ATP (Campbell and reece, 2002). In addition, circulating free fatty acids inhibit the insulin secretion and the glucose uptake in the muscle and stimulate the endogenous glucose production. Since the glycaemia is a result of the balance between the glucose anabolic and catabolic pathways, a hypothesis to explain the observed associations would be that in healthy subjects with higher levels of body fat also have higher levels of free fatty acids, which action overpowers that of insulin and thus resulting in a higher glycaemia. The positive association between uric acid and the weight, the weekly consumption of coffee, the coffee level, the intake of (poly)phenols from coffee preparations and the consumption of vegetables and the negative association with the intake of (poly)phenols from cocoa beverages, suggest a negative effect of coffee polyphenols on the circulating concentrations of uric acid. In two earlier studies, a negative association between uric acid and the consumption of coffee, but not tea,

were reported (Choi and Curhan, 2007, Kiyohara et al., 1999). In the referenced studies, the population size was larger, included a broader population age with an older age average and more subjects were representing the higher intake of coffee. These factors may contribute to a different outcome.

Further significant associations, which did not involve the biomarkers of health and whose analysis did not include adjustment for age, gender and waist-to-hip ratio, were also identified. The time a subject had reported to be a consumer of tea consumption was negatively associated with the systolic blood pressure and the consumption of alcohol. The latter was positively associated with the consumption of fruits, fruits and vegetables and herbs and spices. There is no logical explanation to the remaining significant associations with the consumption of alcohol. These could, nonetheless, be due to a positive association with a different variable not taken into account in the statistical analyses.

No association was detected between the frequency of the bowel movement and the biomarkers of health and this could perhaps be explained by the fact that with a shorter transit time, toxic as well as health-promoting compounds remain for less time in the gastrointestinal tract, and therefore fewer amounts of either would be absorbed. This question may be attempted to be answered in an animal model, by comparing the effect on biomarkers of health of an exposure to "toxic" compounds and to compounds with protective properties. The gastrointestinal tract playing a central role in the absorption and metabolism of (poly)phenolic compounds, the analysis of the microbiota present in healthy subjects and subsequent association testing with biomarkers of health would therefore be of major interest to investigate the influence of populations and their density on the absorption and metabolism of (poly)phenols, as well as on biomarkers of health.

Overall, the variables of coffee consumption (i.e. time of consumption, weekly consumption, coffee level and (poly)phenolic intake) whether combined or individually, were linked to higher levels of total aminothiols, the homocysteine-to-cysteine ratio and uric acid. The variables of tea consumption (i.e. time a subject had reported to be a consumer of tea and weekly consumption) were only associated with lower levels of cysteinylglycine. These could possibly be explained by a higher cleavage rate of cysteinylglycine into cysteine and glycine, as judged by the negative correlation between the consumption of tea and the cysteinylglycine-to-cysteine

ratio, prior to adjustment for age, gender and waist-to-hip ratio. Despite these results, it would be erroneous to conclude with certainty that the consumption of those beverages is responsible for the increase or decrease of the affected variables. Further predictor variables would have to be adjusted for. The lack of associations with individual brews could be due to the fact that not all subjects were regular consumers of all the suggested preparations, thus resulting in a narrower range of consumption for particular brews, when compared to the estimated polyphenolic intake. The advantage of the latter, is that for each reported brew, an index representative of the polyphenolic content was attributed (see section 4.4.3), which allowed a non-null value for each participant on a broader scale and consequently no statistical loss of power.

In general, the lack of significance of some relationships may be explained by the fact that the population for the present study was relatively young and in general good health, resulting in less marked variations in the selected biomarkers. In the present study, the compounds of coffee for which the attention was brought to were (poly)phenols. Other compounds, which include the cholesterol-raising diterpenes kahweol and cafestol, were not considered for the characterization of the beverages reported in the food frequency questionnaires completed by each participant. Furthermore, a positive association has also been reported between caffeine and homocysteine (Jacques et al., 2001). These compounds may thus explain some associations, such as the positive relationship between the level of (poly)phenols ingested with coffee preparations. Finally, for certain pairs tested, different outcomes have been reported in the literature. The main contributors to this difference are a diverging size and characteristics of the study population, a different study design and different analytical procedures. These discrepancies make the comparison with published work challenging. Therefore, the observations of the present study cannot be extrapolated to a population that does not match the selection criteria of the present study.

Chapter 7
Bioavailability of coffee chlorogenic acids and circulating levels of selected biomarkers of health, and the influence of habitual consumption of coffee and other daily habits

7.1 Abstract

In the present chapter, the high inter-individual variation observed in the metabolism of coffee phenolics (Renouf et al., 2010b) was aimed to be exploited for the identification of subjects with good and poor absorption and metabolism of coffee chlorogenic acids. The absorption of coffee chlorogenic acids within and between individuals, assessed by a colleague (Mr. Nicolai U. Kraut), was not consistent between the completed visits, as shown by high coefficients of variation between visits in the urinary excretion of chlorogenic acid-derived metabolites for some of the participants. (Poly)phenols have been reported to have beneficial effects on health, with a reduction of risk of developing some inflammation-derived degenerative diseases, however, the metabolic effect of any compound depends on how well these are absorbed. The impact of the absorption of coffee chlorogenic acids and its intra-individual variation on selected biomarkers of health was thus assessed, to evaluate whether subjects with a higher absorption of coffee chlorogenic acids have a better profile of selected inflammation and cardiovascular health biomarkers. Also, in order to assess whether the habitual consumption of coffee and some lifestyle habits have any impact on the absorption of coffee chlorogenic acids and its intra-individual variation, the relationships between the descriptive variables were assessed. As a measure of coffee chlorogenic acids absorption, six major derived metabolites were quantified in urine up to 36 hours post-consumption of a provided coffee beverage. Age- and/or gender-adjusted analyses only revealed few significant associations when the chlorogenic acid-derived metabolites were analysed together as a combined average, as opposed to when the metabolites were regarded individually. The habitual consumption of coffee, whether analysed by the

volumes or the (poly)phenols ingested, was related to a lower combined appearance of chlorogenic acid-derived metabolites, and when individually analysed, mainly with a lower urinary excretion of dihydrocaffeic acid 3-*O*-sulfate. Little influence of daily habits was detected on the absorption and metabolism of coffee chlorogenic acids. Also, gender, more than the age, was a more important determinant of the relationships of interest. Overall, the data suggested that a habitual consumption of coffee reduces the bioavailability of selected chlorogenic acids, that general daily habit have the potential, however little, to influence the bioavailability of chlorogenic acids and further researches targeting the colonic microbiota population may help understanding the link between the presence of certain chlorogenic acid-derived metabolites and the inflammation and cardiovascular status in subjects.

7.2 Introduction

Evidence for the beneficial role of (poly)phenols in the prevention of degenerative diseases has been reported (Manach et al., 2004, Scalbert et al., 2005, Vauzour et al., 2010, Chong et al., 2010, Higdon and Frei, 2006, Andersen et al., 2006) and the consumption of (poly)phenol-rich products has been encouraged. The bioactivity of a compound, however, does not only depend on the amount ingested, but also on its bioavailability (Williamson and Manach, 2005), which in turn is influenced by numerous determinants, including the structure of the compound, its physicochemical properties, the food matrix and host factors such as the age, gender, medication, health status or microbial populations in the gastrointestinal tract (Manach et al., 2004, D'Archivio et al., 2010, Stalmach et al., 2014, Bohn, 2014, Shivashankara and Acharya, 2010).

(Poly)phenols and hence chlorogenic acids are processed as xenobiotics and therefore not stored in the organism. Upon release from the ingested food matrix, (poly)phenols undergo digestion in the stomach, under the combined action of pepsin, peristaltic movements and a low pH, and in the small intestine, where the increase of pH activates the enzymes secreted by the pancreas and bile (Bohn, 2014, van Duynhoven et al., 2011). While aglycones are readily absorbed by the enterocytes, the majority of the (poly)phenol esters first need to be hydrolysed, and

approximately 70 % of these structurally more complex (poly)phenols that are not absorbed in the small intestine pass through to the colon (Stalmach et al., 2010, Renouf et al., 2010b, Crozier et al., 2009). The absorbed compounds undergo phase I and II metabolism. Phase I metabolism, mainly consisting of a reduction, oxidation, hydrolysis or hydration, increases the hydrophilicity of the compounds and prepares them for phase II reactions by either the addition or the exposure of functional groups (Bohn, 2014, Scheepens et al., 2010). Phase II metabolism primarily includes sulfation, methylation and glucuronidation, and occurs in the intestine in the first place, where glucuronosyltransferases and sulfurotransferases have been identified. Once in the enterocyte, the compounds may be transported back into the intestinal lumen or into the blood. In the circulation, the (poly)phenol-derived metabolites are distributed to the target tissues and organs, where they are available for further metabolism and/or biological activity. In the liver, phase I and II metabolism also takes place. The compounds which are not absorbed or which have been re-excreted into the intestinal lumen via the enterohepatic circulation (i.e. bile) or pancreatic secretions are subject to the enzymatic activity of the intestinal microbiota, which has the ability to deglycosylate, dehydroxylate, demethylate, deconjugate and hydrolyse the compounds amongst other properties. Finally, the excretion of the (poly)phenol-derived metabolites occurs via the urine, mainly, and the faeces (Bohn, 2014). The pharmacokinetics and pharmacodynamics of a compound will depend on the conditions of each stage of this complex chain of events, often abridged ADME for the absorption, distribution, metabolism and elimination processes.

Coffee is highly consumed worldwide and is rich in chlorogenic acids, which are esters formed between one molecule of quinic acid and one or more hydroxycinnamic acid(s). The contents of chlorogenic acids can reach 422 mg for a single espresso serving (Crozier et al., 2012). The three predominant chlorogenic acids in processed coffee are 5-caffeoyquinic acid and its two isomers, namely 3- and 4-caffeoylquinic acid. These three isomers can represent approximately up to 74 % of the total chlorogenic acids present in coffee, as recently reported by Stalmach et al. (2014).

