**UNIVERSITY OF VERONA**

**SCHOOL OF MEDICINE**
**DEPARTMENT OF MEDICINE**
**ITALY**

# "Plasma Proteomics and Lipid Profiles Analysis in Patients Affected by Coronary Artery Disease: Focus on Apolipoprotein C-III"

SSD MED 09

## CYCLE XXIX

**PhD IN BIOMOLECULAR MEDICINE**

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TABLE OF CONTENTS:

## *Riassunto*

**Riassunto:** Negli ultimi dieci anni è stato dimostrato come l'apolipoproteina C-III (Apo C-III), sulla base della forte correlazione che c'è tra i livelli plasmatici di Apo C-III e l'alta concentrazione sierica dei trigliceridi nell'uomo, sia un marker prognostico per il rischio cardiovascolare. A tal proposito la struttura molecolare dell'Apo C-III potrebbe essere rilevante. Tuttavia pochi sono gli studi che hanno cercato di elucidare l'impatto che la concentrazione dell'Apo C-III ed i suoi cambiamenti strutturali (sialilazione) hanno sul metabolismo lipidico delle lipoproteine ed inoltre dimostrare come l'Apo C-III possa influenzare l'andamento dei parametri associati alla patologia coronarica arteriosa (CAD).

**Metodi:** Tre diversi gruppi di pazienti CAD, attentamente selezionati tra i soggetti arruolati nel progetto Verona Hearth Study, sono stati analizzati con tecnologie di proteomica e lipidomica. L'isoelectrofocusing e l'analisi shotgun topdown MS sono state applicate per l'identificazione e la quantificazione delle tre differenti glicoforme dell'Apo C-III. Il mono e bidimensionale western blotting sono stati utilizzati per la validazione delle proteine precedentemente identificate come diversamente espresse in base ai livelli di Apo C-III mediante analisi compartiva.
Il profilo proteomico totale dei pazienti CAD è stato ottenuto tramite l'analisi SWATH, un approccio untargeted. L'analisi di Gas-Cromatografia e Liquid-Cromatografia-MS ha permesso la caratterizzazione lipidomica dei pazienti CAD e CAD free.

**Risultati:** Le tre diverse glicoforme dell'Apo C-III mostrano un andamento particolare, la forma non sialilata presenta, infatti, un andamento negativo con gli altri parametri mentre la monosialilata ha un andamento positivo con tutti i parametri. Nessuna correlazione è stata osservata per la forma disialilata. L'analisi di validazione ha confermato i risultati dell'analisi comparativa. L'analisi SWATH sottolinea un particolare set di proteine associate con bassi ed alti livelli di Apo C-III. Mentre l'approccio lipidomico sottolinea come in base ai livelli di Apo E nei pazienti CAD e CAD free è possibile osservare uno specifico profilo lipidico, in particolare ad alti livelli di Apo E è associata la presenza di esteri del colesterolo nella forma ossidata.

**Conclusioni:** Nonostante lo studio sia stato condotto su una piccola popolazione, permette nei pazienti CAD attentamente selezionati una caratterizzazione del ruolo dell'Apo C-III e della distribuzione delle sue glicoforme. L'analisi SWATH ha rilevato un set di proteine che caratterizza i pazienti con alti e bassi livelli di Apo C-III. In fine l'approccio lipidomico ha sottolineato come nella patologia CAD un ruolo importante potrebbe essere giocato non solo dalla distribuzione delle apolipoproteine ed i comuni parametri lipidici osservati ma anche da peculiari specie lipidiche. Ulteriori analisi, con un a popolazione più grande, sono ancora richieste per validare i nostri risultati

## *Abstract*

**Background**: In the last decade Apolipoprotein C-III (Apo C-III) has been demonstrated to be a prognostic marker for cardiovascular risk on the basis of the strong correlation between Apo C-III plasma levels and high serum concentrations of triglyceride in humans. In such perspective, the molecular structure of Apo C-III may be relevant for its role

Overall, very few studies tried to elucidate the impact of the Apo C-III concentration and modifications (sialylation) on lipoproteins and lipid metabolism and how Apo C-III can affect the outcome associated to Coronary Artery Disease (CAD) parameters.

**Methods:** Three different groups of CAD patients, carefully selected among subjects enrolled in the Verona Heart Study project, were studied by means of proteomics and lipidomics technologies. Isoelectrofocusing and shotgun topdown MS approach were applied for the identification and quantification of the three different Apo C-III glycoforms. Mono and bidimensional western immunoblotting were performed in order to validate protein previously found by comparative analysis differentially expressed according to Apo CIII levels. A total proteomic profile of CAD patients was obtained by SWATH, an untargeted approach, analysis. Gas-Chromatography and Liquid-Chromatography-MS analysis allowed a lipidomic characterization of CAD and CAD free patients.

