Ana Rita Marques Lemos

Licenciada em Biologia

Molecular targeting of proteins by homocysteine: implications in familial and clinical hypercholesterolemia

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Sofia de Azeredo Pereira, Professora Doutora
NOVA Medical School, UNL
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Júri:

Presidente: Prof. Doutora Margarida Casal Ribeiro Castro Caldas Braga
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RESUMO

As doenças cardiovasculares (DCVs) são uma das principais causas de morte mundialmente e um dos factores que está na sua origem é a hipercolesterolémia.

A hipercolesterolémia pode ter uma base genética (hipercolesterolémia familiar, HF) e não genética (hipercolesterolémia clínica, HC), sendo a primeira muito mais severa, originando aterosclerose prematura.

Se por um lado a função patofisiológica da homocisteína (Hcy) na DCV é ainda controversa, por outro, a *S* e *N*-homocisteinilação de proteínas oferece um novo paradigma a ser considerado na patogénese vascular da hipercolesterolémia.

Neste sentido, o presente estudo ambiciona revelar novos conceitos sobre a ligação de Hcy a proteínas, na HC e HF.

Foram incluídos no estudo 187 indivíduos: 65 normolipidémicos e 122 hipercolesterolémicos.

As fracções de Hcy total (tHcy) e livre (fHcy) foram quantificadas em amostras de soro após validação de um método de HPLC-FD, para avaliar a *S*-homocisteinilação. A actividade de lactonase (LACase) da enzima paraoxonase-1 (PON1) foi quantificada por um ensaio colorimétrico para avaliar a *N*-homocisteinilação.

Os níveis de tHcy não diferem entre grupos. Não obstante, a fracção fHcy diminui nos grupos hipercolesterolémicos, com maior evidência para a população HF. Consequentemente, parece haver um aumento de *S*-homocisteinilação, independentemente da terapia de redução lipídica (TRL). Também a actividade LACase é mais baixa neste grupo, mesmo com TRL, por isso, o risco de *N*-homocisteinilação parece ser maior.

Além disso, a diminuição dos rácios LACase/ApoA1 e LACase/HDL na população HF mostra que a lipoproteína de alta densidade (HDL) está disfuncional nesta população apesar da concentração ser normal.

Os resultados suportam a hipótese de que a função patofisiológica da Hcy na hipercolesterolémia pode residir na sua capacidade de fazer modificações pós-traducionais em proteínas. Este fenómeno é particularmente evidente na condição de HF. No futuro será interessante identificar quais as proteínas-alvo envolvidas na progressão da patologia vascular.

PALAVRAS-CHAVE: Hipercolesterolémia; Doença cardiovascular; Homocisteinilação de proteínas; Paraoxonase-1; Actividade lactonase.

ABSTRACT

Cardiovascular diseases (CVDs) are one of the leading causes of death and disability worldwide and one of its underlying causes is hypercholesterolemia. Hypercholesterolemia can have genetic (familial hypercholesterolemia, FH) and non-genetic causes (clinical hypercholesterolemia, CH), the first much more severe, with occurrence of premature atherosclerosis.

While the pathophysiological role of homocysteine (Hcy) on CVD is still controversial, molecular targeting of protein by *S* and *N*-homocysteinylation offers a new paradigm to be considered in the vascular pathogenesis of hypercholesterolemia.

On this regard, the present study aims to give new insights on protein targeting by Hcy in both CH and FH conditions.

A total of 187 subjects were included: 65 normolipidemic and 122 hypercholesterolemic.

Total (tHcy) and free (fHcy) fractions were quantified in serum samples after validation of an HPLC-FD method, to assess *S*-homocysteinylation. Also, the lactonase (LACase) activity of paraoxonase-1 (PON1) was quantified by a colorimetric assay, as a surrogate of *N*-homocysteinylation.

tHcy does not differ among groups. Nevertheless, fHcy declines in the hypercholesterolemic groups, with more evidence to the FH population. Consequently, there seems to be an increase of *S*-homocysteinylation, regardless of lipid lowering therapy (LLT). Also, despite of LLT use, LACase activity is lower in FH, thus the risk for protein *N*-homocysteinylation seems to be higher.

Moreover, the decrease in LACase/ApoA1 and LACase/HDL ratios in FH, shows that HDL is dysfunctional in this population, despite its normal concentration values.

Data supports that the pathophysiological role of Hcy on hypercholesterolemia may reside in its ability to post-translationally modify proteins. This role is particularly evident in FH condition.

In the future, it will be interesting to identify which target proteins are modified and thus involved in vascular pathology progression.

KEYWORDS: Hypercholesterolemia; Cardiovascular disease; Protein homocysteinylation; Paraoxonase-1; Lactonase activity.

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ABBREVIATIONS

5-HETEL	5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid lactone
5,6-DHETL	5,6-dihydroxytrienoic acid-1,5 lactone
δ-iodolactone	5-hydroxy-6-iodo-8,11,14-eicosatrienoic acid δ-lactone
AA	Araquidonic acid
AIDS	Acquired immunodeficiency syndrome
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
AREase	Arylesterase activity
BSA	Bovine serum albumin
CBS	Cystathionine ß-synthase
CH	Clinical hypercholesterolemia
CHD	Coronary heart disease
CV	Coefficient of variation
CVD	Cardiovascular disease
Cys	Cysteine
Cys-Gly	Cysteinyl-glycine
DHC	Dihydrocoumarin
ESC/EAS	European Society of Cardiology / European Atherosclerosis Society
EDTA	Ethylenediaminetetra-acetic acid
FH	Familial hypercholesterolemia
fHcy	Free homocysteine
GSH	Glutathione
Hcy	Homocysteine
HcyTL	Homocysteine-thiolactone
HDL	High-density lipoprotein
HeFH	Heterozygous familial hypercholesterolemia
HHcy	Hyperhomocysteinemia
HLOQ	Higher limit of quantification
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC-FD	High-performance liquid chromatography with fluorescence detection
INSA	National Institute of Health Dr. Ricardo Jorge
LACase	Lactonase activity
LDL	Low-density lipoprotein
LLOQ	Lower limit of quantification
LLT	Lipid lowering therapy
Lys	Lysine
Met	Methionine

MS	Methionine synthase
NAC	N-acetilcysteine
N-Hcy	*N*-homocysteinylation/*N*-homocysteinylated
o-HPPA	3-(o-hydroxyphenyl) proprionic acid
ox-LDL	Oxidized LDL
PBS	Phosphate-buffered-saline
POase	Paraoxonase activity
PON1	Paraoxonase-1
PS	Physiological serum
PUFAs	Polyunsaturated fatty acids
QC	Quality control
ROS	Reactive oxygen species
RT	Room temperature
SBD-F	7-Fluorobenzofurazan-4-sulfonic acid ammonium salt
S-Hcy	*S*-homocysteinylation/ S-homocysteinylated
TC	Total cholesterol
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
tHcy	Total homocysteine
t-PA	Tissue plasminogen activator
WHO	World Health Organization

1. INTRODUCTION

1.1 Cardiovascular disease's burden in the XXI century

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels that include coronary heart disease (CHD), cerebrovascular and congenital heart disease, among others (WHO, 2015).

According to the World Health Organization (WHO), approximately 17 million people die of CVD every year, particularly heart attacks and strokes. This number is increasing each year and it is expected to reach 23.3 million people until 2030 (WHO, 2015). CVDs occur almost equally in men and women. Although heart attacks and strokes are major killers in all parts of the world, 80% of premature deaths from these causes could be avoided by controlling the main risk factors: tobacco, unhealthy diet and physical inactivity (WHO, 2015). CVD is the leading cause of death in developing and developed countries, Portugal included (**Figure 1.1**) (WHO, 2015). It causes more than half of all deaths across the European Region and it represents 46 times the number of deaths and 11 times the disease burden caused by acquired immunodeficiency syndrome (AIDS), tuberculosis and malaria combined, in Europe (WHO, 2015).

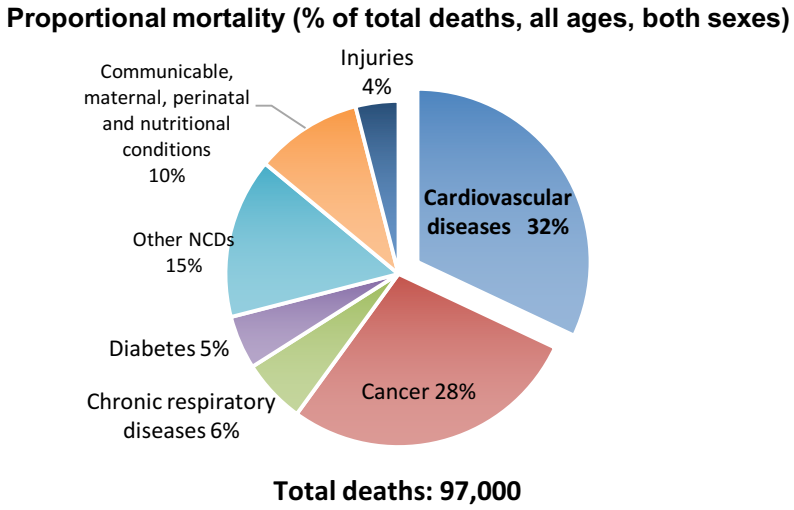

Figure 1.1 Percentage of deaths caused by cardiovascular diseases in Portugal (adapted from WHO, 2014). NCDs: Non-communicable diseases

1.2 Hypercholesterolemia pathophysiology

Hypercholesterolemia is characterized by high serum levels of cholesterol, and its deposition in the artery wall leads to atherosclerosis, the underlying cause of CVD (Çiftçi *et al.*, 2015). Hypercholesterolemia can have genetic (familial hypercholesterolemia, FH) and non-genetic causes (clinical hypercholesterolemia, CH), being the first much more severe, with occurrence of premature atherosclerosis (Marais, 2004).

FH is a monogenetic condition transmitted through an autosomal dominant pattern with an estimated prevalence of 1 in 500 in the heterozygous (HeFH) form in most populations (Hutter *et al.*, 2004) and is characterized by permanent high levels of circulating low-density lipoprotein (LDL)

particles (Medeiros *et al.,* 2014). The homozygous form of the disease is very rare (1 in 1 million) and very severe with onset in the first two years of life (Marais, 2004).