From previous *in vivo* human studies, the analysis of plasma and urine of subjects with an intact colon and the ileal effluent and urine of ileostomy patients has allowed the identification of the absorption sites of a number of chlorogenic

acids and their derivatives (Stalmach et al., 2009, Stalmach et al., 2010). *In vitro* studies have helped understanding some mechanisms involved in the metabolism of ingested chlorogenic acids and their derivatives (da Encarnação et al., 2014, Farrell et al., 2011, Plumb et al., 1999, Scherbl et al., 2014) and in their protective effects on health (Del Rio et al., 2013). Epidemiological studies have suggested beneficial effects of coffee consumption in regards to type 2 diabetes mellitus (van Dam and Hu, 2005, Oba et al., 2010), cardiovascular (Andersen et al., 2006, Mineharu et al., 2011) and inflammation health (Andersen et al., 2006) with the intake of coffee, but to date, no research has investigated the link between the absorption and metabolism of coffee chlorogenic acids and the health status in humans. The aim of the present chapter was thus to assess the impact of the absorption and metabolism of major coffee chlorogenic acids in health human adult subjects on selected biomarkers of health measured in fasting plasma. The influence of habits of consumption of coffee and other daily habits on the bioavailability of coffee chlorogenic acids was also investigated. The novel perspective for studying the health effects of chlorogenic acids was to exploit the previously reported inter-individual variation in the metabolism of coffee phenolics (Renouf et al., 2010b) to rank the subjects from highest to lowest absorption and compare their profile of selected health biomarkers.

7.3 Materials and methods

7.3.1 Data sets and biological samples analysed

For the analyses of the present chapter, only the data sets of subjects which completed the three possible visits (n = 40) were included. However, from the 40 participants, four sample sets were excluded. One of the excluded participants did not have any blood taken on the first visit, due to the fear of needles. The second excluded participant had blood taken on each visit, however, the participant had eaten one banana shortly before the first visit. The third and fourth excluded participants did not collect the urine sample in full, as self-reported.

7.3.2 Biomarkers and metabolites of interest

The biomarkers of health used for the analyses of the present chapter were the total aminothiols cysteine, homocysteine, cysteinylglycine and glutathione, glucose, insulin, the insulin-to-glucose ratio and uric acid. All were quantified in fasting plasma, as described in chapters 3 and 5. The thiol substrate-to-product ratios described in chapter 6, namely the glutathione-to-cysteinylglycine, the cysteinylglycine-to-cysteine and the homocysteine-to-cysteine ratios, were not part of the analyses, as these were judged to be insufficiently good indicators of the enzymatic activity within the transsulfuration pathway. In the transsulfuration pathway, cysteine can be obtained from the hydrolysis of cystathionine and the breakdown of cysteinylglycine in reactions catalyzed by the cystathionine γ-lyase and a peptidase, respectively (Himmelfarb et al., 2002), so that measuring the homocysteine-to-cysteine and cysteinylglycine-to-cysteine ratios does not distinguish between the cysteine obtained by either reaction.

As a reflection of the absorption and metabolism of coffee chlorogenic acids, a selection of chlorogenic acid-derived metabolites were quantified in urine. These were analysed by liquid chromatography linked to mass spectrometry (1200 series Agilent Technologies, Berkshire, UK) in the urine of each participant following the consumption of coffee. The latter consisted of 4 g "Green Blend" Nescafé instant Coffee (Nestlé) dissolved in approximately 300 mL boiling water. The subjects then collected the totality of the urine produced from 0 - 4, 4 - 8, 8 - 12, 12 - 24 and 24 - 36 hours after the consumption of the beverage in separate containers. As notified in chapter 4, all the urine samples were processed and analysed by a colleague PhD student, Mr. Nicolai U. Kraut, and details other than the relevant data for the present chapter are not discussed in this manuscript. Six major urinary metabolites were identified in preliminary analyses by Mr. Nicolai U. Kraut. These were dihydrocaffeic acid-3-*O*-sulfate, feruloylglycine, vanilloylglycine, ferulic acid-4-*O*-sulfate, dihydroferulic acid-4-*O*-sulfate and dihydroferulic acid and were therefore the metabolites of interest in the present study and chapter. For each subject, the chlorogenic acid-derived urinary metabolites were analysed combined and individually. For the analyses with the combined metabolites, the average of each chlorogenic acid-derived urinary metabolite was first summed for each visit. The

values obtained for the three completed visits were then averaged to obtain the variable referred thereafter as the "averaged sum of metabolites". For the analyses of the individual metabolites, the average of each chlorogenic acid-derived urinary metabolite was calculated from the three completed visits. All amounts of excreted urinary metabolites are the average ± standard deviation and expressed in µmol, unless otherwise stated.

7.3.3 Impact of the absorption and metabolism of coffee chlorogenic acids and its intra-individual variation on selected biomarkers of health at fast

To assess the impact of the absorption and metabolism of coffee chlorogenic acids and the intra-individual variation on the general inflammation and cardiovascular health status, simple correlation tests were carried out, followed by age and/or gender adjustment. The simple relationship between the averaged sum of metabolites or the individual metabolites and each biomarker of health was first investigated. As an indicator of the magnitude of intra-individual variation for each subject who completed three visits, the averaged coefficient of variation was obtained from the division by the averaged sum of metabolites of the corresponding standard deviation. The result was then multiplied by 100 to obtain a percentage. Simple correlation tests were then carried out between the coefficients of variation of the combined metabolites or the individual metabolites and each biomarker of health.

7.3.4 Influence of habitual coffee consumption and general daily habits on the absorption and metabolism of coffee chlorogenic acids and its intra-individual variation

The influence of habitual coffee consumption and the influence of general daily habits on the absorption and metabolism of coffee phenolics was first investigated by carrying out simple correlation tests. In a further stage, the analyses were adjusted for the age and gender of the subjects. The information regarding the habits of coffee consumption included details on the time in years a subject had declared to be a coffee consumer, the weekly consumption of coffee, the coffee level

of coffee consumption variable ("coffee level") and the intake of (poly)phenols from coffee preparations ("coffee polyphenolic level"). The information regarding the general daily habits included details on the consumption of alcohol, the physical activity habits ("physical activity score") and the weekly frequency of bowel movements. The information was obtained by analysis of the food frequency questionnaire completed by each participant, as described in chapters 4 and 5.

7.3.5 Statistical analyses

The simple correlation tests carried out consisted of either a parametric Pearson's or its non-parametric equivalent Spearman's correlation test. Bivariate normality was assessed by a Shapiro-Wilk test and the presence of outliers by visual inspection of scatter plots. Outliers were included in the statistical analyses unless these were assessed to be non-genuine unusual points. If bivariate normality was achieved, a Pearson's correlation test was run to assess the relationships of interest. If, on the other hand, bivariate normality was not achieved and/or that one of the analysed variables was ordinal or had any outlier, a Spearman's correlation test was carried out.

The same relationships, adjusted for age and gender, were examined in a model of linear multiple regression. Since the age and the gender are two determinants of the bioavailability of dietary (poly)phenols in humans (D'Archivio et al., 2010), an initial simultaneous adjustment for the age and gender was performed for all the studied pairs. As the number of data sets available was reduced to n = 36, a risk of statistical power loss was present. The pairs for which the *p*-value was < 0.200 were therefore subjected to further analyses with separate adjustment for the age and gender. The distribution of the residuals was assessed by visual inspection of histograms and Q-Q plots. Data points were assessed as influential, when the corresponding distance of Cook D was D > 1. When influential data points were detected, the multiple regression analysis was first repeated upon exclusion of these. If the outcome differed from that obtained with inclusion of the influential data points, then, in order to reduce the effect of these on the trend of the relationship, a transformation was applied to the variable with the influential data points included. For clarity, the results only present the statistically significant detected associations.

All the statistical analyses were performed using the IBM SPSS statistics 20 software package.

7.4 Results

7.4.1 Bioavailability of coffee chlorogenic acids and variability

An average of 882 μmol caffeoylquinic acids (i.e. 3-, 4- and 5-caffeoylquinic acid) were ingested with the consumption of the provided coffee prepared with 4 g "Green Blend" Nescafé. Amongst the six urinary metabolites analysed, dihydrocaffeic acid-3-*O*-sulfate, a metabolite absorbed in the colon, was excreted in highest amounts, with an average of 64.5 ± 53.3 μmol, followed by feruloylglycine with 49.6 ± 22.7 μmol, the newly identified vanilloylglycine with 40.4 ± 30.8 μmol, ferulic acid-4-*O*-sulfate with 38.0 ± 23.0 μmol, dihydroferulic acid-4-*O*-sulfate with 28.0 ± 30.5 μmol and dihydroferulic acid with 17.7 ± 13.7 μmol (Figure 7.1). The averaged sum of metabolites ranged from 68.4 to 613.8 μmol with an average of 238.2 ± 120.4 μmol. On average, a high intra-individual variation was observed and was translated into high coefficients of variation which ranged from an average of 37 % for feruloylglycine, to as high as 54 % for dihydrocaffeic acid-3-O-sulfate. Due to the high intra-individual variability of the bioavailability of coffee chorogenic acids, it was not possible to categorise the study participants into either good or poor absorbers of chlorogenic acids, instead, the total population with three completed visits was ranked from lowest to highest absorbers of coffee chlorogenic acids and studied.

All outliers were assessed to be genuine unusual data points and therefore included in the subsequent statistical analyses. Two subjects showed a particularly high excretion of chlorogenic acid-derived urinary metabolites corresponding to 67.3 and 69.9 %, respectively, of the ingested chlorogenic acids and indicative of a high absorption. Although these values could be a result of a deviation from the imposed dietary restrictions, the values do not exceed 100 % and therefore it would be borderline to exclude the corresponding data sets under the assumption that these participants have not complied with the diet restriction.

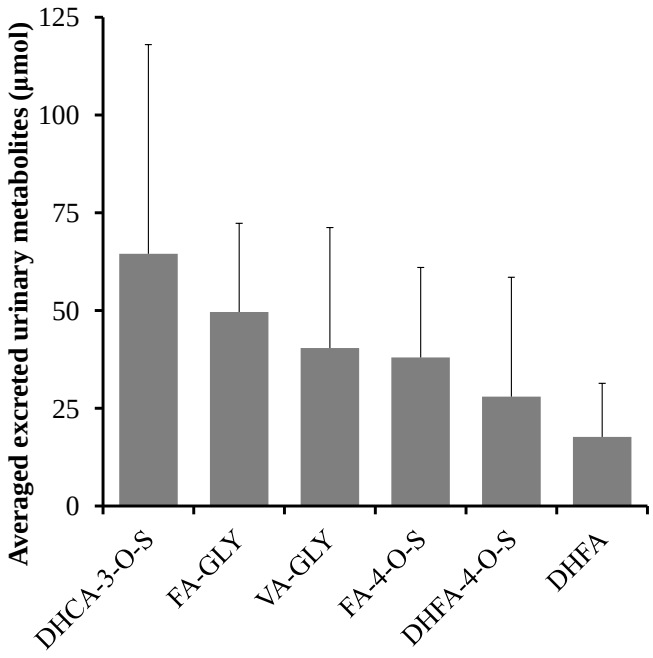


Figure 7.1: Absolute 36-hour excretion of the six major chlorogenic acid-derived urinary metabolites detected upon consumption of 4 g "Green Blend" Nescafé instant coffee (average from 36 participants). The large coefficients of variation (data not shown) and standard deviation error bars reflect a high intra- and inter-individual variation. DHCA-3-*O*-S, dihydrocaffeic acid 3-*O*-sulfate; FA-GLY, feruloylglycine; VA-GLY, vanilloylglycine; FA-4-*O*-S, ferulic acid 4-*O*-sulfate; DHFA-4-*O*-S, dihydroferulic acid 4-*O*-sulfate; DHFA, dihydroferulic acid.