**Results:** The three Apo C-III glycoforms showed a peculiar trend, where the non-sialylated form presented a negative correlation with the others parameters, instead the monosialylated a positive one. No correlations with the disialylated glycoforms were found. The validation analysis confirmed the comparative analysis results. The SWATH analysis underlined a peculiar set of proteins associated with low and high Apo C-III levels. The lipidomic approach underlined how according to Apo E levels in CAD and CAD free patients it is possible to observe a peculiar lipid profile, in particular high levels of Apo E are associated with the presence of cholesteryl ester oxidized.

**Conclusion**: In spite of the relatively small sample size, the study allowed a multifaceted characterization of Apo C-III and Apo C-III glycoforms distribution in CAD patients. SWATH analysis revealed a set of proteins characterizing patients with high and low levels of Apo C-III. The lipidomic approach underlined that not only the apolipoproteins distribution and common lipids parameters but also some peculiar lipid species may play an important role in CAD. To validate the present results, further analysis are however required, possibly on larger populations of patients .

## *Abbreviations list*

*Apo A1: Apolipoprotein A1*

*Apo AV: Apolipoprotein AV*

*Apo B:Apolipoprotein B*

*Apo C-II: Apolipoprotein II*

*Apo C-III: Apolipoprotein C-III*

BHT: *Butylated hydroxytoluene*

*CAD: Coronary Artery Disease*

*CE: Cholesteryl ester*

*DDA: Data Dipendent Analysis*

*DIA: Data Indipendent Analysis*

*GS: Gas Chromatography*

*HDL: High Density Lipoproteins*

*LysoPC: Lysophosphatidylcholine*

*LDL: Low Density Lipoproteins*

*LPL: Lipoprotein Lipase*

*LV: Latent Value*

*OPLS-DA: Orthogonal Principal Component Analysis-Discriminant Analysis*

*PC: Phosphatidylcholine*

*PCA: Principal Component Analysis*

*PLS: Partial Least Squares*

*PLS-DA: Principal Component Analysis-Discriminant Analysis*

*RP-LC-MS: Reverse Phase-Liquid Chromatography-Mass Spectrometry*

*SWATH: Sequential Window Acquisition Of All Theoretical Fragment Ion Spectra*

*TG: Triglycerides*

*TAG: Triacyl Glycerole*

*VHS: Verona Hearth Study*

*VLDL: Very Low Density Lipoproteins*

# *1.Introduction*

## 1.1. Coronary artery disease and risk factors

Coronary artery disease (CAD) is multifactorial condition, involving both genetic and acquired risk factors in turn interacting to determine the development of the disease. Typically, coronary artery disease occurs when part of the smooth, elastic lining inside a coronary artery develops atherosclerosis.

Atherosclerosis is a multifactorial process in whom a stringent relationship between plasma lipid levels and cardiovascular disease (CVD) risk has been widely recognized. (Lagrost, Gambert et al. 1994, Berliner and Heinecke 1996, Sharrett, Ballantyne et al. 2001),

If the crucial role of cholesterol concentrations is a long-lasting, well established notion, triglycerides (TG) levels and TG-rich lipoproteins have been only recently accepted and confirmed as an additional important risk factor for CVD (Luo and Peng 2016). A relevant support for this evidence, it was played by genome wide association studies (GWAS) that have identified some single nucleotide polymorphisms (SNPs) associated with both TG levels and CVD (Do, Willer et al. 2013) In particular, among the candidate genes associated with TG plasma levels, recent observations revealed that loss-of-function (LOF) mutations in the gene encoding apolipoprotein C-III (Apo C-III) are associated with low levels of TG, and decreased CVD risk (40–41 %) (Jorgensen, Frikke-Schmidt et al. 2014, Tg, Hdl Working Group of the Exome Sequencing Project et al. 2014)