There are currently three systems at use for a clinical diagnostic of FH after which people are selected for genetic testing. The diagnostic criteria used by the National Institute of Health Doutor Ricardo Jorge (INSA) for diagnosing a person with HeFH was adapted from the Simon Broome Heart Register Group in the UK (Bourbon and Rato, 2006; Scientific Steering Committee, 1999) and it reflects on LDL and total cholesterol (TC) levels above 190 mg/dL and 290 mg/dL, respectively, as well as family history of hypercholesterolemia and premature CVD (Annex I). Usually, triglyceride levels are normal. If not treated, 50% of male before 50 and 30% of female before 60 years old will likely develop CHD (Kim and Han, 2013). Sometimes there are also physical symptoms that result from cholesterol deposition within macrophages on extra vascular tissues (Bourbon and Rato, 2006; Kim and Han, 2013) and facilitate medical diagnosis, for example, xanthomas in the Achilles tendon, knees or finger tendons, which appear irregular and thickened (Bourbon and Rato, 2006; Marais, 2004), xanthelasmas which are cholesterol deposits on the eyelids and arcus cornealis that appears from cholesterol infiltration around the cornea (Kim and Han, 2013).

In 1980, Goldstein and Brown – Nobel prize recipients for their research on cholesterol metabolism – discovered the molecular basis of the disease which is the mutations occurring on LDL receptor gene (*LDLR*) causing half or total defective LDL receptors (LDLR) at the surface of several cell types, including hepatocytes (Avis *et al.*, 2007; Bourbon and Rato, 2006). The studies that followed over the years, contributed for the discovery of two other genes connected to the FH phenotype: apolipoprotein B (*APOB)* and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) (Bourbon and Rato, 2006).

LDLR mutation is found in most FH cases and more than 1000 different mutations have been described (Kim and Han, 2013; Medeiros *et al.*, 2010). The fact that these malfunctioning receptors can't make the uptake of LDL particles into the cells, particularly the liver, for processing and degradation, leads to the accumulation on vessel walls initiating the atherogenic process and premature CVD (Avis *et al.,* 2007; Medeiros *et al*., 2014).

ApoB is an apolipoprotein associated with LDL and the only particle connecting LDL to its receptor on the liver. When the mutation occurs on the *APOB* gene, the apolipoprotein formed cannot bind LDL, hence, the clinical phenotype is similar to the ones in *LDLR* mutations (Austin *et al.*, 2004; Bourbon and Rato, 2006).

The last gene found to be associated with FH was *PCSK9* in 2004 (Bourbon and Rato, 2006). *PCSK9* codes the information for a serine protease that helps regulate the amount of cholesterol in the bloodstream by controlling the number of LDL receptors at the hepatocytes surface (Maxwell *et al*., 2005). Apparently, the protein does its function by breaking down the receptors before they reach the cell's surface, interfering with their recycling (Horton *et al*., 2007). The mutation changes a single amino acid and provokes a gain of function of the protein that exacerbates its activity, i.e., the degradation rate of the receptors is higher (Cameron *et al.,* 2006). Fewer receptors implicate higher LDL levels on the bloodstream.

A schematic representation of FH onset and its physiological implications can be seen in **Figure 1.2**.

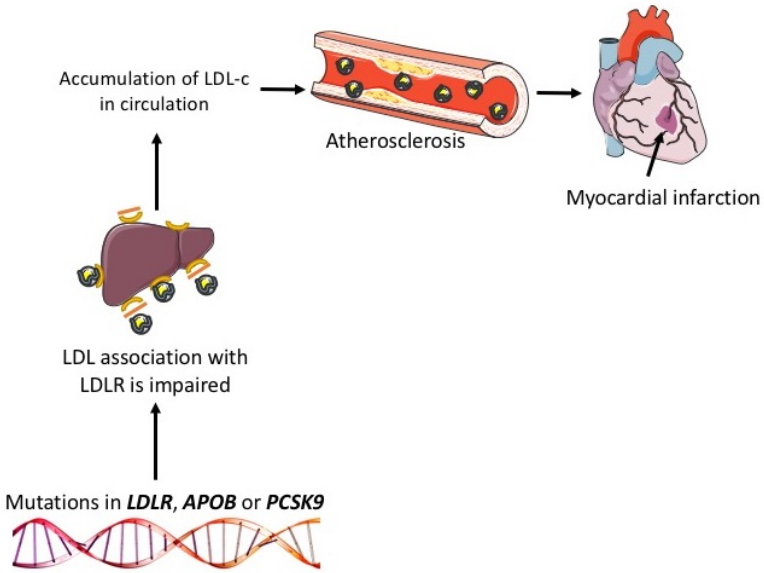

Figure 1.2 Pathophysiology of familial hypercholesterolemia (adapted from Nordestgaard *et al.*, 2013). ApoB: Apolipoprotein B; **LDL**: Low-density lipoprotein; **LDLR**: Low-density lipoprotein receptor; **PCSK9**: Proprotein convertase subtilisin/kexin type 9.

Besides the defects on LDL receptors, patients with HeFH develop a damaged flow-mediated dilatation of the brachial artery (de Jongh *et al.,* 2002) and an increased intima media thickness of the carotid artery (Wiegman *et al.,* 2004) that result in functional and morphological changes on the blood vessels since infancy (Avis *et al*., 2007). In 2005, Sankatsing and co-authors showed that these abnormalities are representative markers of atherosclerotic vascular disease (Sankatsing *et al.,* 2005), so it is important that treatment starts at a young age.

1.3 Protein homocysteinylation and paraoxonase-1 detoxification

Homocysteine (Hcy) is part of a group of amino acids that possesses a thiol group (-SH), called aminothiols, where cysteine (Cys), cysteinylglicine (Cys-Gly) glutathione (GSH) and N-acetilcysteine (NAC) are also members.

Hcy can enter two metabolic pathways. It is either converted by transsulfuration to cystathionine and then into Cys or it is remethylated to methionine (Met). The first process requires the enzyme Cystathionine ß-synthase (CBS) and vitamin B6 as a cofactor; in the second, vitamin B12 and 5,10-methyl-tetrahydrofolate serve as cofactors for methionine synthase (MS) (Hankey and Eikelboom, 1999; Jakubowski, 2006).

The only source of Hcy in the human body is through protein ingestion (Yilmaz, 2012). Normal total Hcy (tHcy) levels in the human body range from 5 to 15 μM and higher values are classified as moderate (16-30 μM), intermediate (31-100 μM) and severe (>100 μM) hyperhomocysteinemia (HHcy) (Hankey and Eikelboom, 1999). The term “total Hcy” refers to the pool of all Hcy fractions in the blood (Yilmaz, 2012).

HHcy is an established risk factor for the onset and development of atherosclerosis in susceptible populations (Olszewski and Mccully, 1991). High levels of fasting tHcy are associated with coronary

atherosclerosis in patients affected by FH in both males and females (Pisciotta *et al*., 2005). Every increase of 2.5 μM in plasma Hcy may be associated with an increase of stroke risk of about 20% (Malinowska *et al.,* 2012). Although severe HHcy is rare, moderate HHcy is a rather frequent factor in the general population (Jakubowski, 2008), which increases the vascular risk and the formation of homocysteine-thiolactone (HcyTL). For instance, tHcy above 20 μM is associated with a nine-fold increase of the myocardial infarction and stroke risk (Malinowska *et al*., 2012).

Despite these observations, pathophysiological role of Hcy on CVD is still controversial. On one hand, tHcy is accepted as an independent risk factor. On the other hand, studies aimed at lowering its plasma concentrations showed no significant reduction on cardiovascular risk (Cheng, 2013). This might indicate that by monitoring tHcy itself, valuable information might be lost. In fact, 80% of the total amount of homocysteine in circulation is bound to proteins (Hankey and Eikelboom, 1999).

Molecular targeting of proteins by *N*-homocysteinylation (due to acylation of free amino groups mediated by Hcy toxic metabolite, HcyTL) or by *S*-homocysteinylation (formation of disulphide bonds with protein's Cys residues mediated by Hcy itself) may have a role on the pathogenesis of hypercholesterolemia (Malinowska *et al*., 2012).

Both *N*- and *S*-homocysteinylation have well-established pathophysiological consequences owed to protein structure modification, as protein inactivation, cell damage, oxidative stress, activation of autoimmune response and enhanced thrombosis (Zinellu *et al.,* 2012) (**Table 1.1**).

There are two different types of disulphides in protein structures that have different functional roles: one type of disulphides stabilizes proteins by cross-linking their polypeptide chains while others are redox reactive and thus may react with free aminothiols, such as Hcy (Jakubowski, 2013). These alterations can change the redox balance of proteins (Malinowska *et al*., 2012) Plasma *S*-thiolated proteins have been detected in healthy humans, in patients with cardiovascular disease and in several cell types after oxidant exposure (Zinellu *et al.,* 2006).

Proteins are *N*-homocysteinylated at rates proportional to the amount of lysine (Lys) residues present, and a physiologic concentration of HcyTL of 10 nM is sufficient to start the process (Jakubowski, 2006; Zinellu *et al*., 2006). *N*-Hcy-proteins levels in human plasma are several times higher than the plasma levels of HcyTL (0-35 nM), varying from 0.1 μM to 13 μM, meaning that most HcyTL formed establishes immediate bonds with the amino groups of proteins (Jakubowski, 2006).

HcyTL formation occurs in all cells tested thus far, including human vascular endothelial cells, when Methionyl-tRNA syntethase selects by error Hcy instead of Met in an editing reaction of protein synthesis (Jakubowski, 2006; Yilmaz, 2012). This process is favoured when the two other pathways are impaired by lack of vitamin B6, B12 and/or folate (B9) intake, by high-Met diet or by genetic alterations of enzymes involved in Cys/Met metabolism – CBS, MS and/or methylenetetrahydrofolate reductase (Jakubowski, 2006).

Generally, the percentage of tHcy that binds to proteins as *N*-Hcy in human plasma of healthy individuals is 3–7% (Jakubowski, 2008). Increased total plasma Hcy concentrations lead to increased production of HcyTL and *N*-homocysteinylation which in turn favours the number of -SH sites available for *S*-homocysteinylation (Zinellu *et al.,* 2006). The opposite also happens. Human serum albumin has been the most studied *N*-Hcy-protein. It was noted that the protein disulphide form established

between Cys34 residue and a free Cys aminothiol was *N*-homocysteinylated faster than mercaptoalbumin (the form with free reduced Cys34 residue) (Jakubowski, 2006). Lys525 residue of albumin is two times more reactive to *N*-homocysteinylation when the protein is in its disulphide form than when Cys34 is reduced, leading to a final product much more susceptible to proteolysis (Jakubowski, 2006).