7.4.2 Impact of the absorption and metabolism of coffee chlorogenic acids on the circulating levels of selected biomarkers of health at fast

Bivariate normality was not obtained by any of the biomarker vs. averaged sum of metabolites pairs tested. A Spearman's rank-order correlation test was thus run to assess the relationships between the averaged sum of excreted chlorogenic acid-derived metabolites in urine and the circulating concentration of cysteine, homocysteine, cysteinylglycine, glutathione, glucose, insulin and uric acid as well as the insulin-to-glucose ratio at fast. When the metabolites were analysed individually against each biomarker of health, bivariate normality was only observed for the cysteine vs. feruloylglycine, glutathione vs. feruloylglycine, and uric acid vs.

feruloylglycine pairs. For these same pairs, no outlier was detected and therefore a Pearson's correlation test was carried out the assess the relationship between these variables. A Spearman's rank-order correlation test was run to analyse the relationship between the remaining biomarkers and individual metabolites pairs.

Only one statistically significant association was identified when the metabolites were analysed as a combined average. This was a moderate positive association between the circulating concentration of insulin and the averaged sum of metabolites, r_s (34) = 0.343, p = 0.041. When the metabolites were regarded individually, homocysteine was weakly positively associated with the excretion of vanilloylglycine, r_s (34) = 0.334, p = 0.047; insulin was moderately positively associated with dihydrocaffeic acid-3-*O*-sulfate, r_s (34) = 0.410, p = 0.013, and ferulic acid-4-*O*-sulfate, r_s (34) = 0.464, p = 0.004; and the insulin-to-glucose ratio was weakly positively associated with dihydrocaffeic acid-3-*O*-sulfate, r_s (34) = 0.377, p = 0.023, and moderately positively associated with ferulic acid-4-*O*-sulfate, r_s (34) = 0.416, p = 0.012.

Upon adjustment for age and gender, analysis of histograms and Q-Q plots indicated that all the residuals followed a normal distribution. Some outliers and leverage points were present, but none of them was influential, as assessed by inspection of Cook's distances (D < 1). The previously detected association between the averaged sum of metabolites and insulin was not significant upon adjustment, whether it was simultaneously adjusted for the age and gender, B = 0.036, t (34) = 1.659, p = 0.107; or individually adjusted for the age, B = 2.139, t (34) = 1.594, p = 0.120; or the gender, B = 2.446, t (34) = 1.745, p = 0.090. The circulating concentration of cysteinylglycine was negatively associated with the amount of excreted ferulic acid 4-*O*-sulfate, when the age and gender were simultaneously adjusted for, B = - 0.079, t (34) = - 2.264, p = 0.031; but not when individually adjusted for either the age, B = - 0.058, t (34) = - 1.456, p = 0.155; or the gender, B = - 0.069, t (34) = - 1.957, p = 0.059. Since the analysed population (n = 36) was rather small and a simultaneous adjustment of the age and gender may result in a slight deviation of the statistical power, it is safer to consider the results obtained from separate adjustment for the age or the gender. The circulating concentration of glutathione was negatively associated with the excreted feruloylglycine upon adjustment of both age and gender, B = - 0.025, t (34) = - 2.224, p = 0.033; and when the age was adjusted for, B = - 0.024, t (34) = - 2.245, p = 0.032; but not when

only the gender was taken into account, B = - 0.015, t (34) = - 1.351, p = 0.186 (Figure 7.2, A). Furthermore, when individual adjustment for either the age or the gender was carried out on the relationships for which $p < 0.200$ was obtained upon simultaneous adjustment for the age and gender, glutathione was also related to an increase in urinary vanilloylglycine upon adjustment for the gender, B = 0.018, t (34) = 2.232, p = 0.033; but not upon adjustment for the age, B = 0.015, t (34) = 1.839, p = 0.075 (Figure 7.2, B); cysteine was related to the measured urinary vanilloylglyine when the influence of age was adjusted for, B = 0.262, t (34) = 2.095, p = 0.044; but not when gender was adjusted for, B = 0.209, t (34) = 1.731, p = 0.093 (Figure 7.2, C); and the insulin-to-glucose ratio was related to an increase in urinary dihydrocaffeic acid 3-*O*-sulfate upon adjustment for the gender, $B = 1.78*10^{-8}$, t (34) = 2.090, p = 0.044; but not for the age, $B = 1.52*10^{-8}$, t (34) = 1.707, p = 0.097 (Figure 7.2, D). The number of significant associations was relatively small, when compared to the relationships analysed. These results, however, indicate that absorption and metabolism of coffee chlorogenic acids may influence the circulating levels of specific biomarkers of health. These results also suggest that the age and gender differentially determine some of these relationships.

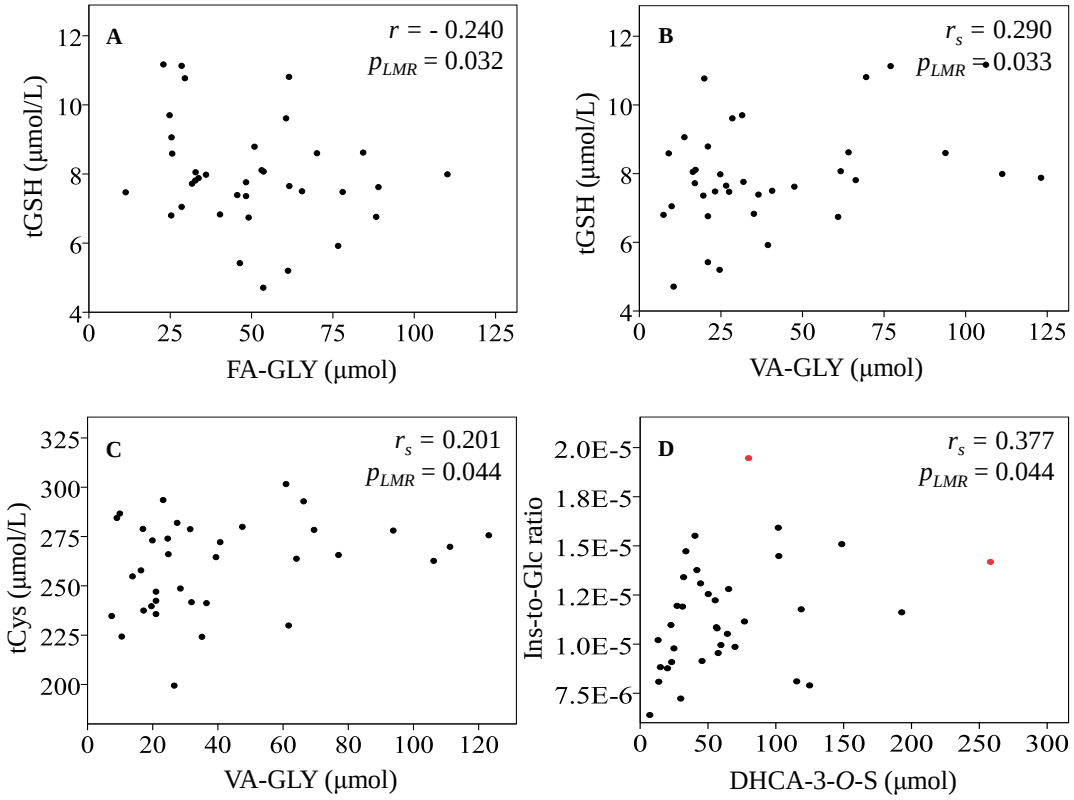


Figure 7.2: Scatter plot representation of the significant relationships between observed biomarkers of health and excreted chlorogenic acid-derived urinary metabolites, upon adjustment for the age and/or gender. The red-marked data points indicate outliers, which were, however, genuine and included in the analysis. p_{LMR}, *p*-value obtained upon multiple regression, tCys, total cysteine; DHCA-3-*O*-S, dihydrocaffeic acid 3-*O*-sulfate; FA-GLY, feruloylglycine; tGSH, total glutathione; Ins-to-Glc, insulin-to-glucose ratio; VA-GLY, vanilloylglycine.

7.4.3 Relationship between biomarkers and the magnitude of intra-individual variability of absorption and metabolism

Bivariate normality was obtained with all the biomarker vs. coefficient of variation of the excreted metabolite pairs analysed, except for the homocysteine vs. coefficient of variation of the excreted metabolite pair. A Pearson's correlation test was therefore run to assess the relationship between the coefficient of variation of the excreted metabolites and the fasting concentration of cysteine, cysteinylglycine, glutathione, glucose, insulin, the insulin-to-glucose ratio and uric acid. A Spearman's test was run to assess the relationship between the coefficient of variation of the

excreted metabolites and the fasting concentration of homocysteine. Analysis of the correlation factors and corresponding *p*-values indicated no significant association within the pairs analysed. There was no significant association detected either between the biomarkers of health and the individual urinary metabolites ($p > 0.05$).

Similar to the influence of the absorption and metabolism of coffee chlorogenic acids on the circulating concentrations of selected biomarkers of health, upon adjustment for age and gender, inspection of histograms and Q-Q plots indicated a normal distribution of all the residuals. Some outliers and leverage points were also present, but none of them was influential, as assessed by inspection of Cook's distances ($D < 1$). None of the biomarkers of health was significantly related to the intensity of the intra-individual variability of the averaged sum of metabolites, whether the adjustment for the age and gender ($p \geq 0.154$) was simultaneous or performed in separate analyses ($p \geq 0.151$) and when the intra-individual variation for each individual metabolite was regarded, only the circulating concentrations of cysteine was associated with an increased intra-individual variation of urinary vanilloylglycine, when the gender, $B = 0.292$, $t\ (34) = 2.054$, $p = 0.048$; but not the age, $B = 0.260$, $t\ (34) = 1.720$, $p = 0.095$ was adjusted for (Figure 7.3). These results suggest that the intra-individual variation in the absorption and metabolism of coffee chlorogenic acids has very little to no influence on the circulating concentrations of the selected biomarkers of inflammatory and cardiovascular status of a healthy adult individual.