## 1.2. Apolipoprotein C-III

In the last decade Apolipoprotein C-III (Apo C-III) has been demonstrated to be a prognostic marker of cardiovascular risk (Olivieri, Stranieri et al. 2002, Olivieri, Bassi et al. 2003, Olivieri, Martinelli et al. 2010). Apo C-III is a small 8.8kDa- 79 amino acids, protein, synthetized in the liver and in a lesser extent in the intestine; although in different proportions, it is present on the surface of the low density lipoprotein (LDL), very low density lipoprotein (VLDL) and high density

lipoproteins (HDL) (Tg, Hdl Working Group of the Exome Sequencing Project et al.). Overall, it is more abundant on Triglyceride-rich particles/lipoprotein (TRL) and for this reason it is considered mainly a marker of these lipoproteins and a primary player of their metabolism (Figure 1). In particular, Apo C-III affects the TRL metabolism through 3 different actions: a) by interfering with Apo B binding to hepatic Apo B/E receptors, as demonstrated by experiments in mouse models (Aalto-Setala, Fisher et al. 1992, de Silva, Lauer et al. 1994, Aalto-Setala, Weinstock et al. 1996) and in humans (Zheng, Khoo et al. 2007, Mendivil, Zheng et al. 2010, Zheng, Khoo et al. 2010); b) by inhibiting the clearance of TRL by lipoprotein lipase (LPL) *in vitro* (Brown and Baginsky 1972, Wang, McConathy et al. 1985, Yamamoto, Morita et al. 2003) and *in vivo*, although the finding is much more controversial; c) by stimulating VLDL liver secretion (Sundaram, Zhong et al. 2010, Yao 2012).

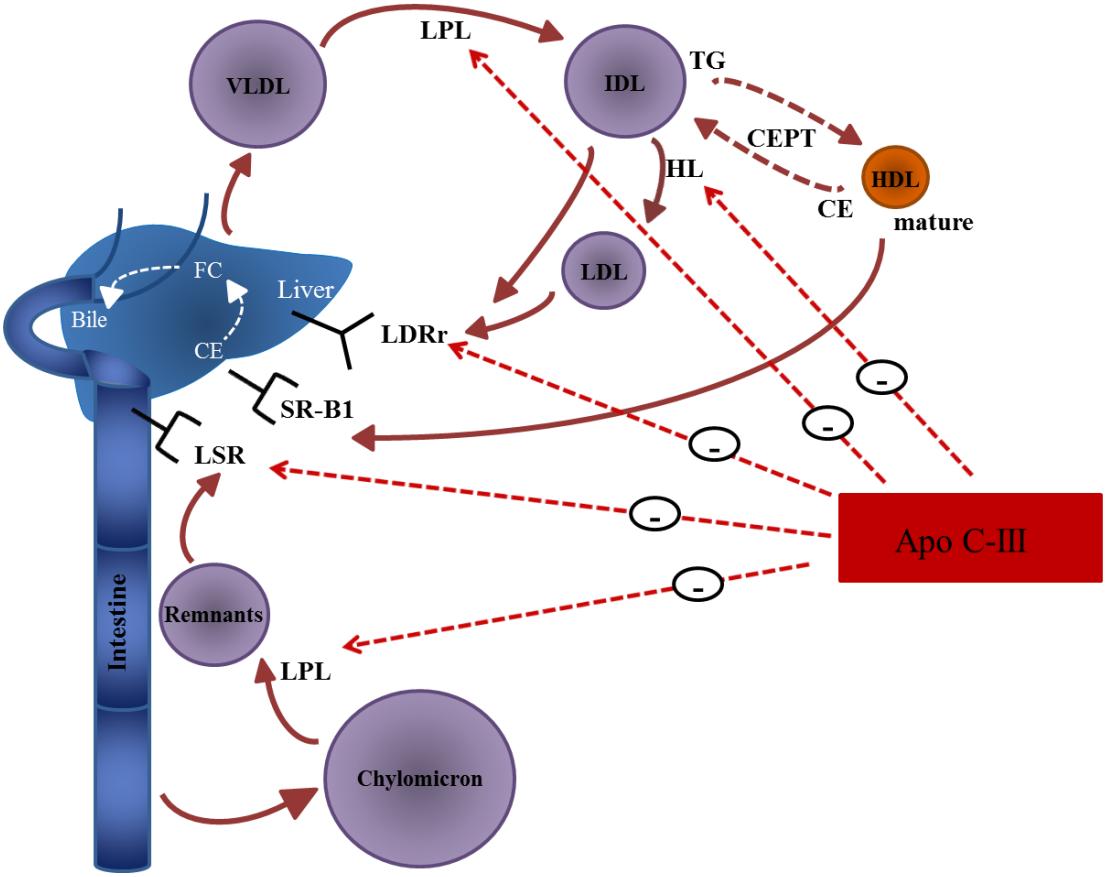


**Figure 1**. Apo C-III effects on the lipoproteins metabolism

All of these findings support a strong correlation between Apo C-III plasma levels and high serum concentrations of TG in humans (Batal, Tremblay et al. 2000, Cohn, Patterson et al. 2004, Zheng, Khoo et al. 2007, Zheng, Khoo et al. 2010).