Table 1.1 Cardiovascular consequences of *S* and *N*-homocysteinylated proteins.

***S*-homocysteinylated protein**	**Pathogenic role**	**Reference**
Albumin	Modification of the intracellular redox potential	Sengupta *et al.,* 2001
ApoB-100 (major component of LDL)	Increase of reactive oxygen species (ROS) production and cytotoxicity	Jakubowski *et al.,* 2013
Fibrillin	Structural changes that lead to protein misfolding	Jakubowski *et al.,* 2013
Fibronectin	Function and fibrin interaction are impaired; pro-thrombotic effect and vascular occlusion	Gluschenko and Jacobsen, 2007
Factor Va	Resistance to inactivation by activated protein C – pro-thrombotic tendency	Lentz *et al.*, 2002; Gluschenko and Jacobsen, 2007
Annexin A2	Decrease of tissue plasminogen activator (t-PA) ligation - pro-thrombotic phenotype	Zinellu *et al*., 2006
Transthyretin	Potential role on amyloid deposition	Gluschenko and Jacobsen, 2007

***N*-homocysteinylated protein**	**Pathogenic role**	**Reference**
Albumin	Increasing of oxidative stress and proteolysis	Perna *et al.,* 2006
Fibrinogen	Impaired fibrinolysis – pro-thrombotic tendency	Sauls *et al.,* 2011
ApoA1	Reverse cholesterol transport dysfunction – atherosclerosis lesions	Ishimine *et al.,* 2010
Insulin	Protein aggregation and fibrillation	Yousefi et al. 2012

ApoA1: Apolipoprotein A1; **ApoB**: Apolipoprotein B

High-density lipoprotein (HDL) and LDL have also been subject of homocysteinylation studies. LDL interaction with HcyTL causes aggregation of the lipoprotein and higher uptake by cultured macrophages (Zinellu *et al*., 2006). Ferretti and co-workers theorized that the oxidative stress caused by Hcy on human aortic endothelial cells could be explained by the internalization of *N*-

homocysteinylated LDL by membrane receptors with intracellular release of Hcy after hydrolytic degradation (Ferretti *et al.,* 2004). The authors have also reported that LDL incubation with 100 μM HcyTL for two hours causes *N*-homocysteinylation of ≈10% of apoB-100 Lys residues (Ferretti *et al*., 2004).

As to HDL, it is the major carrier of hydroperoxides in plasma and that happens as consequence of oxidation at inflammation sites and re-entry into circulation or by transfer of oxidized lipids from LDL to HDL (Ferretti *et al.,* 2010). An increase in lipid hydroperoxides and other oxidation markers destabilizes the lipoprotein structure, facilitating HcyTL access to HDL amino groups (Ferretti *et al.,* 2010).

HcyTL is detoxified by the lactonase (LACase) activity of Paraoxonase-1 (PON1), an HDL-associated enzyme, hence avoiding protein *N*-homocysteinylation and its nefarious effects on the organism (Yilmaz, 2012) (**Figure 1.3**).

LACase is nowadays considered to be the native function of this enzyme in mammal's organisms (Jakubowski, 2000). It was first proposed by Jakubowski that PON1 evolved to metabolise HcyTL (Jakubowski, 2000). Further on, other investigators found that it is also able to hydrolyse other classes of endogenous lactones, specifically, aromatic and aliphatic lactones that arise from oxidation of polyunsaturated fatty acids (PUFAs) and result in modulation of the local anti-inflammatory response (Draganov *et al.,* 2005; Khersonsky and Tawfik, 2005). *5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid lactone* (5-HETEL) and *5,6-dihydroxytrienoic acid-1,5 lactone* (5,6-DHETL) are lactones originated through one of araquidonic acid (AA) oxidative pathways, the lipoxygenase pathway (Draganov and Teiber, 2008). 5-HETEL inhibits platelet neutrophil phospholipase A2, an inflammatory mediator that unchains the AA cascade, and cycloxygenase, the enzyme involved in the oxidative pathway that originates thromboxane A2 and prostaglandins (Draganov and Teiber, 2008). 5,6-DHETL is a potent vasodilator that is rapidly metabolised by PON1 to 5,6-dihydroxyeicosatrienoic acid (5,6-DHET), though it is not known what the physiological effect of this metabolism *in vivo* might be (Draganov and Teiber, 2008). *5-hydroxy-6-iodo-8,11,14-eicosatrienoic acid δ-lactone* (δ-iodolactone) inhibits signal transduction pathways that are activated by local growth factors (e.g. epidermal and fibroblast) (Draganov and Teiber, 2008).

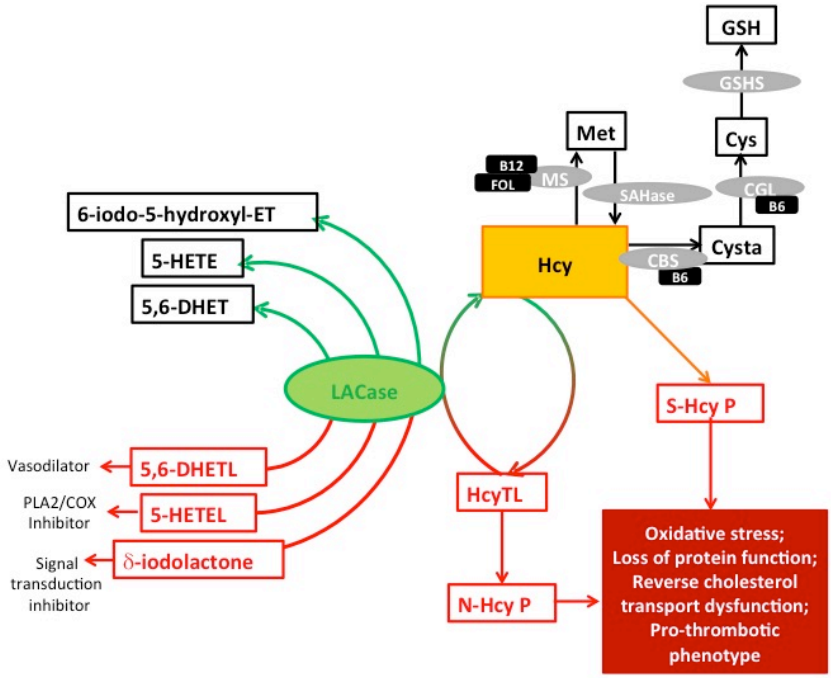

Figure 1.3 Protein targeting by homocysteine and vascular protection conferred by lactonase activity (adapted from Dias *et al.,* 2015). **5-HETEL**: 5-hydroxy-eicosatetraenoic acid lactone; **5,6-DHETL**: 5,6-dihydroxytrienoic acid-1,5 lactone; **δ-iodolactone**: 6-Iodo-5-hydroxy-8,11,14-eicosatrienoic trienoic acid δ-lactone; **CBS**: Cystathionine ß-synthase; **CGL**: cystathionine γ-lyase; **Cys**: Cysteine; **Cysta**: Cystathionine; **ER**: Endoplasmic reticulum; **FOL**: Folate; **GSH**: Glutathione; **GSHS**: Glutathione synthetase; **Hcy**: Homocysteine; **HcyTL**: Homocysteine-thiolactone; **LACase**: Lactonase; **Met**: Methionine; **MS**: Methionine synthase; ***N*-Hcy P**: *N*-homocysteinylated protein; ***S*-Hcy P**: *S*-homocysteinylated protein; **SAHase**: S-adenosylhomocysteine hydrolase.

Human PON1 is a calcium-dependent glycoprotein, one calcium ion with a structural role and the other with a catalytic function, mainly expressed in the liver (Perła-Kaján and Jakubowski, 2012). It was firstly classified as an aryldialkylphosphatase (EC 3.1.8.1) and later, it was also designated by Aldrige, in 1953, an A-esterase for its ability to hydrolyse organophosphates (Mackness and Mackness, 2013). It retains the hydrophobic sequence on the N-terminal region, which allows the link with HDL (Draganov *et al.,* 2005; Farid and Horii, 2012). 95% of PON1 content exists on a subclass of HDL, HDL_3, which is responsible for most of HDL antioxidant capacity and contains also apolipoprotein A1 (ApoA1) and clusterin (Farid and Horii, 2012; Gugliucci *et al.,* 2012; Mackness and Mackness, 2013). PON1 activity largely depends on its association with apoA1 and phospholipids in HDL, although a minor, free form can be found (Gugliucci *et al*., 2012).

It is believed to have antiatherogenic and antioxidant properties and a broad substrate specificity going from drug metabolism and nerve agents detoxification (sarin and soman) to the metabolism of organophosphates (paraoxon, diazoxon), aromatic esters (phenyl acetate) and several classes of lactones (HcyTL, PUFA lactones and statins with lactone structure) (Draganov *et al*., 2005; Perla-Kaján and Jakubowski, 2012).

1.4 Objectives

General aim

Molecular targeting of protein by Hcy offers a new paradigm to be considered in the pathogenesis of hypercholesterolemia. This work aimed to give new insights on protein targeting by Hcy in hypercholesterolemia condition with genetic (familial hypercholesterolemia, FH) and non-genetic aetiology (clinical hypercholesterolemia, CH).

Specific aims

- ✓ To optimize and validate a method for Hcy fractions quantification;
- ✓ To compare tHcy and its fractions (fHcy and S-Hcy-P) in normolipidemic, CH and FH patients;
- ✓ To explore *S*-protein bound fraction in normolipidemics, CH and FH patients;
- ✓ To assess *N*-protein bound fraction through the use of LACase activity as a surrogate;
- ✓ To ascertain the effect of lipid lowering drugs use in LACase and in tHcy and its fractions, in the three populations;

2. MATERIALS AND METHODS

2.1 Study population and patient's inclusion

The Portuguese Familial Hypercholesterolemia Study has been implemented in 1999 at the INSA following WHO recommendations for a universal FH screening, in 1998 (WHO, 1998). People with a clinical diagnosis of FH have been recruited by clinicians all over the country to perform genetic tests to identify a possible mutation (Medeiros *et al.*, 2015). Parallel to this, there is a national study being developed at INSA named "eCOR" that was designed to estimate the prevalence of major cardiovascular risk factors in the general Portuguese population.