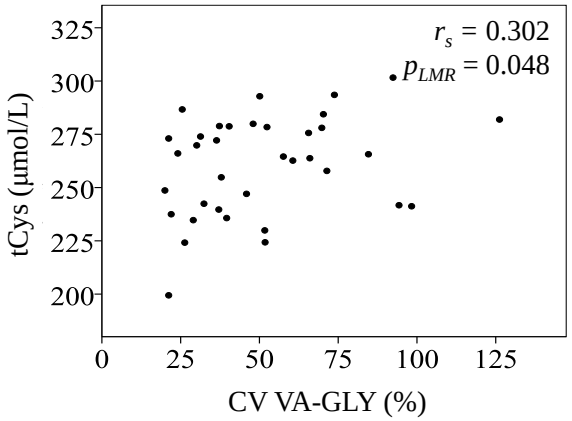


Figure 7.3: Scatter plot representation of the only significant association detected between the coefficient of variation in the excretion of metabolites and the biomarkers of health. CV, coefficient of variation; p_{LMR}, *p*-value obtained upon multiple regression, tCys, total cysteine; VA-GLY, vanilloylglycine.

7.4.4 Influence of habitual consumption of coffee on the absorption and metabolism of coffee chlorogenic acids

Bivariate normality was not obtained by any of the analysed pairs and therefore a Spearman's rank-order correlation test was run to assess the relationships of interest in this category. Although some relationships were close to achieve statistical significance, no association was detected between the averaged sum of metabolites in urine and the time one subject had been a coffee consumer, r_s (31) = - 0.053, p = 0.768; the weekly consumption of coffee, r_s (34) = - 0.283, p = 0.094; the coffee level, r_s (34) = - 0.321, p = 0.056; or the (poly)phenolic intake with coffee preparations, r_s (34) = - 0.326, p = 0.052. When the metabolites were analysed individually against each variable of coffee consumption, the weekly consumption of coffee was negatively associated with the urinary excretion of dihydrocaffeic acid-3-*O*-sulfate, r_s (34) = - 0.438, p = 0.008; and dihydroferulic acid-4-*O*-sulfate, r_s (34) = - 0.377, p = 0.023. The "coffee level" variable was also negatively associated with dihydrocaffeic acid-3-*O*-sulfate, r_s (34) = - 0.462, p = 0.005; and dihydroferulic acid-4-*O*-sulfate, r_s (34) = - 0.388, p = 0.019. The additional variable characterizing the (poly)phenolic intake from coffee preparations, namely the "coffee polyphenolic level" variable, was negatively associated with dihydrocaffeic acid-3-*O*-sulfate, r_s (34) = - 0.499, p = 0.002; and dihydroferulic acid-4-*O*-sulfate, r_s (34) = - 0.402, p = 0.015; as well as with dihydroferulic acid, r_s (34) = - 0.331, p = 0.049.

Upon simultaneous adjustment for the age and gender, inspection of histograms and Q-Q plots indicated a normal distribution of all the residuals. Some outliers and leverage points were present, and some, as assessed by inspection of Cook's distances (D > 1), were also influential data points. The data treatment in the case of influential data points is discussed in the relevant section. When adjusted for both the age and gender, the intake of (poly)phenols from coffee preparations described by the "coffee (poly)phenolic level" variable was identified to be significantly associated with the averaged sum of metabolites, B = - 1.189, t (34) = - 2.447, p = 0.020. Upon separate adjustment for the age or gender, the relationship remained significant, independently of whether the analysis was adjusted for the age, B = - 1.135, t (34) = - 2.330, p = 0.026; or for the gender, B = - 1.208, t (34) = - 2.348, p = 0.025 (Figure 7.4, A). When the analysis was adjusted for the gender, an influential data point was detected and removed from the data set for a subsequent analysis. Upon exclusion of the influential data point the outcome was not

influenced, B = - 2.145, t (33) = - 2.436, p = 0.021. A few new significant associations were also identified. The weekly consumption of coffee was negatively associated with the averaged sum of metabolites, when the influence of gender, B = - 2.677, t (34) = - 2.065, p = 0.047; but not when the age B = - 2.075, t (34) = - 1.611, p = 0.117 was taken into account (Figure 7.4, B). The "coffee level" variable was significantly negatively related to the averaged sum of metabolites, when adjusted for the gender, B = - 1.053, t (34) = - 2.115, p = 0.042; but not when adjusted for the age, B = - 0.769, t (34) = - 1.542, p = 0.133 (Figure 7.4, C).

When the impact of habitual consumption of coffee was assessed on the excreted individual chlorogenic acid-derived metabolites, the weekly consumption of coffee was associated with a decrease in urinary dihydrocaffeic acid 3-*O*-sulfate, when the age and gender were adjusted for simultaneously, B = - 1.162, t (34) = - 2.033, p = 0.05. The relationship remained significant when the gender, B = - 1.322, t (34) = - 2.349, p = 0.025; but not the age, B = - 1.110, t (34) = - 1.932, p = 0.062, was taken into account, suggesting that the age, more than the gender is a determinant of this relationship (Figure 7.4, D). The intake of (poly)phenols from coffee preparations, described by the "coffee polyphenolic" variable, was associated with a decrease in urinary dihydrocaffeic acid 3-*O*-sulfate, B = - 0.491, t (34) = - 2.203, p = 0.035 (Figure 7.4, E); and feruloylglycine, B = - 0.227, t (34) = - 2.474, p = 0.019, upon simultaneous adjustment for the age and the gender. When the age and gender were adjusted for separately, the "coffee polyphenolic" variable remained negatively associated with the urinary dihydrocaffeic acid 3-*O*-sulfate (age-adjusted: B = - 0.464, t (34) = - 2.072, p = 0.046; gender-adjusted: B = - 0.498, t (34) = - 2.169, p = 0.037), and the exclusion of an influential data point, identified when the analysis was adjusted for the gender, did not influence the outcome, B = - 1.065, t (33) = - 2.778, p = 0.009. The relationship between the "coffee polyphenolic" variable and the urinary levels of feruloylglycine was not significant upon separate adjustment for either the age or gender ($p > 0.05$). As notified in section 7.4.2, the analysed population (n = 36) was rather small and a simultaneous adjustment for the age and gender may result in a slight deviation of the statistical power. For this reason, it is wiser to consider the results obtained from separate adjustment for the age or the gender. When individual adjustment for either the age or the gender was carried out on the relationships for which $p < 0.200$ was obtained upon simultaneous adjustment for the age and gender, the weekly consumption of

coffee was associated with lower amounts of urinary feruloylglycine upon adjustment for the gender, B = - 0.555, t (34) = - 2.318, p = 0.027; but not the age, B = - 0.447, t (34) = - 1.838, p = 0.075 (Figure 7.4.F).

The time that a subject has been a consumer of coffee, as self-reported, did not have any impact on the absorption and metabolism of coffee chlorogenic acids, however, the identified relationships suggest a potential effect of a habitual consumption of coffee preparations, on the absorption and metabolism of coffee chlorogenic acids, with a lower urinary excretion being associated with higher intakes of coffee preparations (i.e. volume and (poly)phenols).

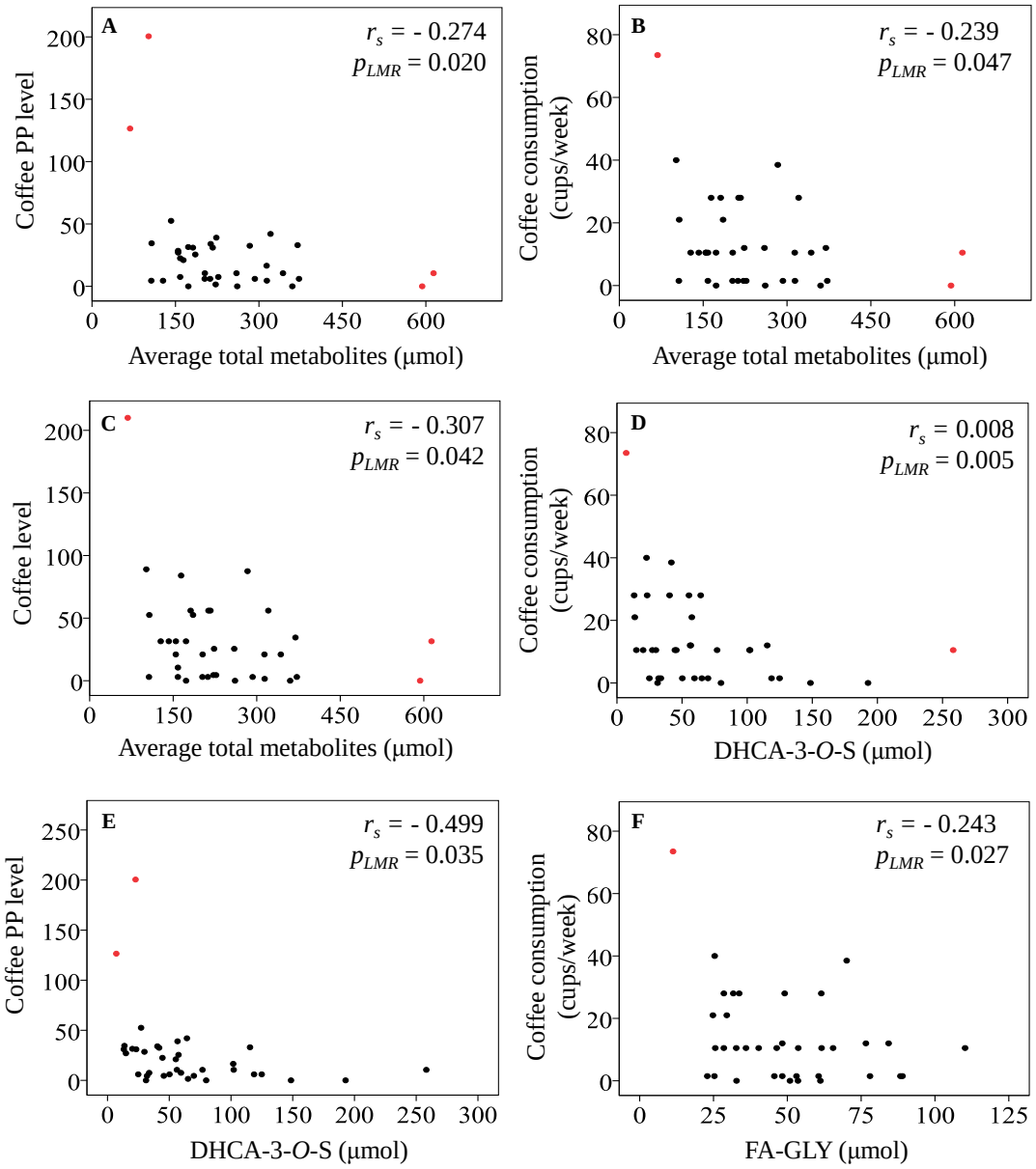


Figure 7.4: Influence of a habitual consumption of coffee on the absorption and metabolism of chlorogenic acid upon adjustment for the age and/or gender. The red-marked data points indicate the presence of outliers, which were included in the analyses, as these were considered to be genuine unusual data points. p_{LMR}, *p*-value obtained upon multiple regression, DHCA-3-*O*-S, dihydrocaffeic acid 3-*O*-sulfate; FA-GLY, feruloylglycine.