In addition to the "lipid" effects, several pieces of evidence support a pro-inflammatory role of Apo C-III in atherogenetic process, i.e by stimulating monocytes adhesion to endothelial cells and by inducing inflammatory mediators production in these cells (Kawakami, Aikawa et al. 2006, Kawakami, Aikawa et al. 2006).

### 1.3 Relation of apolipoprotein C-III, with other players: TG, PUFA, LPL, Apo AV

Apo C-III seems to be able to delay the clearance of TRLs by inhibiting lipoprotein lipase (LPL) (Gangabadage, Zdunek et al. 2008, Ooi, Barrett et al. 2008). For this reason, it is a key regulator not only of fasting but also of postprandial TG levels (Windler and Havel 1985, Zheng, Khoo et al. 2010). On the other hand it has been shown that polyunsaturated fatty acids (PUFA) have the ability to decrease the Apo C-III concentrations by means of their agonist activity on peroxisome proliferator receptor α (PPAR-α) (Jump, Tripathy et al. 2013), an inhibitor of *APOC3* gene transcription (Ooi, Barrett et al. 2008). Previous in vitro study showed, in fact, evidence of an Apo C-III-lowering effect generally exerted by n-3 FAs (Schoonjans, Staels et al. 1996). It could be supposed that this Apo C-III-lowering effect could cause a consequent decrease in TG plasma levels avoiding the development of the atherosclerosis lesions. Nevertheless not all the patients take advantages by these beneficial effects of PUFAs because individuals carrying some genetic variants (i.e. T/C -455 on *APOC3*) on the promoter of *APOC3* gene appear insensitive to the inhibitory modulation of PUFA (Olivieri, Martinelli et al. 2005).

Another candidate gene that plays an important role in the metabolism of TG is Lipoprotein Lipase (LPL). LPL has both pro- and anti-atherogenic roles. The anti-atherogenic role is mostly due to the plasmatic LPL, whereas the pro-atherogenic role is mediated by LPL derived from vessel endothelial cells and macrophages (Goldberg 1996).

The activator apolipoprotein C-II (Apo C-II), located on the substrate lipoproteins, and glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1), an endothelial membrane protein, are needed for translocation of LPL from parenchymal cells (e.g. myocytes, adipocytes) where it is produced to the vascular surface of the capillary endothelium, where the enzyme acts (Young and Zechner 2013). Lack of any of these components results in severely impaired lipoprotein catabolism with massive hypertriglyceridemia (Hultin, Savonen et al. 2013). Several other proteins influence these interactions, notably Apo C-III which inhibits and Apo AV which enhances lipolysis (Hultin, Savonen et al. 2013, Young and Zechner 2013).

*APOA5* gene is located closely to *APOC3* gene on chromosome 11q23 in the so called "*APOA1-C3-A4* gene cluster" (Pennacchio, Olivier et al. 2001); since its discovery, apolipoprotein AV (Apo AV) has been recognized to be a potent factor affecting plasma TG concentrations in humans and mice. Apo AV is present in TG rich (chylomicrones and VLDL) and in HDL particles. In comparison with other apolipoproteins, Apo AV plasma concentrations in humans are very low– about 100 μg/l (O'Brien, Alborn et al. 2005). Others studies have shown that the binding with Apo AV enhances the activity of LPL (Fruchart-Najib, Bauge et al. 2004, Schaap, Rensen et al. 2004) and that the polymorphic variants in the *APOA5* gene are associated with increased plasma TG levels (Pennacchio, Olivier et al. 2002, Lai, Demissie et al. 2004, Wright, Young et al. 2006, Grallert, Sedlmeier et al. 2007, Johansen, Wang et al. 2010, Zhao and Zhao 2010). Furthermore, mutations in the *APOA5* gene leading to Apo AV deficiency are associated with severe hypertriglyceridemia in humans (Kluger, Heeren et al. 2008). *APOA5* gene variants have been associated to insurgence of CAD and other manifestations of atherosclerosis (Hsu, Ko et al. 2006, Willer, Sanna et al. 2008, Laurila, Naukkarinen et al. 2010, Triglyceride Coronary Disease Genetics, Emerging Risk Factors et al. 2010).