The eCOR project has so far, a total of 1685 individuals included and approximately 20% are under lipid lowering therapy. Randomized samples have been collected from 5 Portuguese sub-regions defined for statistic means (North, Centre, Lisboa, Alentejo and Algarve) and stratified by age and sex in order to be representative of the Portuguese adult population, aged between 18-79 years old. Data collection includes an individual survey, physical examination and clinical history. Serum samples were collected after a 12-hour fasting, by venipuncture, in healthcare centres across the country and sent to INSA.

The current work was conducted in accordance with the Declaration of Helsinki. The study protocol and database have been approved by the National Institute of Health Ethics Committee and the National Data Protection Commission, respectively. Written informed consent was obtained from all participants before their inclusion in the study.

A subgroup has been selected and divided into three groups according to their TC levels to quantify LACase activity and Hcy fractions: subjects from the eCOR with normal cholesterol levels; patients previously diagnosed with CH and patients diagnosed with FH. Both hypercholesterolemic groups were diagnosed at INSA, following Simon Broome criteria (Annex I). Lipid reference values adopted in this study were followed according to the guidelines established by the European Society of Cardiology for the general population (ESC/EAS, 2011) (**Table 2.1**).

Table 2.1 Lipid reference values based on ESC guidelines for the management of dyslipidemias.

Parameter	Recommended value (mg/dL)
Total cholesterol	< 190
Low-density lipoprotein	<115
High-density lipoprotein	>40 for men; >50 for women
Apolipoprotein B	< 120
Apolipoprotein A1	>120 for men; >140 for women
Triglycerides	< 150

2.2 Homocysteine quantification: total, free and *S*-homocysteinylated protein fraction

I. RATIONAL

Like other aminothiols, Hcy exists mostly in the protein-bounded form (70-80%) due to the reactivity of its sulfhydryl group (-SH) but it also circulates in the blood in its free oxidized form as homocysteine dimers or mixed disulphides (20-30%) and, in less extent, in a free reduced form (1%) (Hankey and Eikelboom, 1999).

Molecular targeting of proteins by Hcy may occur by *N*-homocysteinylation or by *S*-homocysteinylation (Malinowska *et al*., 2012).

In most studies and even in clinical routine analysis, the contribution of Hcy to CVD relies on tHcy quantification, which gives limited knowledge, hence probably leading to the loss of valuable information. In this sense, it would be important to understand the contribution from each Hcy fraction in CVD. To achieve this, a high sensibility quantification method is required. High-performance liquid chromatography with fluorescence detection (HPLC-FD) allows the detection of free and total Hcy, having the advantage of differentiating from other thiols present in the sample (Cys, Cys-Gly, GSH and NAC). *S*-Hcy-protein fraction is extrapolated by subtracting the free to the tHcy concentration (Przemysław *et al.,* 2011). By adapting the conceptual idea for fHcy separation from Przemyslaw and co-workers and the analytical method described by Nolin and colleagues (Nolin *et al.,* 2007), a new method that allowed the quantification of Hcy fractions was optimized.

Whenever available, the values of tHcy obtained were compared to those obtained in clinical setting.

II. STANDARDS PREPARATION FOR THE CALIBRATION CURVE AND QUALITY CONTROL

Stock solution

A L-Homocysteine (*Sigma-Aldrich*) 10mM stock solution was prepared by dissolving the appropriate amount of the solid analyte in 1mL of deionized and distilled water.

Standards preparation

Three calibration curves were prepared for validation purposes.

The standards for the calibration curve were prepared by successive dilutions of the stock solution in phosphate-buffered-saline (PBS) in order to obtain ten different concentrations of L-Homocysteine ranging from 0.625 to 50 µM.

The quality control (QC) samples were prepared from distinct stock solutions by successive dilutions in PBS to obtain final concentrations of Hcy of 37.50, 18.75 and 4.375 µM (QC1, QC2, QC3 respectively).

III. METHOD VALIDATION

The validation criteria were defined according to guidebooks regarding the validation of bioanalytical methods (US-FDA, 2001; EMA, 2011).

Linearity of the method: Three calibration curves were prepared from different stock solutions and using 10 standards within the concentration range: 0.625 µM (lower limit of quantification, LLOQ) to 50 µM (higher limit of quantification, HLOQ).

The calibration curves were plotted by linear regression of the chromatographic peak area (mV*min) as function of analytes concentration (µM) to assess the linearity of the method. Also, the slopes and Y-intercept (Y0) of the curves where compared in order to access reproducibility. The average back-calculated concentrations were also assessed.

Lower limit of quantification: The LLOQ for Hcy was established at 0.625 µM. In order to validate the LLOQ, five runs of this standard were analysed for accuracy and inter-assay precision studies.

Accuracy: Accuracy was defined as the closeness to the theoretical concentration of the QC and LLOQ samples. Mean concentration of each QC sample analysed should be within 100±15% of the theoretical concentration, except for LLOQ, where 100±20% is acceptable. This parameter was calculated using the equation 1.

$$\text{Accuracy (\%)} = \frac{\text{Obtained concentration}}{\text{Exact concentration}} \times 100 \qquad (1)$$

Inter-assay precision: Inter-assay precision was assessed by the coefficient of variation (CV) obtained from the analysis of QC samples in five runs on different days. The CV of QC should not exceed 15%, except for LLOQ for which a 20% variation is acceptable.

Stability: To understand the stability of the main solutions used in this protocol, standards with Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 7-fluorobenzofurazan-4-sulfonic acid ammonium salt (SBD-F) were frozen at -80 ºC, overnight and analysed on the next day. Stock-solutions were also submitted to three freeze-thaw cycles.

The stability of Hcy was also tested on two separate 50 µM standards after being submitted to two different conditions: a) -20 ºC and b) 4 ºC, both overnight.

Selectivity and interference: Interference with other endogenous compounds (Cys, Cys-Gly, GSH and NAC) was tested.

Carryover: Sample *carryover* was evaluated by inserting vials of methanol after every five samples.

IV. STANDARD OPERATING PROCEDURE

Sample preparation: The method herein described allows the quantification of tHcy and was adapted from a method previously developed by Nolin and colleagues (Nolin *et al*., 2007). Briefly, the analytes present in 50 µL of serum samples were first reduced with 5 µL of TCEP (100g/L, *Sigma-Aldrich*) and incubated at room temperature (RT) for 30 minutes. After that, proteins were precipitated with 45 µL of trichloroacetic acid solution (TCA, 100 g/L) (*Roth*), containing 1.0 mM of ethylenediaminetetra-acetic acid (EDTA, *Sigma-Aldrich*). The resulting mix was centrifuged, the pellet discarded and a volume of 25 µL of the supernatant was collected and added to a new tube containing 5 µL of 1.55 M sodium hydroxide (NaOH, *VWR*), 62.5 µL of 0.125 M sodium tetraborate buffer (pH 9.5, *Sigma-Aldrich*) with 4.0 mM EDTA and 25 µL of SBD-F (1 g/L, *Sigma-Aldrich*) in borate buffer (0.125 M with 4.0 mM EDTA). Samples were vortexed and incubated in the dark, at 60 ºC during 1 hour, for derivatization. A final volume of 30 µL of the derivatized solution was transferred to microvials and placed in the refrigerated autosampler (8 ºC). A 10 µL aliquot was injected into the HPLC system for analysis.

The main procedure steps order was changed for the fHcy fraction extraction, based on Przemyslaw methodological concept (Przemysław *et al.,* 2011): first precipitation and removal of proteins, and then reduction and derivatization of the supernatant containing the free fraction (**Figure 2.1**).

Equipment and chromatographic conditions: HPLC analysis was performed on a *Shimadzu* system (LC-10AD VP) using a reversed-phase C18 *LiChroCART* 250-4 column (*LiChrospher* 100 RP-18, 5µm, VWR) at 29 ºC. Detector was set at excitation and emission wavelengths of 385 and 515 nm, respectively. Mobile phase consisted of acetate buffer 0.1 M (pH 4.5, *VWR*) and methanol (*VWR*) [99:1 (v/v)]. The analytes were separated in an isocratic elution for 20 min at a flow rate of 0.8 mL/min.

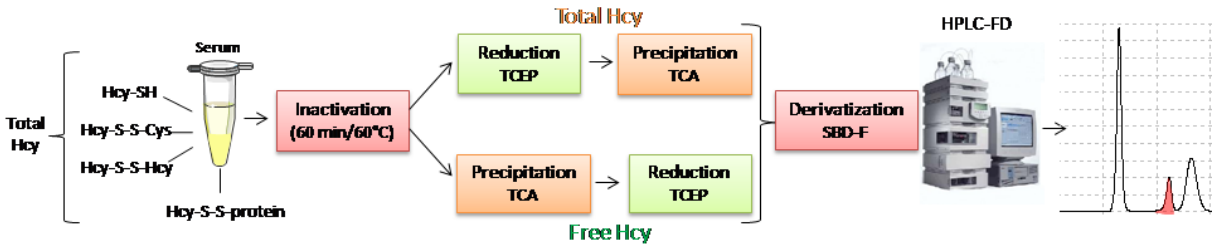

Figure 2.1 Quantification of total and free homocysteine fraction in serum.

HPLC-FD: High performance liquid chromatography with fluorescence detection; **SBD-F:** 7-fluorobenzofurazan-4-sulfonic acid ammonium salt; **TCA:** Trichloroacetic acid; **TCEP:** Tris(2-carboxyethyl)phosphine hydrochloride.

2.3. PON1 lactonase activity assessment

I. RATIONAL

LACase activity is still the least studied PON1 activity, possibly because it was the last PON1 activity to be discovered and because previous methods have disadvantages that hamper a practical clinical application (Billecke *et al.*, 2000; Gaidukov and Tawfik, 2005; Rock *et al.*, 2008). On the other hand, it is also the one that raises most interest because of increasing evidence that it is the endogenous PON1 activity. Over the years, different authors came to the conclusion that PON's family has a high affinity for lactones so it is nowadays proposed that the native function of PON1 is the metabolism of endogenous lactones, namely HcyTL and other arisen from oxidation of polyunsaturated fatty acids (PUFAs) (Draganov *et al.*, 2005; Jakubowski, 2000; Khersonsky and Tawfik, 2005). This activity is responsible for HcyTL detoxification converting it back to Hcy, which can then enter Met and Cys metabolism, hence avoiding HcyTL deleterious effects (Yilmaz, 2012). Therefore, LACase activity might be a surrogate of protein *N*-homocysteinylation.