7.4.5 Influence of a habitual consumption of coffee and other (poly)phenol-rich products on the variation of the absorption and metabolism of coffee chlorogenic acids

Bivariate normality was not obtained by any of the analysed pairs. A Spearman's rank-order correlation test was therefore run to assess the relationships of interest. The correlation factors and corresponding *p*-values revealed no significant association in the pairs analysed and consequently, no indication of an influence of the habits of coffee consumption on the variation of the absorption and metabolism of tested coffee chlorogenic acids. The same was observed when the variation of each metabolite was analysed individually.

Upon adjustment for the age and gender, visual inspection of histograms and Q-Q plots indicated that all the residuals followed a normal distribution. Some outliers and leverage points were present, but none of them was identified to be influential, as assessed by inspection of Cook's distances ($D < 1$). No significant associations were detected within this category, indicating no influence of the habitual consumption of coffee on the variability of absorption and metabolism.

7.4.6 Influence of general daily habits on the absorption and metabolism of coffee chlorogenic acids

Bivariate normality was not obtained by any of the analysed pairs. Also, due to the ordinal nature of the "frequency of bowel movement" variable, the three associations were tested by carrying out a Spearman's rank-order correlation test. The results indicated no association between the absorption and metabolism of selected coffee chlorogenic acids and the consumption of alcohol, r_s (34) = - 0.193,

p = 0.258; the physical activity, r_s (33) = - 0.228, p = 0.187; or the frequency of bowel movement, r_s (34) = 0.068, p = 0.694. When regarded individually, no urinary metabolite was associated to either the consumption of alcohol, the habits of physical activity or the frequency of bowel movement ($p > 0.05$).

Upon adjustment for the age and gender, visual inspection of histograms and Q-Q plots indicated that all the residuals followed a normal distribution. Some outliers and leverage points were present, but none of them were influential, as assessed by inspection of Cook's distances (D < 1). The consumption of alcohol, the level of physical activity and the frequency of bowel movement remained unrelated to the amounts of excreted urinary metabolites, with B = - 9.454, t (34) = - 1.636, p = 0.112; B = - 5.186, t (33) = - 1.767, p = 0.087; and B = 2.204, t (34) = 0.346, p = 0.732, respectively. Since a p-value < 0.200 was obtained for the pairs that included the consumption of alcohol and the physical activity, a multiple regression with separate adjustment for the age or the gender was carried out. The outcome was not influenced for the impact of the alcohol consumption habits (age-adjusted B = - 6.222, t (34) = - 1.140, p = 0.262, gender-adjusted B = - 5.303, t (34) = - 0.884, p = 0.383) or the level of physical activity (age-adjusted B = - 4.244, t (33) = - 1.462, p = 0.116, gender-adjusted B = - 5.904, t (33) = - 1.950, p = 0.060) on the absorption and metabolism of coffee chlorogenic acids. When the impact of daily habits was assessed on the individual excreted chlorogenic acid-derived metabolites, however, the consumption of alcohol was associated with lower amounts of urinary ferulic acid 4-*O*-sulfate upon simultaneous adjustment for the age and gender, B = - 2.705, t (34) = - 2.458, p = 0.020; but not upon separate adjustment ($p > 0.05$) and the relationship was therefore not considered. When individual adjustment for either the age or the gender was carried out on the relationships for which $p < 0.200$ was obtained upon simultaneous adjustment for the age and gender, a higher level of physical activity was associated with a lower excretion of urinary dihydroferulic acid 4-*O*-sulfate, when the gender, B = - 1.163, t (33) = - 2.097, p = 0.044; but not when the age was adjusted for, B = - 0.894, t (33) = - 1.583, p = 0.123 (Figure 7.5). These results indicate little influence from the tested daily habits on the absorption and metabolism of coffee chlorogenic acids.

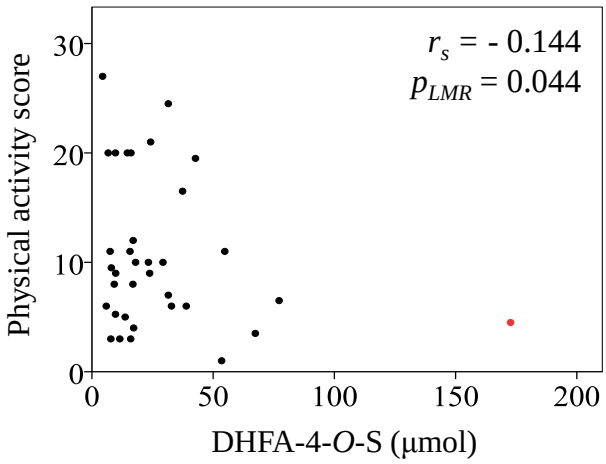


Figure 7.5: Impact of daily habits on the absorption and metabolism of chlorogenic acids upon adjustment for the age and/or gender. The red-marked data point indicates the presence of a genuine outlier and was therefore included in the analysis. p_{LMR}, *p*-value obtained upon multiple regression, DHFA-4-*O*-S, dihydroferulic acid 4-*O*-sulfate.

7.4.7 Influence of general daily habits on the variation of the absorption and metabolism of coffee phenolics

Bivariate normality was not obtained by any of the pairs analysed and all the associations were therefore tested by carrying out a Spearman's rank-order correlation test. The results indicated no association between the variation of the urinary excretion of selected coffee chlorogenic acids and the consumption of alcohol, the level of physical activity, or the frequency of bowel movement ($p > 0.05$). One outlier present in the variable of frequency of bowel movement was excluded due to the unlikelihood of the value (i.e. one bowel movement per week). When the influence of the alcohol consumption habits, physical activity and bowel was assessed on the intra-individual variation of individual urinary metabolites, the consumption of alcohol was negatively associated with the variation of excretion of dihydrocaffeic acid 3-*O*-sulfate, r_s (34) = - 0.410, p = 0.013; and that of dihydroferulic acid 4-*O*-sulfate, r_s (34) = - 0.455, p = 0.005. Also, the frequency of bowel movement was negatively associated with the variation of excretion of dihydrocaffeic acid 3-*O*-sulfate, r_s (34) = - 0.342, p = 0.041; feruloylglycine, r_s (34) = - 0.394, p = 0.017; and ferulic acid 4-*O*-sulfate, r_s (34) = - 0.345, p = 0.039.

Upon adjustment for the age and gender, visual inspection of histograms and Q-Q plots indicated that all the residuals followed a normal distribution. The relationship between the variability of the combined metabolites and the consumption of alcohol, the level of physical activity or the frequency of the bowel movement remained non-significant. During the analysis with the "physical activity" variable, an influential data point was identified (Cook's D > 1). The exclusion of the latter did not influence the outcome, however, as an important decrease of the *p*-value was observed, a subsequent analysis with inclusion of the influential data point and separate adjustment for the age and gender was performed. The results indicated the same outcome, with the relationships remaining non-significant, when the age or gender were adjusted for. When the variability of the urinary excretion was regarded individually for each metabolite, the frequency of bowel movement was negatively associated with dihydrocaffeic acid 3-*O*-sulfate, B = - 2.776, t (34) = 2.103, p = 0.043. This relationship remained significant upon adjustment of either the age, B = - 2.896, t (34) = - 2.175, p = 0.037; or the gender, - 2.797, t (34) = - 2.153, p = 0.039 (Figure 7.6, A). The frequency of bowel movement was also negatively associated with ferulic acid 4-*O*-sulfate, B = - 2.090, t (34) = - 2.112, p = 0.043, with the relationship remaining unchanged upon adjustment of either the age, B = - 2.030, t (34) = - 2.063, p = 0.047; or the gender: - 2.087, t (34) = - 2.145, p = 0.039 (Figure 7.6, B). When both the age and the gender where taken into account, no significant association between the frequency of bowel movement and feruloylglycine was identified, but due to the low *p*-value of 0.055, further investigations were carried out with individual adjustment for either the age or the gender. The results revealed a significantly negative relationship between both variables when either the age, B = - 1.890, t (34) = - 2.057, p = 0.048; or the gender, - 1.840, t (34) = - 2.011, p = 0.035 were corrected for (Figure 7.6, C).

These results may be suggestive of an influence of the frequency of bowel movement on the intra-individual variation in the absorption and metabolism of coffee chlorogenic acids, however, the range observed in the frequency of bowel movement within the study population was very narrow. Also, 44 % of the participants shared the same frequency of bowel movement. Altogether, it may not be appropriate to conclude any relationship between the influence of the frequency of bowel movement on the variation of the absorption and metabolism of chlorogenic acid.