Since the identification of Apo AV as an important factor in plasma TG metabolism, three hypotheses explaining Apo AV action have been proposed: (1) Apo AV acts through an intracellular mechanism involved in VLDL synthesis and/or secretion;. (2) Apo AV accelerates the

hydrolysis of TRL by affecting LPL activity directly or by indirectly modifying other regulatory apolipoproteins of LPL, such as Apo C-III. (Atwood, Cheng et al.); 3) Apo AV acts as a ligand for lipoprotein receptors or proteoglycans and thereby promotes receptor-mediated endocytosis of lipoproteins.

### 1.4. Apolipoprotein C-III glycoforms

In the functional perspective above described, the molecular structure of Apo C-III may be of relevance. Apo C-III is present mainly in 3 isoforms termed Apo C-III0, Apo C-III1, and Apo C-III2, depending on the number of sialic acid molecules (0 to 2) at the oligosaccharidic portions of the protein (Nicolardi, van der Burgt et al. 2013). It has been estimated that each isoform may physiologically contribute, respectively, to approximately 10, 55, and 35% of the total circulating Apo C-III levels (Nicolardi, van der Burgt et al. 2013), but some doubts about such proportions as well as their relative impact on the functional activity of the TRLs still remain. In fact, overall, very few studies tried to elucidate the impact of the Apo C-III sialylation on lipoproteins and lipid metabolism.

Results of GWAS have identified GALNT2 as a candidate gene in lipid metabolism and as a link between glycosylation and atherosclerosis GALNT2 codifies for N-acetylgalactosaminyltransferase 2 (ppGalNAc-T2 ) that transfers an N-acetyl galactosamine to the hydroxyl group of a serine or threonine residue in the first step of O-linked oligosaccharide biosynthesis (Bennett, Weghuis et al. 1998). The ppGalNAc-T2 belongs to a family of ppGalNAc transferases comprising 20 members in humans (Kosmas, Christodoulidis et al. 2014), all catalyzing the transfer of GalNAc residues onto proteins.

SNPs in intron 1 of GALNT2 were found to be associated with plasma high-density lipoprotein cholesterol (HDL-c) and triglyceride levels (Ten Hagen, Fritz et al. 2003). A missense mutation in GALNT2 causing a reduction of ppGalNAc-T2 catalytic activity has been associated with reduced TG, increased HDL and improved postprandial TG clearance in carriers of the mutation. The data suggest that

this enzyme mediates these effects through glycosylation of Apo C-III, an established inhibitor of LPL. Since Apo C-III is a specific substrate for ppGalNAc-T2, it was studied whether sialylation of the sole O-linked glycan of Apo C-III might affect the enzyme affinity. It was shown that the neuraminidase treatment resulted in a shift from the acidic Apo C-III isoforms to Apo C-III0 due to the loss of sialic acids, resulting in a significant reduction of the potential of Apo C-III to inhibit human recombinant LPL activity (Kosmas, Christodoulidis et al. 2014).

Thus, ppGalNAc-T2 can affect plasma lipids through posttranslational modification of Apo C-III. Taking into account these consideration the evaluation of the isoforms abundance could be useful to get new insights into the interplay between the molecules involved in the lipid metabolism and CAD. The process of sialylation of Apo C-III could be able to modulate VLDL and LDL particle size, and in turn their metabolism, leading to a longer residence time of TRL and TRL remnants in the circulation. Moreover, the dynamic process determining the relative amounts of Apo C-III and Apo E carried by TRL may be influenced by the presence of sialylated isoforms of Apo C-III, due to the fact that Apo C-III is generally physiologically associated with relevant amount of Apo E on TRL (Campos, Perlov et al. 2001, Zheng, Khoo et al. 2007, Mendivil, Zheng et al. 2010, Zheng, Khoo et al. 2010). In comparison to Apo C-III, Apo E plays indeed balanced and opposite roles on circulating LPL and with regards to its receptor ligand activity, as Apo E favors the lipolytic process and the clearance of TRL (Handattu, Nayyar et al. 2013, Mendivil, Rimm et al. 2013, White, Garber et al. 2014). Since Apo E and Apo C-III are not uniformly distributed among Apo B-containing VLDL and LDL, the corresponding ratio with Apo B may also change according to the amount and characteristics of the Apo C-III isoforms present.