The LACase method is based on the stoichiometric production of 3-(o-hydroxyphenyl) propionic acid (o-HPPA) resulting from the hydrolytic reaction of the substrate dihydrocoumarin (DHC) (**Figure 2.3**). This reaction is monitored spectrophotometrically at 405 nm, following the colour change of the pH indicator phenol red (from red to yellow). This method was developed and validated at *Translational Pharmacology* lab (Dias *et al*., submitted).

II. STANDARDS PREPARATION FOR THE CALIBRATION CURVE

The stock solutions were prepared by adding the appropriate amount of o-HPPA (*Sigma-Aldrich*) to freshly prepared HEPES buffer (2.0 mM, pH 8.0) (*Roth*), containing calcium chloride ($CaCl_2$, 1.0 mM) (*BDH Chemicals Ltd Pool England*) and 0.005% albumin from bovine serum (BSA, *Roth*). Six standard samples were prepared for the calibration curve by diluting the stock solutions in physiological serum (PS). For correction of the non-enzymatic hydrolysis of o-HPPA, a blank sample was prepared using PS and HEPES buffer.

The accuracy and precision of two QC concentrations (QC1 - 4.92 mM and QC2 - 6.55 mM of o-HPPA), as well as the LLOQ (1.29 mM of o-HPPA) and the HLOQ (10.24 mM of o-HPPA) were evaluated using a previously defined criterion (Dias *et al.,* 2014).

III. STANDARD OPERATING PROCEDURE

LACase activity was assayed by measuring the extent of the hydrolysis of DHC, using a spectrophotometric method adapted to a 96-well microplate (**Figure 2.3**). Briefly, serum samples were diluted in the proportion of 1:5 in PS and incubated during 10 minutes, at 37 °C, as well as the previous prepared standards. Then, 190 µl of freshly prepared HEPES buffer (2.0 mM, at pH 8.0) containing $CaCl_2$ (1.0 mM), BSA (0.005%), phenol red (106 µM, *Fluka*) and DHC (1.0 mM, *Sigma-Aldrich*) were added per well. After 1 minute of incubation at RT, the absorbance was measured at

405 nm on a microplate reader (*Biotrack II* plate reader, *Amersham Biosciences*). The activity was directly obtained from the calibration curve and expressed as kU/L, which is the amount of enzyme producing 1 mM of o-HPPA per minute. As DHC is toxic by inhalation or skin contact substrate (Ceron *et al.*, 2014), proper clothing was used.

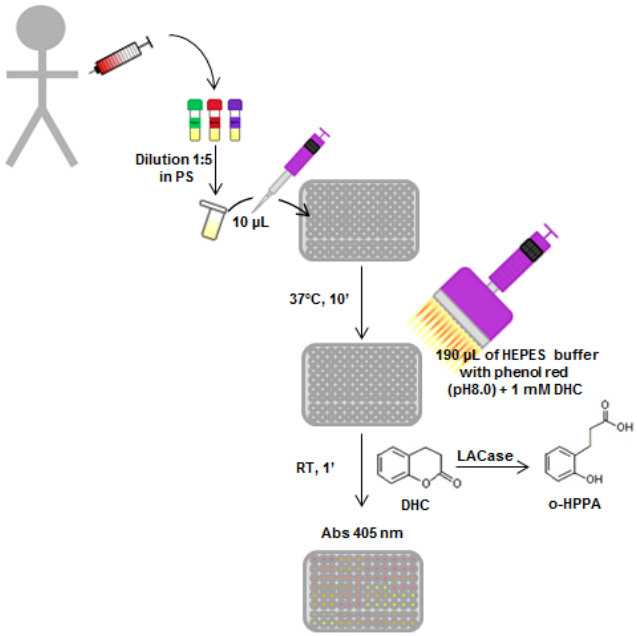

Figure 2.2 Quantification of lactonase activity in serum (*N*-Homocysteinylation surrogate) (adapted from Dias *et al.*, 2015). **DHC**: dihydrocoumarin; **LACase:** Lactonase activity; **o-HPPA:** 3-(o-hydroxyphenyl) propionic acid; **PS**: physiological serum; **RT**: Room temperature.

2.4 Statistical analysis

Statistical analysis was performed using *GraphPad Prism*® version 6.0 (GraphPad Software Inc., CA). Data was expressed as median [interquartile range, IQR] or frequencies (%), whenever applicable. Normality of groups was tested using *D'Agostino & Pearson* and *Shapiro-Wilk test*. *Chi-Square* test was used to assess data independence. Correlations between parameters were explored using *Pearson* or *Spearman's rank* correlation test according to the normality of groups. Comparisons between groups were performed using *One-way ANOVA* or *Unpaired t test* for normal distributions and *Kruskal-Wallis* or *Mann-Whitney U* for groups that did not pass the normality test. Values of $p<0.05$ were considered significant. Comparisons with more than two variables were performed using *Two-way ANOVA test*. The *F*-test was used to explore differences between the slopes and the elevations of the calibration curves in the validation of the Hcy method. Variability among data was expressed in variation coefficient (CV), expressed as %.

3. RESULTS

3.1 Development and validation of a homocysteine quantification method

I. LINEARITY OF THE METHOD

The linear regression model showed to be the most suitable for fitting a function to the points obtained (*p* value of *Run Tests*>0.05 and deviation from linearity non significant). There were no differences between the slopes and the elevations of the calibration curves (*p* value of *F tests* <0.001).

The correlation coefficient, r^2, was higher than 0.999, which is a good indicator of adjustment of all calibration curves. The 95% confidence interval for the Y-intercept when X=0 contained zero. The average back-calculated concentrations obtained were close to the expected theoretical values at each tested concentration and presented differences lower than 14%.

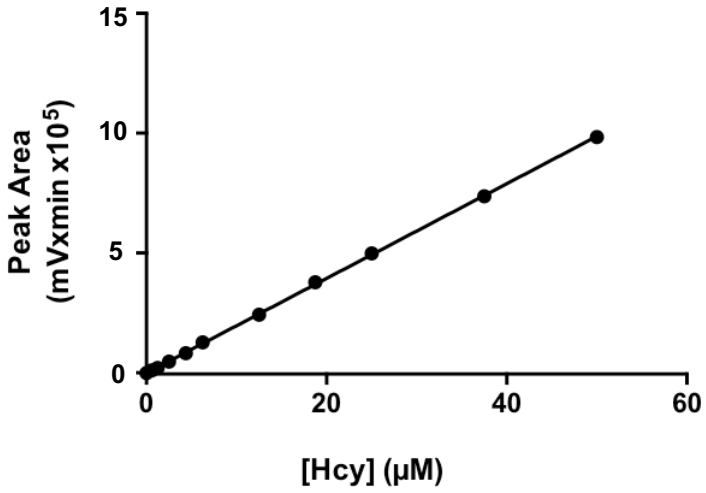

Figure 3.1 Homocysteine calibration curve. m= 19750±84; y(0)= -583±1817.

II. LOWER LIMIT OF QUANTIFICATION

The LLOQ for Hcy was 0.625 µM. The accuracy and inter-assay precision results are presented in **(Table 3.1)**.

III. ACCURACY

The accuracy values obtained for QC1, QC2 and QC3 ranged between 96% and 98% **(Table 3.1)**.

IV. INTER-ASSAY PRECISION

The inter-assay precision for all samples evaluated was higher than 94% **(Table 3.1)**.

Table 3.1 Accuracy and inter-assay precision frequencies for the LLOQ and QC samples of homocysteine quantification method.

Standard (µM)	Accuracy (%)	Inter-assay precision (%)
LLOQ (0.625)	93	94
QC3 (4.357)	98	98
QC2 (18.750)	98	97
QC1 (37.500)	96	97

LLOQ: lower limit of quantification; **QC1**: quality control 1; **QC2**: quality control 2; **QC3**: quality control 3.

V. STABILITY

Stability performance showed no interference for using SBD-F and TCEP defrost one time (*Kruskal-Wallis test*).

Three freeze–thaw cycles had no effect on the stability of tHcy present in the stock-solutions.

Hcy standards can be maintained overnight at 4 ºC and -20ºC (*Kruskal-Wallis test*).

VI. SELECTIVITY AND INTERFERENCE

Hcy retention time was not affected by the presence of other analytes in all standards tested (**Figure 3.2)**.

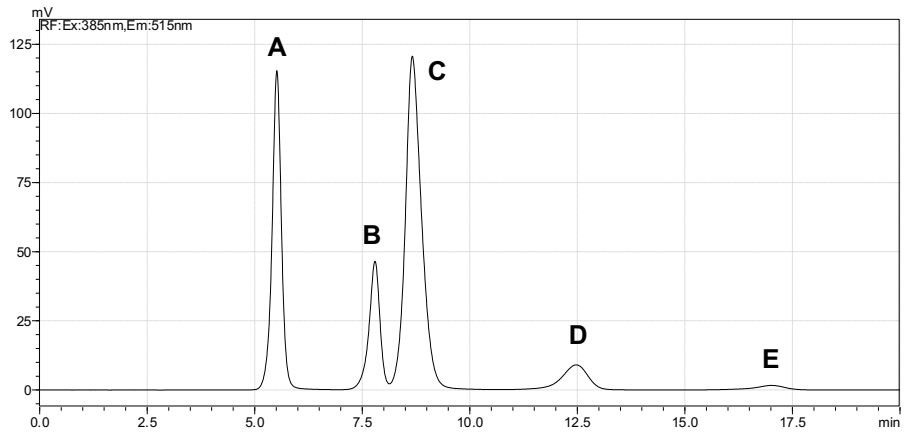

Figure 3.2 Representative chromatogram of a standard sample with 50 µM of homocysteine.
Legend: A – Cysteine peak (5.5 min); **B –** Homocysteine peak (7.8 min); **C –** Cys-Gly (8.7 min); **D –** GSH (12.5 min); **E –** NAC peak (17 min).