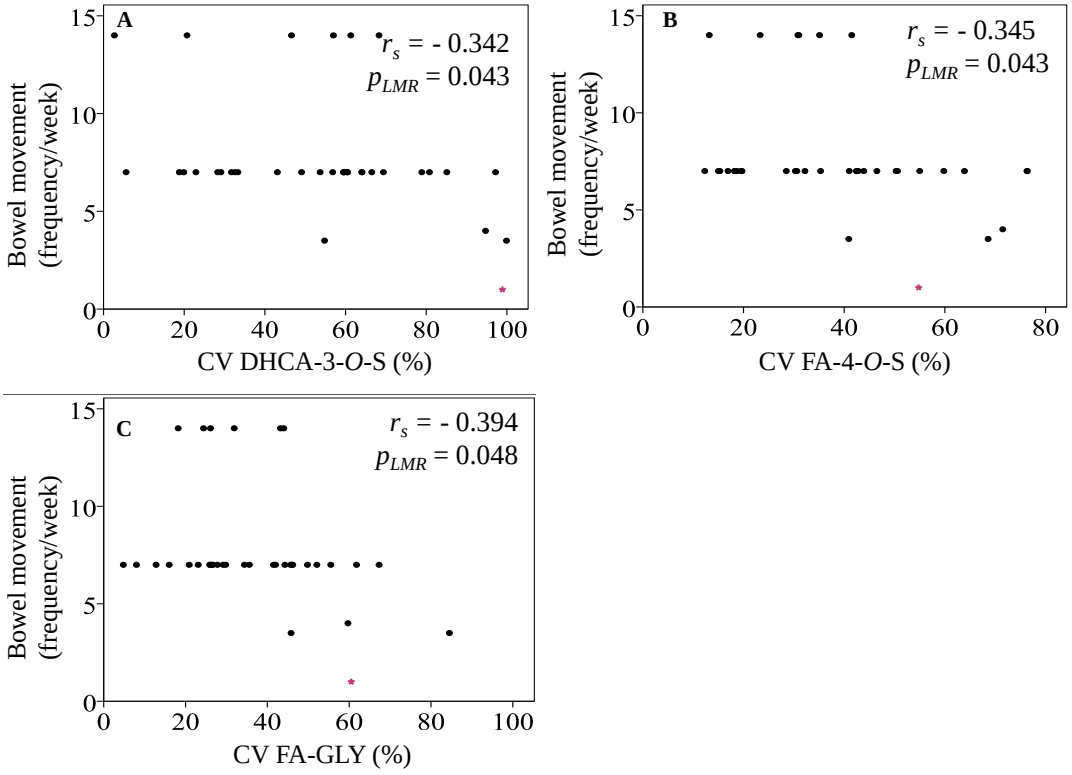


Figure 7.6: Scatter plot representation of significant relationships between selected daily habits and the variation of the absorption and metabolism of coffee chlorogenic acids. Purple-marked *, excluded outlier, due to the unlikelihood of the bowel frequency. CV, coefficient of variation; p_{LMR}, *p*-value obtained upon multiple regression, DHCA-3-*O*-S, dihydrocaffeic acid 3-*O*-sulfate; DHFA-4-*O*-S, dihydroferulic acid 4-*O*-sulfate; FA-GLY, feruloylglycine; FA-4-*O*-S, ferulic acid 4-*O*-sulfate.

7.5 Discussion and conclusion

Whether a compound has biological effects will partly depend on its bioavailability, to what extent it is absorbed and metabolized. Studying the bioavailability of compounds is therefore important in order to understand their effect(s) on the organism. In the present chapter, the influence of the absorption and metabolism of coffee chlorogenic acids on the circulating concentrations at fast of selected biomarkers of inflammation and cardiovascular health was assessed. In a study by Renouf et al. (2010b), high inter-individual variations were reported in the

bioavailability of coffee phenolics. The inter-individual variation was aimed to be exploited in the present study in order to categorise the participants into good or poor absorbers of coffee chlorogenic acids. Due to the high intra-individual variation in the excretion of coffee chlorogenic acid-derived metabolites amongst the three completed visits, the categorisation of the study population was not possible and instead it was analysed as a single group. One possible explanation for this high intra-individual variation is the diet during the study. Although the diet of each participant was limited to low-(poly)phenol products, the same diet was not imposed upon the participants during the three visits. A plethora of factors, whether endogenous or exogenous, have been reported to influence the bioavailability of (poly)phenols (Bohn, 2014) and include, but not exclusively, the food matrix (e.g. fat contents, protein contents and other (poly)phenols), the structure of the compound, the amount of food simultaneously ingested and microbiota population. The latter is very likely one major cause behind the intra-individual variations observed. Recent research has indicated that diet is able to significantly affect the human microbiota (i.e. difference in diversity and metabolic activity), even in the short-term (David et al., 2014). Similar observations had previously been reported in a study by Jaquet et al. (2009), where, upon consumption of coffee at higher doses, over a period of three weeks, modifications of the microbiota population were observed, with an increase in number of *Bifidobacterium* spp. and, for some subjects, in metabolic activity. A standardization of the accompanying diet may therefore stabilize the microbiota populations, reduce the intra-individual variation and improve the identification of good and poor absorbers of coffee chlorogenic acids. To test this, the analysis of the populations, density and metabolic activity of the microbiota present in the faeces may give a better understanding of the differential metabolism observed for coffee chlorogenic acids and other (poly)phenols.

In further analyses, the relationships between habits of consumption of coffee and some general daily habits on the bioavailability of coffee chlorogenic acids and its intra-individual variability were investigated. Simple correlations tests were complemented by multiple regression analyses with adjustment for the age and/or gender, two known endogenous determinants of drugs and (poly)phenols bioavailability (D'Archivio et al., 2010).

When the influence of the absorption and metabolism of coffee chlorogenic acids, on the circulating levels of selected biomarkers of health was assessed, no significant association was detected when the metabolites were analysed as an averaged sum, whether the analyses were adjusted for the age and/or the gender. When the relationships with individual chlorogenic acid-derived metabolites were assessed, glutathione was associated with higher urinary vanilloylglycine levels (gender-adjusted) and negatively associated with the feruloylglycine levels in urine (age- and gender-adjusted, and age-adjusted only). Cysteine was associated with higher levels of urinary vanilloylglycine (age-adjusted). Finally, the insulin-to-glucose ratio was associated with higher levels of urinary dihydrocaffeic acid-3-*O*-sulfate (gender-adjusted). Considering the number of associations tested, the significant associations were relatively few, however these are still suggestive of an influence of the bioavailability of coffee chlorogenic acids on specific biomarkers of health. All the associations detected between the selected biomarkers of health and coffee chlorogenic acid-derived metabolites involved colonic metabolites. The colon is home to about 10^{12} bacteria per gram of contents in the colon (Sekirov et al., 2010). The gastrointestinal microbiota was shown to be a determining factor for the health status of the host metabolism (Tremaroli and Backhed, 2012) and may therefore also play an important in the profile of the selected biomarkers in the present study. Since about 70 % of the chlorogenic acids reach the colon, the bacteria responsible for the catabolism of these, could very well have an influence on the general health biomarkers. This would have to be tested by simultaneous analysis of selected biomarkers of health, microbiota in the faeces and their metabolic activity on (poly)phenols.

Given the reported benefits of (poly)phenols on health, it would be coherent that a high bioavailability of such compounds would result in a lower presence of biomarkers of degenerative diseases and/or higher presence of biomarkers of "good" health. In the present study, few associations were identified. As the study population was a group of healthy adults, it is possible that smaller variations in the biomarkers were observed than if a group of subjects suffering from an inflammatory or cardiovascular condition had also been included.

A negative association between the absorption and metabolism of coffee chlorogenic acids and the intake of (poly)phenols from coffee preparations was detected upon the simultaneous or separate adjustment for the age and gender and

with or without inclusion of a detected influential data point. The weekly consumption of coffee and the intake of (poly)phenols from coffee preparations (i.e. "coffee level" and "coffee polyphenolic level" variables) were all negatively associated with the total excretion of metabolites in adjusted analyses. The "coffee polyphenolic level" variable was also associated with a lower urinary excretion of dihydrocaffeic acid-3-*O*-sulfate (age- and/or gender-adjusted), the major urinary metabolite of coffee chlorogenic acids. A higher weekly consumption of coffee was associated with a lower urinary excretion of dihydrocaffeic acid-3-*O*-sulfate (age- and gender-adjusted, and gender-adjusted only) and feruloylglycine (gender-adjusted). Although a relatively broad time of long-term coffee consumption (1 - 40 years) was observed and was likely to be sufficient to observe any effect, if there were any, the time that a subject had declared to be a consumer of coffee did not have any impact on the absorption and metabolism of coffee chlorogenic acids. This outcome could be explained by the fact that a subject that has been consuming coffee for a longer time does not necessary have a higher intake of coffee. As an example, one subject may consume three coffees per week and be a coffee consumer for 10 years, while a different subject may consume twenty-one coffees per week, and be a coffee consumer for 4 years. The consumption of coffee may have a more important effect on the second subject. The identified relationships are suggestive of a potential effect of a habitual consumption of coffee preparations, in terms of volume ingested and (poly)phenolic intake, on the absorption and metabolism of coffee chlorogenic acids, with a lower urinary excretion being associated with higher intakes of coffee preparations. In a recent research investigating the impact, although acute, of the dose of ingested chlorogenic acids with a coffee beverage on the bioavailability of coffee chlorogenic acids, a higher dose was associated with a reduced urinary excretion of chlorogenic-derived metabolites. Taking into account the previously reported data from Erk et al. (2012), Stalmach et al. (2014) suggested a reduced bioavailability of coffee chlorogenic acids upon consumption of higher levels of chlorogenic acids. The results obtained in the present study would be in line with those of Stalmach et al. (2014) and it could additionally be suggested that habitual consumption of higher amounts of coffee and stronger coffee preparations in terms of (poly)phenolic contents are associated with a reduction of bioavailability of coffee chlorogenic acids in healthy adults. The obtained results could, however, also indicate that there is a shift toward the metabolism of other metabolites that

were not measured. The negative correlation observed between the variables of coffee consumption and dihydrocaffeic acid-3-*O*-sulfate could partly be explained by the ability of coffee compounds, to inhibit the SULT1A1 and SULT1A3 (Okamura et al., 2005), the two main sulfotransferases responsible for the sulfation of dihydrocaffeic acid (Wong et al., 2010).

No association between the consumption of alcohol, the habits of physical activity or the frequency of bowel movement and the absorption and metabolism of the combined metabolites was identified. However, when regarded individually, a lower urinary excretion of dihydroferulic acid 4-*O*-sulfate was associated with a higher level of physical activity. These results indicate that although the impact may be limited, daily habits are susceptible to influence the absorption and metabolism of coffee chlorogenic acids. Since the absorption of chlorogenic acids and derived metabolites occurs in the gastrointestinal tract, a significant relationship was expected between the frequency of bowel movement and the metabolites, whether regarded as a summed average or individually. This was not the case. One possible explanation would be the relatively narrow range of frequencies observed in the study population.

Finally, the intra-individual variation in excretion of metabolites had little to no influence on the circulating biomarkers and was not influenced by the habitual consumption of coffee, alcohol, the level of physical activity or the frequency of bowel movement.