### 1.4.1 Methods for Apolipoprotein C-III isoforms assay

The first reports regarding Apo C-III isoforms were based on the use of Isoelectric focusing (IEF) (Catapano, Jackson et al. 1978) on the basis of the observed unsatisfactory resolution in Apo C-III

isoforms separation gave by polyacrylamide gel electrophoresis in either SDS or 8M urea (Kane, Sata et al. 1975, Carlson and Ballantyne 1976, Schonfeld, Weidman et al. 1976).

In more recent years other researchers applied IEF for the study of Apo C-III isoforms, (Wopereis, Grunewald et al. 2003, Wada, Kadoya et al. 2012), connecting the IEF to immunoblotting by diffusion. Nevertheless, electrophoresis techniques like IEF are only semiquantitative and lack the capability to differentiate electrically neutral sugar moieties, electrically neutral amino acid substitutions, oxidation, and some others post-translation modifications (Haase, Menke-Mollers et al. 1988). Due to these limitations, other technologies were applied to the analysis of Apo C-III isoforms; in particular mass spectrometry was proven successful in identifying all the isoforms species (Bondarenko, Cockrill et al. 1999, Zhang, Sinaiko et al. 2012, Jian, Edom et al. 2013, Nicolardi, van der Burgt et al. 2013).In spite of this methodological success, the occurrence and the role of the different isoforms in patients or healthy subjects is still a matter of debate since not many studies are available so far (Yassine, Trenchevska et al. 2015, Koska, Yassine et al. 2016).

### 1.5. Omics approaches for biomarkers identifications in cardiovascular diseases

Omics technologies such as proteomics, matabolomics and lipidomics can be very useful for the discovery and measurements of biomarkers in cardiovascular diseases (Hoefer, Steffens et al. 2015). These approaches can be directly applied to the study of existing samples collections (Hoefer, Steffens et al. 2015).

#### 1.5.1. Proteomics

Proteomics has recently benefit from advances in method development, especially bioinformatics and instrument speed, sensitivity and resolution of mass spectrometers, for the prediction of the onset of CVD (Beck HC 2015). Several proteomics platforms are nowadays available and their selection is dependent on the type of sample and proteins to investigate (Tunon, Martin-Ventura et al. 2010, Langley, Dwyer et al. 2013, Hoefer, Steffens et al. 2015, Mesaros and Blair 2016). There

are, in general two mass spectrometry (MS) based approaches: the untargeted discovery approach (analysis with no *a priori* assumption) and the targeted MS approach (analysis of pre-selected panel of proteins with high precision, e.g. Multiple Reaction Monitoring). A combination of these two approaches is still the most comprehensive strategy for the discovery of new biomarkers by proteomics. In cardiovascular disease, plasma proteomics analysis has been applied for the discovery and measurements of new potential biomarkers (Beck HC 2015, von Zychlinski A 2015).

### 1.6. Lipidomics and CAD

Lipidomics or the "system-level analysis and characterization of lipids and factors that interacts with them" (Wenk 2005, Lisa, Cifkova et al. 2011) is an emerging area within the field of "omics" sciences. Lipids are involved in many cellular processes and aberrant lipid metabolism are the basis of some important diseases, such as diabetes, atherosclerosis, obesity, Alzheimer's disease and some cancer types (Wenk 2005). This is the reason why the analysis of the lipidome and the complete set of lipids within a cell, tissue or organism, is of great interest.

Among circulating metabolites in plasma, lipid molecules have a critical role in atherosclerosis and a profound effect on the development of cardiovascular disease (Parish, Offer et al. 2012). The application of lipidomic technologies to the study of cardiovascular disease will increase our understanding of the pathophysiological process providing a deeper insight into the patient's lipidome and possibly lead to identification potential lipid biomarkers to develop new therapeutic strategies (Giovane, Balestrieri et al. 2008, Ekroos, Janis et al. 2010).

Mass spectrometry (MS) is the most commonly used analytical technique in lipidomics research; gas chromatography mass spectrometry (GC)-MS and liquid chromatography mass spectrometry(LC)-MS, are mainly applied, each method having its own advantages and disadvantages.

GC-MS is better for the analysis of lipids such as free fatty acids (FFAs) and steroids. Generally, FFAs and steroids are analyzed by transforming the compounds into volatile esters. For the analysis

of phospholipids, neutral lipids, and sphingolipids, which are greater in molecular weight and less volatile than FFAs and steroids, LC-MS is mainly used.

## 2. AIM OF THE THESIS

As Apo C-III plays an important role in the determination of the risk of CAD in both settings of primary and secondary CVD prevention, this research project had, as its principal aim, the molecular characterization of CAD patients stratified according to Apo C-III levels.