A chromatogram from a patient sample is shown for comparison effects (**Figure 3.3**).

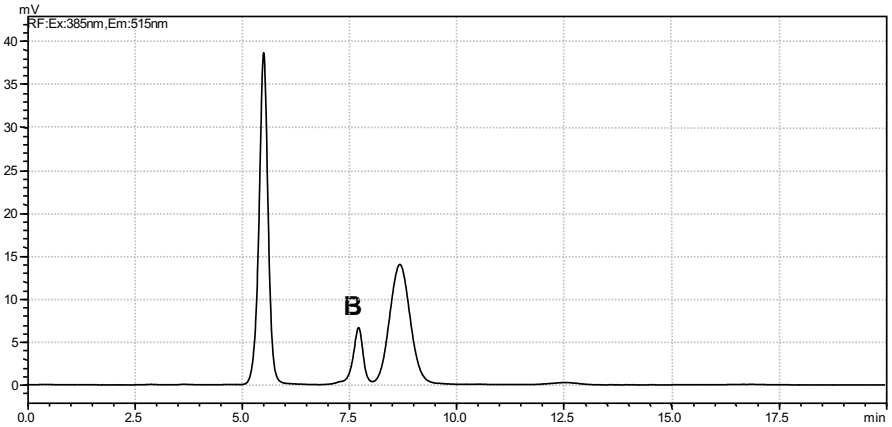

Figure 3.3 Representative chromatogram of a patient sample.

Legend: B – Homocysteine peak (7.9 min).

VII. CARRYOVER

There was no evidence for *carryover* phenomenon.

As a further validation step of the method, tHcy concentrations herein obtained were compared with Hcy values provided by the INSA lab, whenever available (**Figure 3.4**).

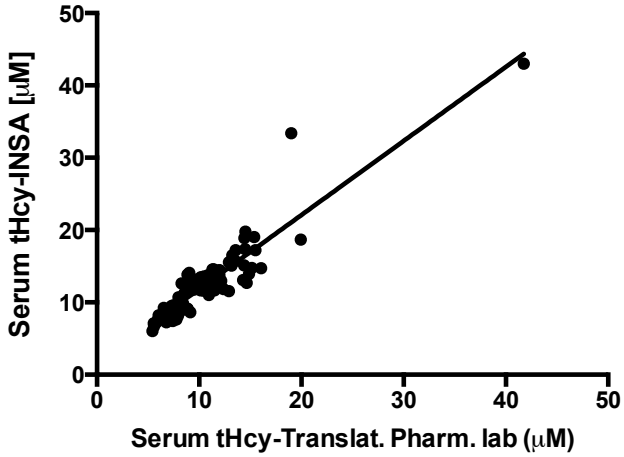

Figure 3.4 Comparison between serum total homocysteine concentration measured at INSA laboratory and the concentration measured at the *Translational Pharmacology* laboratory. (*Spearman* r=0.892, p<0.001).

3.2 Anthropometric and clinical data of the included patients

A total of 187 individuals (53% men) were included in the study: The normolipidemic group (TC <190 mg/dL) included 65 subjects; the clinical hypercholesterolemia group (CH) was composed by 76 patients and familial hypercholesterolemia group (FH) by 46 patients.

The characterization of the studied population is presented on **Table 3.2**. No sex (*Chi-square* test) or age (*Kruskal-Wallis test with Dunn's multiple comparison* test) differences were found between groups.

Table 3.2 Anthropometric and clinical data.

Parameter (Unit)[a]	Normolipidemia	CH	FH	*p* value[b]
n	65	76	46	
Men (%)	49	62	46	ns
Age (years)	44 [39-50]	44 [36-57]	43 [35-50]	ns
LLT (%)	0	47	74	<0.001

[a]Data is presented in number, % or Median [IQR]; [b]*Chi-square test*. **CH**: Clinical hypercholesterolemic; **FH**: Familial hypercholesterolemic; **LLT**: under lipid lowering therapy; **n:** number of subjects.

The number of patients on lipid lowering therapy (LLT) varied significantly among groups, reaching approximately half of the CH patients and almost 75% of the FH population (*Chi-square test*, p<0.001).

Table 3.3 describes the number of patients under the different LLT regimens.

Table 3.3 Lipid lowering therapy use by hypercholesterolemic population.

Parameter[a]	CH	FH	Total
Statins	24	16	40
Fibrates	1	0	1
Statin + Nicotinic acid	1	0	1
Statin + Ezetimibe	4	17	21
Statin + Fibrates	2	0	2
Fibrates + Ezetimibe	1	0	1
Omega 3	1	0	1
Unknown	2	1	3
Total (n)	36	34	70

[a]Data is presented in number. **CH**: Clinical hypercholesterolemic; **FH**: Familial hypercholesterolemic; **n**: number of subjects.

Statins is the most prescribed class of drugs in both populations, as single prescription and also in combination with ezetimibe. Considering the fraction where they are used as single therapeutic, the prevalence is of 67% in the CH group, and 47% in the FH. This fraction is less expressive in the FH group because of a combined therapy of statins plus ezetimibe that reaches 50% of the patients.

Simvastatin and rosuvastatin seem to be the most administered statins in both groups and in both types of prescriptions (single and combined), followed by atorvastatin, pravastatin and pitavastatin.

Considering that, the data herein obtained were analysed comparing patients on treatment vs. patients not receiving any LLT medication (**Table 3.4**).

Table 3.4 Lipid and lipoproteins profile: exploring the effect of disease and treatment.

Parameter (mg/dL)[a]	Normo lipidemia	CH		FH		*p* value[b]
		Non-LLT	*LLT*	*Non-LLT*	*LLT*	
n	65	40	36	12	34	
TC	164 [153-177]	226[212-253]***	241[210-275]***	234[214-261]***	256[224-299]***	$^{+}$
LDL	92 [83-108]	154[139-173]***	162[134-180]***	166[149-182]***	179[156-226]***	$^{+}$
HDL	50 [37-61]	38 [33-48]*	56 [40-67]	44 [36-47]	50 [44-57]	##
ApoA1	138[119-165]	127[115-150]	169[139-185]**	135[119-141]	147[134-160]	###

[a]Data presented as number or Median [IQR]; **Kruskal-Wallis with Dunn's multiple comparisons* test vs normolipidemic; [b]*Two-way ANOVA with Bonferroni post test*: $^{+}$*CH vs FH* and $^{\#}$ *non-LLT vs LLT in CH*.

ApoA1: Apolipoprotein A1; **CH**: Clinical hypercholesterolemia; **FH**: Familial hypercholesterolemia; **HDL**: High density lipoprotein; **LDL**: Low density lipoprotein; **LLT:** under lipid lowering therapy; **Non-LLT:** not under lipid lowering therapy; **TC:** Total cholesterol

CH patients have, as expected, higher levels of TC and LDL than normolipidemic patients (*Kruskal-Wallis* test, $p<0.001$). CH non-LLT group has a significantly lower HDL concentration (*Kruskal-Wallis* test, $p<0.05$) and CH LLT group has higher ApoA1 levels (*Kruskal-Wallis* test, $p<0.01$).

FH patients had also elevated TC and LDL comparatively to normolipidemics (*Kruskal-Wallis,* $p<0.001$) and to CH disease group (*Two-way ANOVA,* $p<0.05$), while HDL and ApoA1 levels did not differ.

LLT use affected particularly HDL and ApoA1, with higher levels in the treated groups (*Two-way ANOVA*, $p<0.01$).

The percentage of patients with HDL<40 mg/dL was higher in CH non-LLT patients (68%), followed by FH non-LLT (33%), 32% in normolipidemic, 25% in CH LLT and 18% on FH LLT.

3.3. Serum homocysteine levels

Table 3.5 Total homocysteine levels in the studied groups.

Parameter (Unit)[a]	Normolipidemia	CH		FH		*p* value
		Non-LLT	*LLT*	*Non-LLT*	*LLT*	
Serum tHcy (μM)	10 [8-13]	9 [7-12]	9 [8-12]	9 [6-10]	8 [6-10]	ns

[a]Data presented as Median [IQR]. **CH**: Clinical hypercholesterolemia; **FH**: Familial hypercholesterolemia; **LLT:** under lipid lowering therapy; **Non-LLT:** not under lipid lowering therapy; **NS**: Non-significative; **tHcy**: total homocysteine

Serum tHcy levels did not differ among the studied groups. Furthermore, most of patients had tHcy levels within the normal range (5-15 μM).

Five patients (2 Normolipidemic and 3 CH) presented moderate HHcy (16-30 μM) and two (1 CH and 1 FH) had intermediate HHcy (31-100 μM). On the selected population, no severe HHcy (>100 μM) was found.

None of the samples presented a tHcy concentration higher than the HLOQ (50 μM).

The levels of fHcy varied greatly among groups, and it was not quantifiable (<0.625 μM) in 9% of normolipidemia condition and in about 90% of FH patients (*Chi-square test*, p<0.001) (**Figure 3.5**).

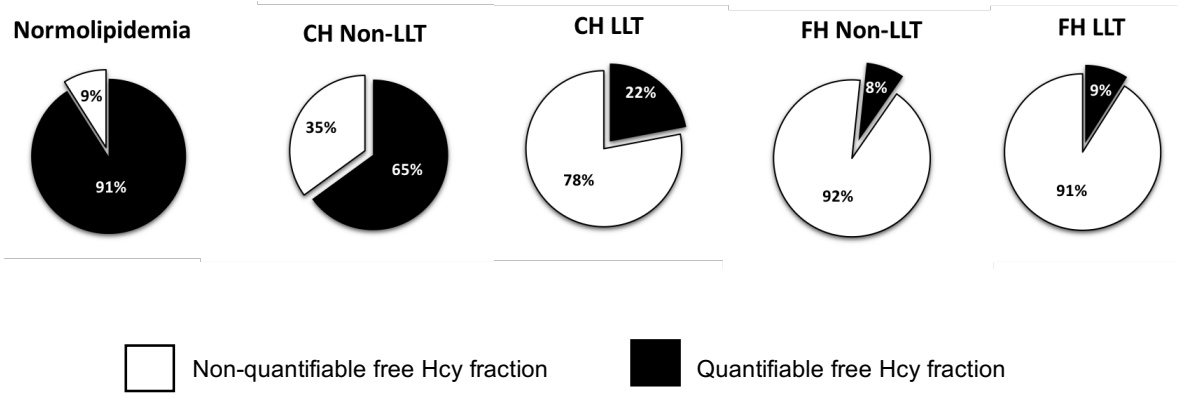

Figure 3.5 Percentage of patients with non-quantifiable levels of free homocysteine (<0.625 μM). *Chi-square test,* p<0.001. **CH:** Clinical Hypercholesterolemia; **FH:** Familial Hypercholesterolemia; **LLT**: Lipid lowering therapy; **Non-LLT**: Not under lipid lowering therapy

Moreover, while the effect of therapy in FH did not influence the percentage of quantifiable fHcy, in CH patients the values dropped from 65% in non-LLT to 22% in LLT group.