The simultaneous adjustment of the age and gender is important, however, only 40 subjects completed the three possible study visit and 4 data sets were excluded. The simultaneous adjustment for the age and gender can result in a loss of statistical power and missing significant associations. In order to overcome this issue, a separate adjustment for the age and gender was performed for the pairs for which $p < 0.200$ was obtained upon a simultaneous adjustment of the age and gender. The results revealed a number of new and interesting significant associations. The results from the present study emphasize the importance of studying the bioavailability of (poly)phenols to understand the health effects of these phytochemicals. Standardizing of the accompanying diet may reduce the intra-individual variability observed through the excreted coffee chlorogenic acid-derived metabolites. Also, a study of the colonic microbiota will be relevant to assess

whether these can explain the relationships observed between selected biomarkers of health and some colonic coffee chlorogenic acid-derived metabolites. Finally, in vitro experiments testing the ability of different coffee compounds to inhibit SULT1A1 and SULT1A3 will help understanding whether a higher consumption of coffee can explain the decrease in chlorogenic acid-derived sulfated metabolites or whether other dietary factors are also significant contributors. The focus in the present study was on coffee chlorogenic acids, however it is likely that similar relationships would be observed if other (poly)phenols were targeted.

Chapter 8
Summary, future perspectives and conclusion

8.1 Purpose of the investigations and outcome

(Poly)phenols are plant secondary metabolites playing an important role in the protection from external stresses such as UV radiation, infection by pathogenic microorganisms, or protection from herbivores and have often been associated with a reduction in the risk of developing certain chronic conditions (Crozier et al., 2006b, Manach et al., 2004, Petti and Scully, 2009). The effect of their consumption on health has thus attracted increased attention. Coffee, one of the most consumed beverages worldwide, is rich in chlorogenic acids, a family of quinic acid esters formed with one or more molecules of hydroxycinnamic acids (Clifford, 1985, Fraga, 2010). A regular consumer of coffee can easily reach a daily intake of 1 g of chlorogenic acids (Clifford, 2000), but these, like any other compound, need to be absorbed in order to have an influence on the metabolism. The importance of conducting bioavailability studies in humans is undeniable. Since the intact (poly)phenols are hardly absorbed in their unmodified chlorogenic acid form, their metabolites are most likely the compounds responsible for the biological activity. As a brief reminder, once ingested, chlorogenic acids are released from the food matrix. The first site of absorption has been reported to be the stomach, where esterase activity has been detected (Farrell et al., 2011). The compounds which are not absorbed reach the small intestine where a higher pH of 6 - 7 favours the activation of enzymes secreted by the pancreas and from the bile and thus allowing the absorption of further compounds. Finally, the chlorogenic acids which are not absorbed in the small intestine (approximately 70 %) or which have been absorbed and re-excreted into the gastrointestinal lumen reach the colon, where an abundant population of microbiota efficiently cleaves the majority of chlorogenic acids. The absorbed compounds undergo phase I and II metabolism which render the compounds more water-soluble and therefore facilitate their excretion, usually in the urine, but also in the faeces. The compounds that have not been absorbed in any of the segments of the gastrointestinal tract will be excreted via the faeces (Bohn, 2014, van Duynhoven et al., 2011). Measuring the metabolites excreted in urine, mainly,

and in faeces is thus a useful tool to evaluate the bioavailability of chlorogenic acids as these are a good reflection of the absorption and metabolism of the parent compounds. In the present thesis, the effect on health of a habitual consumption of popular (poly)phenol-rich products, with emphasis on coffee, was investigated.

The questions raised and aimed at to be answered with the present PhD thesis were described in section 1.3 (Chapter 1). No investigations on the effect of roasting or decaffeination of the free phenolic profile in coffee was previously reported, neither was the contribution of free phenolics present in a regular cup of coffee to the early appearance (i.e. up to 3 hours upon consumption of the beverage) of derived metabolites in plasma. In an attempt to answer questions 1 and 2, chapter 2 reports the analysis of six major hydroxycinnamic acids in five different soluble coffees. These were, although present in much lower amounts than chlorogenic acids (Hoelzl et al., 2010), present in higher amounts than their respective limit of detection and for some, the limit of quantification. As observed with chlorogenic acids and other (poly)phenols, processing affects the (poly)phenolic profile of edible plant-derived products (Clifford, 1999, Manach et al., 2004), and decaffeination and roasting of coffee seems to result in a lower content of free phenolic acids. Using pharmacokinetic modelling, the contribution of these same free phenolic acids present in coffee to the early presence of respective metabolites in plasma following the consumption of a regular cup of coffee was assessed to be non-significant. Additional *in vitro* work performed by Mr. Nicolai U. Kraut and former colleague Dr. Tracy Farrell with Ms. Alexandra Ryder investigating the esterase activity of pancreatic secretions and preparations of Caco-2 cells monolayers on coffee and major chlorogenic acids suggested that the hydrolysis of more complex compounds, such as chlorogenic acids, are major contributing mechanisms to the pool of plasma metabolites observed up to three hours following the consumption of the beverage. The hydrolysis of 5-caffeoylquinic acid by pancreatic esterases and 3-caffeoylquinic acid by the small intestinal brush border esterases was the most abundant. Although a minimal contribution of free hydroxycinnamic acids to the pool of corresponding free metabolites in plasma was expected, no research had previously been done on whether these were present in sufficient quantities in order to significantly contribute to the presence of derived metabolites in plasma resulting from a pre-colonic absorption.

To answer questions 3 to 7 the most appropriate methods with high repeatability and recovery were sought. Chapters 3 and 5 described the optimisation and validation of the different methods used for the recruitment of the study population using a health assessment questionnaire, the assessment of their dietary habits using food frequency questionnaire, the record of their anthropometrics, and the withdrawing, processing and analysis of the blood samples. When compared to the method of Pfeiffer et al. (1999), used as a reference for the optimisation of the aminothiol detection method, the chromatographic separation was improved. Overall, the methods specific for the biomarkers of interest had a good reproducibility and recovery, and particularly for the measurement of glucose and uric acid, were time efficient.

Question 3 was answered in chapter 4, where the recruited population was characterized in terms of the habitual consumption of coffee, tea and cocoa beverages, three popular non-alcoholic (poly)phenol-rich products. The recruited population had a weekly consumption of coffee higher than the estimated UK average, a higher total tea consumption when compared to coffee, corresponding to the estimated average consumption in the UK and a minimal cocoa consumption. Overall, in the studied population, subjects with a higher (poly)phenolic intake from a particular product were more likely to consume more of other (poly)phenol-rich products.

Question 4 was answered in chapter 5, with the characterization of the study population in terms of non-dietary daily habits, anthropometric and biomarkers of health. The study population was in general good health, as assessed by their circulating fasting levels of inflammation and cardiovascular health biomarkers. All the anthropometric features were stable over a period of at least 8 weeks and only two of the biomarkers, namely cysteinylglycine and glutathione, were not consistent over the study period. However, the obtained coefficients of variation were suggestive of a little intra-individual variation. It can thus be concluded that the tested biomarkers of health were in general stable over a period of at least 8 weeks.

Question 5 was addressed in chapter 6, where the habitual consumption of popular (poly)phenol-rich products such as coffee, tea and cocoa beverages, as well as fruits, vegetables and supplements was analysed for associations with the different inflammation and cardiovascular health biomarkers levels in plasma. From

the obtained results, it cannot be concluded that a higher consumption of (poly)phenol-rich products is associated with a better profile for the selected biomarkers of health. Overall, a higher consumption of (poly)phenol-rich products was not associated with a healthier profile of glucose metabolism, which is in line with observations from van Dieren (2009) et al., Kempf et al. (2010) and Carter et al. (2010) for the consumption of coffee, tea (mainly black) and fruit and vegetables. Yamaji et al. (2004), however, reported a lower fasting glucose for consumers consuming ≥ 5 cups coffee per day inflammation or cardiovascular health. Of note, in the present study, only 4 subjects had a regular daily coffee consumption ≥ 5 cups. Grubben et al. (2000) and later Kempf et al. (2010), reported higher levels of homocysteine and some biomarkers of inflammation, respectively, for relatively high consumption of coffee. These were observed after a daily consumption of 1 L unfiltered coffee for 2 weeks, in a healthy adult population (Grubben et al., 2000) and 8 cups filtered coffee for 1 month, in a healthy but at higher risk of developing T2DM adult population. Although the study designs differ, the results of the present study are in line with these observations and suggests that a regular lower consumption is also able to increase the levels of biomarkers of inflammation and cardiovascular health. From two combined cohort studies, De Koning Gans et al. (2010) reported a lowest hazard ratio for coronary heart disease for a daily consumption of 2.1 to 3 cups coffee, but found no association with the incidence of stroke. On the contrary, in a meta-analysis of 11 prospective cohort studies, Larsson and Orsini (2011) reported a U-shape relationship between the consumption of coffee and the incidence of stroke, with the lowest reduction of relative risk for a daily consumption of 3 - 4 cups. In regards to the habitual combined consumption of fruit and vegetables and its impact on selected biomarkers of health, no association was detected with any of the biomarkers tested in the present study. This is in agreement with a meta-analysis carrier out by Carter et al. in regards to the combined consumption of fruit and vegetables, however, a higher consumption of green leafy vegetables was associated with a lower incidence of type 2 diabetes mellitus (Carter et al., 2010). In the present study, the specific effect of habitual consumption of green leafy vegetable was not investigated and therefore, a different outcome cannot be excluded if this had been analysed.

Questions 6 and 7 were addressed in chapter 7, where the associations between variables describing the habitual consumption of coffee and the excretion of

major chlorogenic acid-derived metabolites were investigated. Although few significant associations were identified, the detected associations may suggest a possible influence of the bioavailability of coffee chlorogenic acids on specific biomarkers of health, in healthy adults. As the human gastrointestinal microbiota has shown variations shortly after dietary changes and due to the fact that these are important determinants of the host metabolism and immune system, it is suggested here that the microbiota is an important link between the bioavailability of coffee chlorogenic acids or other (poly)phenols and specific biomarkers of health. Further analyses described in chapter 7 suggested that habitual consumption of coffee at a higher level, but not the time a subject had declared to be a consumer of coffee, may reduce the bioavailability of coffee chlorogenic acids, a hypothesis that is in line with recent observations reported by Erk et al. and Stalmach et al. (Erk et al., 2012, Stalmach et al., 2014). Also, the inhibition of sulfotransferases by coffee compounds, or other habitual dietary foods correlated with the consumption of coffee but not tested in this study, may contribute to the negative association observed between the variables of coffee consumption and dihydrocaffeic acid-3-*O*-sulfate, a major colonic chlorogenic acid-derived metabolite. Finally, from the daily lifestyle habits tested, only the physical activity seemed to have an impact on the bioavailability of the coffee chlorogenic acids. The obtained data are illustrative of the complexity of the field and indicate that the bioavailability of (poly)phenols and their impact on health result from the combined action of multiple factors and compounds, which as observed in this study, do not equally affect the biomarkers of health.

The chart presented in Figure 8.1 summarizes the major significant associations between the studied variables in the present study. Not presented in the chart are the associations with gender or age and the substrate-to-product ratios of aminothiols that were assessed (chapter 6) not to be suitable indicators of the enzymatic activity of specific reactions in the transsulfuration pathway.