This characterization was obtained by the accomplishment of the following main objectives:

√ to analyze Apo C-III glycoforms initially by isoelectric focusing and subsequently by high resolution mass spectrometry in CAD patients stratified according to total concentration of Apo C-III.

√ To validate by Western Immunoblotting 5 proteins (fibrinogen γ and γ' chain, fibrinogen β, complement C3, serum amyloid protein) found to be modulated in CAD patients characterized by high and low levels of Apo C-III and PUFA

√ To study the lipoprotein lipase activity in CAD patients and correlate it with its major interacting factors (Apo C-III, Apo AV, etc)

√ To analyze by "bottom up approach" the plasma proteomic profiles of CAD patients stratified according to total concentration of Apo C-III

√ To investigate the plasma lipidomic profiles of patients (CAD and CAD free) carefully selected according to triglycerides, Apo C-III and Apo E levels.

# 3.Material and Methods

## 3.1. Selection of patients

We selected plasma samples of unrelated patients affected by CAD, who were previously enrolled in the Verona Heart Study (VHS) a cross-sectional and prospective population study with angiographically documented presence/absence of CAD (Olivieri, Martinelli et al. 2013).

All the samples were collected following a standardized protocol. The plasma samples were prepared by collecting blood from each person after an overnight fast. Venous blood was collected into Vacutainer® tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged at 2800 g (3500 rpm) for 15 minutes at 4˚C. The plasma was separated from cellular elements (erythrocytes, leukocytes and platelets) and stored with protease inhibitors at -80˚C until analysis. The lipid distribution (total cholesterol, triglycerides, HDL, LDL) was determined on each patient's plasma, according to routine standard procedures. At the time of blood sampling, a complete clinical and pharmacological history, including the presence or absence of cardiovascular risk factor such as smoking, hypertension and diabetes mellitus, was obtained from the patients.

The study was approved by the institutional review boards of the Azienda Ospedaliera Integrata of Verona. Written informed consent was obtained from all the patients.

We selected three different groups of patients belonging to the VHS population, accordingly to the different study aims:

- For the analysis of the distribution of the three Apo C-III glycoforms by isoelectrofocusing and the validation of the differentially expressed protein by western blotting, we selected 26 CAD patients (100% male; mean age 54.96±10.98). These patients were also subdivided in four groups according to total Apo C-III plasma concentration and lipid profile i.e PUFA plasma concentrations.
- For the MS quantitative analysis of the three Apo C-III glycoforms and SWATH analysis for the total proteomic profile study, a group of 51 CAD patients (mainly males 90.2%,

mean age 59.6 ± 8.1 years), subdivided according to total plasma Apo C-III concentrations, was selected.

- For the lipidomic analysis a group of 39 patients (17 CAD and 22 CAD free patients) (48.7% male, mean age 65.59±7.91), were selected. Such population was stratified according to Apo C-III, Apo E and TG plasma level creating six different groups of survey with different combinations of the three different selected parameters.

### 3.2. Apolipoprotein assays

Plasma concentration of total Apo C-III was determined using an automated turbidimetric immunoassay (Olivieri, Martinelli et al. 2010); the reagents were obtained from Kamiya (**KAI-006** (Tris buffer + goat antiserum) Seattle, WA, USA), and the procedure recommended by the manufacturer was implemented on an auto analyzer **COBAS e501** (Roche). Imprecision was assessed on three pools of control sera with low, medium, and high concentrations of Apo C-III; intra-assay variation coefficients were 1.84%, 2.02%, and 1.98%, and inter-assay variation coefficients were 4.4%, 3.4%, and 2.29% for low, medium, and high concentration, respectively (Olivieri, Bassi et al. 2003). Based on the distribution of the apolipoprotein on the whole VHS population, values <9.2 mg $dL^{-1}$ (25th percentile) and ≥ 12.6 mg $dL^{-1}$ (75th percentile) were considered "LOW" and "HIGH" values, respectively. Briefly sample values were determined by interpolation of two spectrophotometric wavelengths measurements on a logit, five-points, calibration curve, covering the range 0.0-20.0 mg $dL^{-1}$; for concentrations of 20-30 mg $dL^{-1}$, a smaller sample volume was automatically rerun by the instrument, whereas for concentrations >30 mg $dL^{-1}$, the sample was manually diluted (Olivieri, Stranieri et al. 2002).

By nephalometric assay on a nephelometer BNII Siemens (Erlangen, Germany) we also evaluated the Apo A-I, Apo B and Apo E plasma concentrations.