In the normolipidemia group, the median [IQR] value of fHcy was 1.5 [1.1-2.1] μM, which corresponds to 15% of tHcy and the median [IQR] value of S-Hcy-protein was 9 [7-11] μM, 85% of tHcy.

3.4 PON1 lactonase activity

Accuracy, intra and inter-assay precision data of the method were higher than 94, 93 and 92%, respectively, for the QCs, LLOQ and HLOQ. Calibration curves were linear (r^2= 0.999 ± 0.0002) and there were no differences between their slopes and elevations (*F*-test).

LACase results are shown in **Table 3.6**.

Table 3.6 Lactonase activity in the study population.

Parameter (Unit)[a]	Normo lipidemia	CH		FH		*p value*[b]
		Non-LLT	***LLT***	***Non-LLT***	***LLT***	
LACase (kU/L)	8 [5-10]	10 [8-12]	10 [8-12]	5 [4-7]	7 [3-9]	$^{+++}$
LACase/ tHcy	0.9[0.5-1.2]	1.1 [0.8-1.3]	1.0 [0.7-1.4]	0.6 [0.4-1.2]	0.7 [0.5-1.2]	ns
LACase/ HDL	0.15[0.11-0.19]	0.22[0.16-0.33]**	0.18[0.12-0.24]	0.11[0.09-0.17]	0.13[0.06-0.19]	$^{+++}$ #
LACase/ ApoA1	0.05[0.04-0.07]	0.07[0.05-0.10]*	0.06[0.04-0.08]	0.04[0.03-0.05]	0.04[0.02-0.06]	$^{+++}$ #

[a]Data presented as Median [IQR]; **Kruskal-Wallis with Dunn's multiple comparisons* test vs Normolipidemic; [b]*Two-way ANOVA with Bonferroni post test*: $^{+}$*CH vs FH* and $^{\#}$ *non-LLT vs LLT in CH.*

ApoA1: Apolipoproteina A1; CH: Clinical hypercholesterolemia; **FH**: Familial hypercholesterolemia; **HDL:** High density lipoprotein; **LACase**: Lactonase; **LLT:** under lipid lowering therapy; **Non-LLT:** not under lipid lowering therapy; **NS**: Non-significative; **tHcy:** total homocysteine

Regarding the LACase activity, the FH group had a lower activity than CH patients (*Two-way ANOVA,* p<0.001) (**Table 3.7**).

CH non-LLT patients had higher LACase/HDL and LACase/ApoA1 ratios than normolipidemic patients (*Kruskal-Wallis*, p<0.01) and presented differences in both ratios towards the treated CH group (*Two-way ANOVA*, p<0.05).

Also, FH patients had lower ratios than CH, regardless of therapy (*Two-way ANOVA*, p<0.001).

4. DISCUSSION

In the present work, the differences in plasma fractions of Hcy among normolipidemic and hypercholesterolemic patients – CH vs FH - were explored by using serum samples from a national survey for the study of cardiovascular disease in Portugal (eCOR and FH Portuguese study). In this regard, an HPLC-FD method for the tHcy and fHcy quantification was optimized and validated.

Most patients had normal tHcy levels and the tHcy concentration was similar among the three study groups. A decrease in the fHcy fraction in hypercholesterolemic populations was found, which was particularly evident in FH, suggesting an increased *S*-homocysteinylation of proteins in this population.

Susceptibility to *N*-homocysteinylation of plasmatic proteins is plausibly also higher in FH population, due to the decreased LACase activity observed, despite normal HDL and ApoA1 levels and regardless of lipid lowering therapy use. This evidence is also indicative that, while HDL quantity is not diminished in this population, its antioxidant and anti-inflammatory function might be compromised.

The data presented herein points towards a higher protein targeting by Hcy in FH as well as the presence of dysfunctional HDL and suggest a role for protein homocysteinylation in hypercholesterolemia pathobiology.

The method developed by Nolin and colleagues (2007) and adapted at the *Translational Pharmacology* lab, used an initial volume of sample of 100 µL. However, in multi-parameter measurement studies, the volume of sample available becomes a limiting step and is imperative to use the least possible volume. So, by necessity of magnifying the volume of sample available for future use, an attempt was made to apply the less volume of biological fluid possible. The reduction was first tested on standards with the initial volume set to 50 µL and half the volumes applied in Nolin's method, for all reagents. The reduction in sample volume represents an advantage of the optimized method.

Although not being very cost-effective relatively to the reagents used, volumes of reagents are in the range of few µL and the HPLC device allows a daily quantification of 50 samples (plus 10 standards and 10 blank samples). It is also a relatively simple method with high reproducibility and a short run time for each sample (20 min). High accuracy, inter-assay precision and stability of solutions assured the validation of the method.

Besides Hcy, four more aminothiols were simultaneously quantified for the 187 samples (Cys, Cys-Gly, GSH and NAC), whose results are not presented herein but represent future work considering the interconversion that exists among these aminothiols and that confers relevance to a simultaneous quantification.

The range of Hcy standard concentrations (0.625 µM to 50 µM) was set according to normal human plasma concentrations detected in different studies. Normal tHcy ranges from 5 to 15 µM and intermediate to severe HHcy conditions (>31 µM) are rare (Hankey and Eikelboom, 1999; Jakubowski, 2008). In populations with known tendency for HHcy such as chronic kidney disease (Zinellu *et al.*, 2010), higher standard concentrations should be considered. However, to the present study it was not necessary and the method demonstrates a good sensitivity towards tHcy quantification.

Considering that 20 to 30% of tHcy circulates in its free oxidized form (homodimers and mixed disufides) a concentration of free oxidized Hcy ranging from 1 to 4.5 μM is expected in circulation (Hankey and Eikelboom, 1999; Jakubowski, 2006; Mudd *et al.*, 2000). The free reduced Hcy fraction, however, is much lower, around 0.1 μM (Jakubowski, 2006) and this could explain the lack of sensitivity to assess the lower concentrations of fHcy present in the hypercholesterolemic populations. Thus, further effort should be put into achieving a LLOQ more adequate to that kind of concentrations.

This method was applied to a cohort of normolipidemic subjects and two hypercholesterolemic populations with genetic and non-genetic background. The study groups were homogenous regarding sex and age of individuals but differed greatly in the percentage of patients under lipid lowering therapy, the highest proportion of treated patients resided at the FH population. More than 70% were under treatment in the FH group. This proportion diminished to approximately 50% in the clinical dyslipidemia.

Statins are one of the most prescribed drug classes nowadays, not only as single medication but also in a combined form with other drugs and this is reflected also in the hypercholesterolemic populations of this study. Statins inhibit cellular cholesterol synthesis and are widely used to reduce LDL in persons with high levels of this lipoprotein and cardiovascular risk (Mackness and Mackness, 2015). Their target is the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Avis *et al.*, 2007), the enzyme that catalyzes the step of HMG-CoA conversion to mevalonate in the cholesterol synthesis pathway and the rate-limiting step of the process (Avis *et al.*, 2007). Unlike general dyslipidemias, it is difficult to treat FH with only dietary habits, physical activity and statin medication. In some cases, a combined therapy of statins with other drugs may be needed to achieve the target levels (Marks *et al.,* 2003). In FH group, a combination of statins and ezetimibe was applied in a greater number of persons than statins only, and that is explained by the fact that LDL levels are more difficult to lower in these patients because of the genetic cause associated that implicates higher LDL levels in circulation than a clinical dyslipidemia. A combined therapy of statins and ezetimibe may reduce LDL levels in 10 to 20% more than a single statin therapy (Kim and Han, 2013) and that is due to ezetimibe mechanism of action that suppresses dietary and biliary cholesterol absorption on the intestine, lowering the income to the liver (Kim and Han, 2013; Marks *et al.,* 2003).

Although the groups were divided according to their TC levels, when analysing **Table 3.4**, it could be observed that, at least in CH and FH groups, TC levels were mostly influenced by raised LDL levels, as opposite to HDL levels that were similar to normolipidemics with exception of non-LLT CH that had a median value lower than the recommended. ApoA1, the apolipoprotein associated with HDL also presented similar values to the normolipidemia condition. In the treatment groups, HDL concentration is restored to normal levels (>40 mg/dL) and ApoA1 even surpasses the levels of normolipidemia condition and remains above the recommended levels (>120 mg/dL – ESC/EAS, 2011). Some hypothesis could explain this raise in HDL and ApoA1 levels. Avis and colleagues reported a “mild but significant elevation” of HDL and ApoA1 levels (3.6 and 2.4%, respectively) based on a pooled data of six studies of statin therapy in FH children (Avis *et al.*, 2007). Three of those studies showed a significant increase for both HDL and ApoA1 after atorvastatin 20 mg (4.6 and 1.9%,

respectively), simvastatin 40 mg (4.7 and 4.8%) and pravastatin 40 mg treatment (9.4 and 5.1%), all of which were also prescribed to CH and FH patients in this study (see Avis *et al.,* 2007). Statins effect on HDL, however, is not unanimous. They are widely prescribed to lower LDL and TC since there are other compounds more suitable when the goal is to raise HDL levels. The drugs of choice for the treatment of hypertriglyceridemia and low HDL are fibrates (fibric acid synthetic derivatives), n3-fatty acids (*eg.* omega 3), nicotinic acid, or derivatives from this compound, which act primarily on the adipocytes (Dierkes *et al*, 2007). Despite the use of fibrates in four CH patients, omega 3 and nicotinic acid also in one each, these frequencies would probably not influence HDL and ApoA1 levels in CH. Besides, in FH group these were not prescribed drugs. The fact that ezetimibe reduces cholesterol absorption from the intestine to the liver influencing hepatic storage and raising cholesterol clearance from the blood, might be an explanation for higher HDL levels. At least two studies involving FH subjects detected a significant raise in HDL and ApoA1 concentration after ezetimibe treatment only (Dujovne *et al*., 2002) and a combined treatment with statin (Gagné *et al*, 2002).