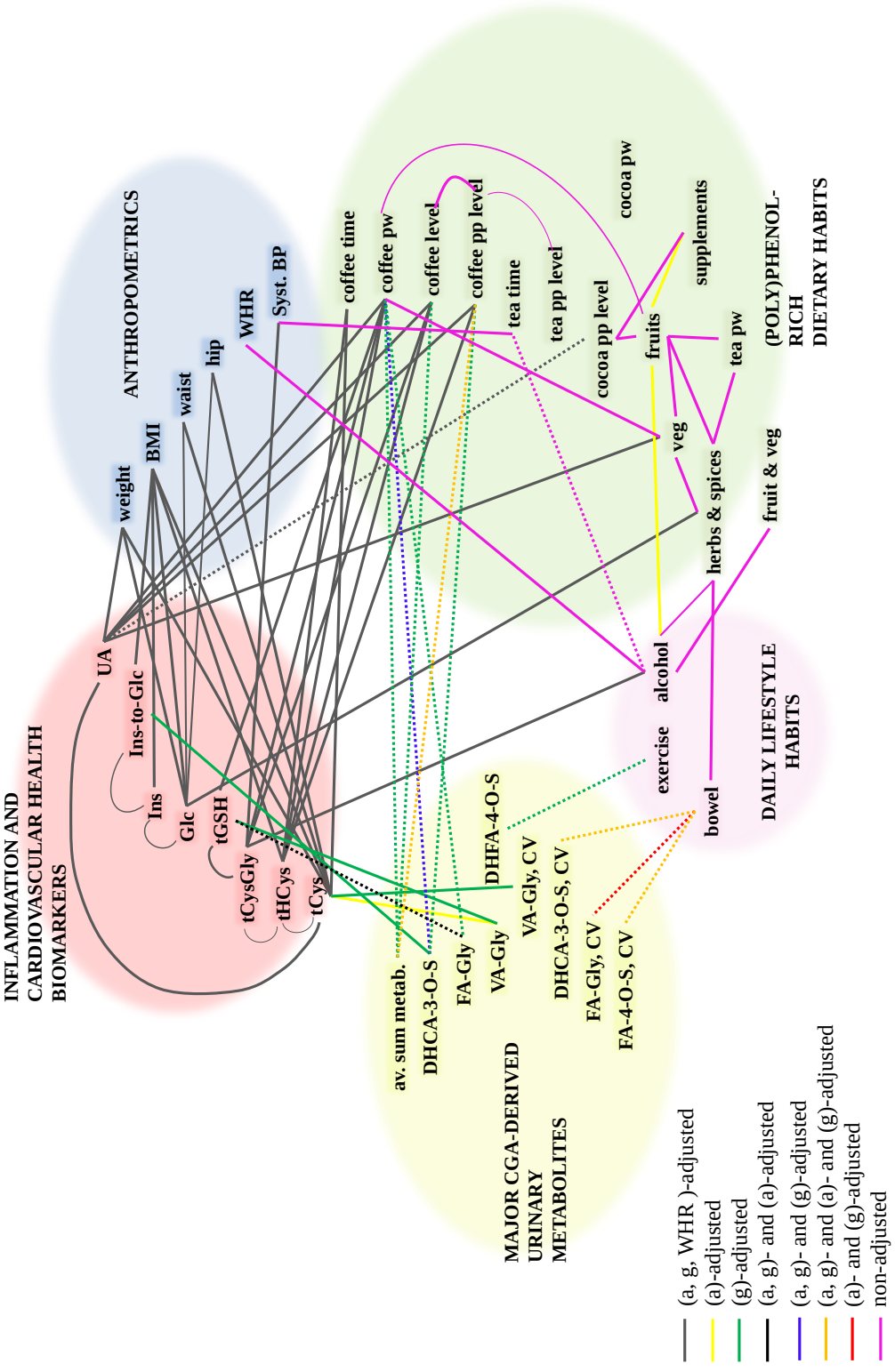


Figure 8.1: Significant associations detected in the present research study between the studied variables, upon adjustment for potential confounders. Bold lines represent positive associations and dashed lines represent negative associations. a, age; g, gender; WHR, waist-to-hip ratio.

8.2 Strengths of the present study

- A major strength of the present study is the novelty of the approach for understanding the health effects of particular (poly)phenols in the long-term, by studying the link between their habitual consumption from diet, their absorption and general health biomarkers.

- Another strength, when compared to some published works (de Bree et al., 2001, Nygard et al., 1997, Nygard et al., 1998, Rasmussen et al., 2000) is the analysis of fasting plasma for the measurement of the health biomarkers, which excludes any post-prandial effect on the target biomarkers.

- An improvement compared to previous studies, was the increase of the washout period length from 24 hours to 36 hours to ensure the elimination of metabolites resulting from the consumption of (poly)phenol-rich foods consumed prior the commencement of the study. As seen in previous pharmacokinetic studies, some participants only reach baseline values for particular phenolic acids after 24 hours following coffee consumption (raw data from Renouf et al. (2013)).

- The study population size of 62 subjects was appropriate, as determined by the sample size calculation, although a higher sample size would have allowed simultaneous adjustment of further confounders.

- The adjustment of the associations which were between the measured biomarkers of health or chlorogenic acid-derived metabolites and other variables for known confounders reduced the influence of these on the trend and strength of the relationships. However, the possibility of confounding by unmeasured factors cannot be ruled out.

- The urine samples have not been treated to hydrolyse the chlorogenic acids by any esterase, glucuronidase or sulfatase, so that the integrity of the compounds was maintained, representing the effective relative amount of metabolites. Also, the quantification was improved by the use of standards.

8.3 Limitations and adjustments that may be implemented for future studies

- Although, this may give rise to disagreements regarding ethical issues, a 100% standardized and controlled diet for the study participants with overnight stays during the study period, including the washout and urine collection period following the consumption of coffee, may help reduce the inter- and most importantly the intra-individual variation in the bioavailability of coffee chlorogenic acids and allow a better identification of subjects with good or poor absorption (of chlorogenic acids). Due to lack of facilities for an overnight stay and financial means, this was unfortunately not feasible for the present study.

- The influence of genetics on the bioavailability of could be excluded if monozygotic twins with different or not habitual consumption of coffee, dietary and lifestyle habits would be the subjects of analysis.

- The analysis of faeces for microbiota and metabolites will certainly be useful. Microbiota are a major factor in the metabolism of consumed products and studying the microbiota populations in the faeces of participants will allow the construction of a microbiota profile and assess if these represent an important link between the circulating metabolites and the general health status. The analysis of metabolites excreted in the faeces would allow an estimation of the elimination by the biliary route. Although the urinary route elimination is the predominant, adding both the urinary and biliary route elimination fractions would provide a more complete estimation of the bioavailability of coffee chlorogenic acids in the subjects.

- In vitro investigations of the ability of coffee compounds to inhibit sulfotransferases responsible for the sulfation of chlorogenic acid derivatives will contribute to the understanding of why lower levels of dihydrocaffeic acid-3-*O*-sulfate are associated with variables of higher habitual consumption of coffee is linked.

- A coffee beverage prepared with 4 g coffee was given to each subject. In order to take into account the difference in available body tissue surface, it may be a good idea to administrate a coffee beverage prepared from an amount of coffee per kg bodyweight.

- The food frequency questionnaire was asked to be completed once for each participant. A repeated dietary assessment for each completed visit may help assessing the consistency of dietary habits for each study participant and minimizing the misestimation of the habitual food consumption by each participants.

8.4 Concluding remarks

As much as it is important to understand the impact of widespread (poly)phenol-rich products on human health and the mechanisms behind it, studying the health effects of popular products such as coffee is an great challenge that requires a clever approach in order to be overcome. The study of new or sparse products, as well as selected (poly)phenols present in only certain foods, are more easily tested, since a totally naïve population may still be isolated and the exclusion of products containing the compounds of interest can easily be done with fewer dietary restrictions. Coffee is one of the most consumed beverages worldwide and therefore, finding non-consumers is very difficult and finding non-consumers that are willing to consume coffee for a research study is even more. The innovative approach used for running this project was based on the fact that an important inter-individual variation in the bioavailability of (poly)phenols was observed, as reported by Renouf et al. (2010b) that obtained broad ranges of C_{max} and T_{max}, for intestinal phenolic acid equivalents. Each participant underwent a 36-hour wash out period prior to each visit to allow the clearance of (poly)phenolic-derived metabolites from any (poly)phenol-rich product consumed up to this point. Besides the challenges with the study design, all researchers conducting human studies are faced with the honesty and reliability of the participants. Although a food diary was kept by each participant, the only method of control used in this study was the profile of urinary metabolites at a fasting state and during the 36 hours following coffee consumption. Particularly urinary dihydrocaffeic acid-3-*O*-sulfate and feruloylglycine, which have been proposed to be sensitive biomarkers for the consumption of even small amounts of coffee (Stalmach et al., 2009).

Overall, the results obtained in the present investigations support previous reports and if not, this could be due to the characteristics of the present study population. The present research suggests that both roast and decaffeination reduce

the amount of free phenolics in coffee, as has previously been reported for the chlorogenic acid content (Clifford, 1999, Manach et al., 2004). Also, previous studies on the absorption and metabolism of chlorogenic acids have assumed that following consumption of coffee hydrolysis of chlorogenic acids was the major pathway for release of free phenolic acids metabolites. The data obtained in the current investigation further support this idea.

A higher consumption of (poly)phenol-rich products was not associated with a healthier profile of glucose metabolism, and the lack of association could be due to the fact that only 4 subjects had a regular daily coffee consumption ≥ 5 cups. The results of the present study suggest that a regular consumption coffee at the level reported by the study population (≤ 5 cups per day) is able to increase the levels of biomarkers of inflammation and cardiovascular health.

Finally, the data from the bioavailability part of this study are suggestive of a lower absorption of chlorogenic acids with a higher consumption of coffee, which is consistent with recent results from Erk et al. (2012) and Stalmach et al. (2014). A higher absorption of chlorogenic acids was associated with a varied outcome in regards to the profile of health biomarkers.

Interesting and promising results contributing to the knowledge of the impact of coffee and other (poly)phenol-rich products to the human health were revealed from this study. Studying the bioavailability of (poly)phenols and their influence on health is, however, a complex research field with still a lot remaining to be explored. A combination of a few adjustments to the design of future studies will complement the obtained results and provide more information about the complex and variable metabolism of (poly)phenols and further improve the understanding of the effects of coffee on human health.

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