THcy differences among groups were not perceptible, and HHcy was not significant in the study population (only 7 patients presented moderate to intermediate HHcy). Every increase of 2.5 μM in plasma Hcy may be associated with an increase of stroke risk of about 20%.

Although HHcy is a context of concerning, Hcy levels below that mark can also be damaging. For instance, a plasma Hcy concentration of 11 μM is able to inhibit tissue plasminogen activator (t-PA) binding to annexin II (its major endothelial cell receptor) by 50%, a critical loss of fibrinolytic potential and an important factor in the aetiology of vascular disorders (Malinowska *et al*., 2012).

When considering *N*-homocysteinylation, hyperhomocysteinemic concentrations (>15 μM) in the blood are required to favour the HcyTL formation and its reaction with Lys residues of proteins (Yilmaz, 2012). Despite that, when Hcy enters HcyTL pathway, only 10 nM of HcyTL are required to start the acylation of amino residues (Jakubowski, 2008), a concentration quite inferior to the ones required for *S*-homocysteinylation, corroborating the theory that this metabolite is more reactive and toxic than the Hcy thiol itself and more proatherogenic (Yilmaz, 2012).

Even at acceptable tHcy concentrations, a sharp difference among hypercholesterolemia groups when considering Hcy fractions was herein found. There is a very clear loss of fHcy fraction towards the hypercholesterolemic groups, and this is even more evident in the FH population where only one subject from the non-LLT and three from the LLT had fHcy above the LLOQ (0.625 μM).

Therefore, the FH population is particularly prone to protein targeting by *S*-homocysteinylation. In fact, in 1991, Olszewski and McCully had already found that all lipoprotein fractions of hypercholesterolemic subjects contained a considerably higher level of Hcy as opposed to normolipidemic ones, with the largest difference seen in the LDL fraction (Olszewski and McCully, 1991). Hcy incorporation creates altered proteins with newly acquired interactions that can change their structure and be detrimental to the organism (Jakubowski, 2008). It can have several consequences, depending on the protein homocysteinylated. For example, *S*-homocysteinylation of ApoB, a major component of LDL, leads to an increased production of reactive oxygen species (ROS) and cytotoxicity (Jakubowski, 2013).

PON1 has unique characteristics not only protecting LDL and HDL against oxidation, but also its anti-inflammatory and anti-thrombotic functions that are manifested through its different catalytic activities. Interestingly, PON1 has a single free sulfhydryl group at position 284 constituted by a Cys. Cys284 is associated with the enzyme's activity, although it is not part of the active site (Tavori *et al*., 2010). In 1998, Aviram and co-workers suggested that Cys284 participates in LDL oxidation detoxification (Aviram *et al.,* 1998). Thus, PON1 free Cys residue in position 284 might be a target for *S*-homocysteinylation, with consequent inactivation of its antioxidant capacity of protecting LDL against oxidation (**Figure 4.1**).

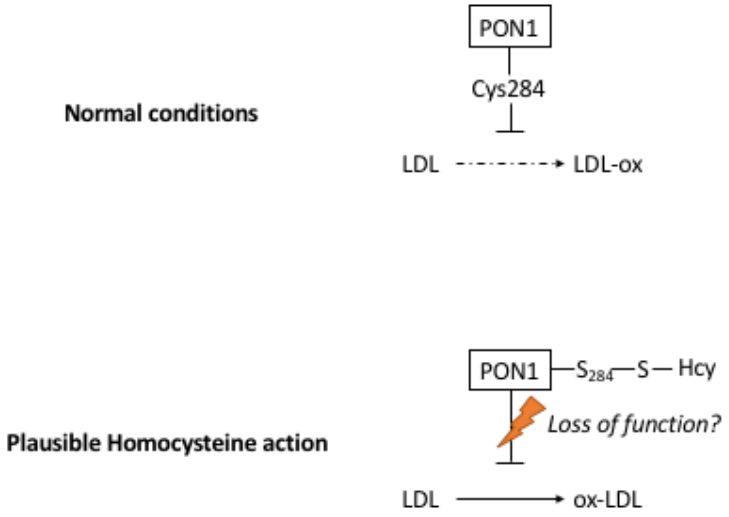

Figure 4.1 Hypothesis of paraoxonase-1 as a target for homocysteine and a plausible explanation for the loss of activity in familial hypercholesterolemia. Cys284: Cysteine residue in position 284; **Hcy:** Homocysteine; **PON1:** Paraoxonase-1; LDL: Low-density lipoprotein; **ox-LDL:** Oxidized low-density lipoprotein.

Dierkes and colleagues did an extensive review about lipid lowering drugs effects on Hcy levels (Dierkes *et al.,* 2007). With respect to statins, the few studies made, came to the same conclusion: they do not influence Hcy levels substantially. A one year long prospective study with lovastatin resulted in a small Hcy reduction and another with concurrent supplementation of vitamins and statins had the same degree of reduction in both groups, revealing the lack of relevance of the statin component.

As to fibrates, numerous short and long-term studies on fenofibrate and other fibric acid derivatives revealed an elevation of Hcy from 40 to 50% (see Dierkes *et al*., 2007). This review enclosed also nicotinic acid and n3-fatty acids, both giving less clear responses of their action over Hcy.

Unfortunately, there are no studies concerning the effects of LLT on the free and protein-bound fractions of Hcy. Plausibly, *S*-Hcy-protein targeted therapeutic would have a role in hypercholesterolemia treatment.

The assessment of LACase activity existent in PON1 enzyme, using a method previously developed and validated at *Translational Pharmacology* lab (Dias *et al.*, submitted) revealed significant differences between disease groups ($p<0.001$).

Currently, it is known that HDL functionality is a far better indicator of its ability to protect against atherogenesis than its concentration levels (Mackness and Mackness, 2013). This concept first arose from observations that some individuals with high or normal HDL were more susceptible to CHD development if they had low PON1 activity than others with high PON1 activity even if HDL concentration was low (Navab *et al.*, 1997).

The FH population showed a normal HDL and ApoA1 concentration that was slightly improved by LLT but in opposite had decreased LACase activity. The LACase/HDL and LACase/apoA1 ratios corroborate the presence of equal quantity of HDL but dysfunctional performance regarding LACase activity.

Considering the importance of this enzyme to detoxify HcyTL deleterious action in the organism and other endogenous lactones, specifically its pro-thrombotic and pro-inflammatory effects and the fact that this population has, by default, atherogenic factors in circulation, leads to the observation of a very sensitive and unprotected group of subjects, as documented by Ferretti and co-workers who observed that an HDL with low PON1 activity is more susceptible to homocysteinylation than an HDL with high PON1 activity (Ferretti *et al*., 2003).

Not only is this population more susceptible to *S*-homocysteinylation of proteins, as was concluded before, but is also clearly unprotected towards *N*-homocysteinylation. *N*-Hcy-proteins elicit an autoimmune response in humans that is enhanced in cardiovascular disease patients (Jakubowski, 2006). Unlike tHcy, HcyTL is effectively eliminated in the urine. HcyTL concentrations in the urine (11-485 nM) are around 14 times greater than plasma HcyTL levels (0-35 nM) (Jakubowski, 2006). Urinary excretion is consistent with the hypothesis of HcyTL being a toxic metabolite in the organism.

Several research groups have been dedicated to evaluate statins effect on PON1 activity, specifically the two most studied, paraoxonase (POase) and arylesterease (AREase) activities. However, to our knowledge, there are no studies indicating the effect they would have on LACase activity. In the future, it would be pertinent to understand what kind of effect, if any, they have on this activity of PON1, in a wider population.

The data herein obtained supports that the pathophysiological role of Hcy on hypercholesterolemia may reside in its ability to post-translationally modify tissue proteins through *S* or *N*-homocysteinylation at acceptable tHcy concentrations. It also emphasizes the limited value of tHcy concentration by itself.

In the future, it will be interesting to identify which target proteins are modified and thus involved in vascular pathology onset and progression. Also, the data herein obtained show that protein molecular targeting by Hcy differs among clinical and familial hypercholesterolemic conditions, giving future perspectives for its use as biomarkers to predict risks and to guide therapeutic decisions.

Adding to these selected 187 subjects, 1106 individuals from four regions of the eCOR study have all three PON1 activities quantified: LACase, POase and AREase. Both POase and AREase protocols are very similar to the one described in this thesis, with a very small volume of sample required to do a colorimetric assay. Briefly, POase is the activity that metabolises paraoxon, the toxic metabolite of the insecticide parathion that has a potent acetylcholinesterase inhibition effect on animals and humans (Kim *et al*., 2013). The AREase activity facilitates the detoxification of aromatic esters, being phenyl acetate one of its best substrates (Draganov *et al.*, 2005). All three activities have characteristics that confer PON1 a significant relevance in HDL's antioxidant effects on the organism.

In order to fully validate the accuracy and utility of these biomarkers, the future work comprises the quantification of all Hcy fractions in the remaining subjects and the correlation of those values with PON1 activity results and clinical data.

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6. ANNEXES

Annex 1. Familial hypercholesterolemia diagnostic criteria based on Simon Broome Heart Research Trust (adapted from Bourbon and Rato, 2006).

<table>
<tr><th></th><th colspan="2">Possible Familial Hypercholesterolemia</th><th colspan="2">Confirmed Familial Hypercholesterolemia</th></tr>
<tr><td>Children under 16</td><td>TC>200 mg/dL or LDL>120 mg/dL</td><td rowspan="2">• Family history of myocardial infarction before the age of 50 in grandparents, aunts or uncles or before the age of 60 in parents, siblings or children
• Family history of hypercholesterolemia</td><td>TC>200 mg/dL or LDL>120 mg/dL</td><td rowspan="2">• Tendon xanthoma in the index case or relative
• Genetic evidence of a mutation in the LDL receptor gene or APOB
• Family history of hypercholesterolemia</td></tr>
<tr><td>Adults</td><td>TC>290 mg/dL or LDL>190 mg/dL</td><td>TC>290 mg/dL or LDL>190 mg/dL</td></tr>
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