### 3.3. Fatty acids assay by gas-chromatography

**Plasmatic FA extraction and analysis**

For the fatty acids extraction 100 µl of plasma sample were added to 10 µl of internal standard (C:17, Sigma) to avoid the possible loss of small quantity of sample that could occur during the procedure making the results comparable. Three standard solution, as calibrator, were also prepared adding, to the 10 µl of the internal standard, 100 µl of a mix solution of known quantity of fatty acids.

To each sample 2 ml of reaction solution with 1.9 ml of methanol and BHT (butyl-hydroxy-toluene 50 mg/L, Sigma) and 100 µl of acetyl-chloride were added. After an incubation of one hour at 100˚C, to allow the trans methylation reaction, and a cooling phase at RT, 1 ml of water and 1 ml of esane were added to each sample. Then the samples were vortexed and centrifuged at 2500 rpm per 2 min. During this passage we have two phases formation, the hydrophilic one on the bottom and the hydrophobic one in the upper side. The supernatant was collected and underwent another step of fatty acids extraction through esane to collect all the traces of fatty acids present. Then the total solution of extraction was collected and dried at 37˚C by UniVapo (GeneVac); the dried pellet was solubilized in 500 µl of esane and transferred into gas-chromatography vials.

Analyses of the plasma phospholipids fatty acids were performed on total lipids extracted using a gas-chromatographic method as de[Hewlett Packard 5890 chromatograph equipped with an HP-FFAP phase column (length 25m, internal diameter 0.2 mm, phase column 0.3 µm); Hewlett Packard, Palo Alto, CA, USA] based on a direct fatty acid transesterification technique, as previously described (Olivieri, Stanzial et al. 1994). The peaks were identified and quantified by comparison with commercially available reference fatty acids (Sigma,St. Luis, MO, USA). Fatty acid C17:0 was used as the internal standard. The areas of the peaks were measured and subsequently quantified using a PC Vectra QS/16S equipped with HP-3365 Chem Station software (Hewlett Packard, Palo Alto, CA, USA) working with the operative system Microsoft Windows 3.0

(Malerba, Schaeffer et al. 2008). The GC analysis was obtained by applying the following instrument settings:

Column: HP-INNOWax Polyethylene Glycol 30.0 m x 25 μm x 0,25 μm

Gas carrier: Elium

Solvent: Esane

Oven Initial Temperature: 100 °C

Ramp 1: 3˚C/min,

Final temperature: 200˚C for 15 min

Ramp 2: 3˚C/min

Final temperature: 240˚C

Oven final Temperature: 240 °C per 40 min

Carrier pressure: 31,39 psi

Column Flow: 29,4 ml/min

Detector: FID-Flame Ionization Detector

Hydrogen Flow to FID: 30 ml/min

Air Flow to FID: 400 ml/min

Temperature FID: 250 °C

Injection Volume : 5 µl

Injection: on-line

Injector: split

Split ratio: 1:10

He Split Flow: 24,3 ml/min

### 3.4.Apolipoprotein C-III isofocusing

Pooled plasma samples from 4 patients belonging to the different four experimental groups, were analysed twice (two technical replicates) by Apo C-III isofocusing according to the method

described in ref. (Wopereis, Grunewald et al. 2003) with minor modification. Briefly, after a prefocusing step (30 min at 100V), 2 µl of sample in 20µl of IEF buffer was loaded to the gel, and then it was isoelectrofocused by applying 200V for 30min, 500V for 15min, 750V for 15min, and 1000V for 75min (Wada, Kadoya et al. 2012). After IEF, gel was fixed in 12%TCA and 3.5% sulfosalycilic acid for 1h, then it was washed and rebalanced in transfer buffer (4M Guanidinium Chloride (Sigma), 0.05 M Tris-HCl (Bio-rad) pH 8, 1mg/ml DTT (Sigma)). The gel was covered with a nitrocellulose membrane (GE-Helthcare Amersham) and kept for 1h at 60˚C for blotting the proteins by diffusion. The membrane-attached gel was then soaked in 50g/l of non-fat dried milk dissolved in TBS containing 0.05% (v/v) Tween-20 for the removal of the membrane as well as blocking. The primary rabbit anti-human Apo C-III antibody (Abcam) was diluted 10000-fold, and the secondary goat anti-rabbit HRP-conjugated antibody (Abcam) was diluted 20000-fold in TBST. The proteins were detected by ECL Western blotting detection system (Euroclone). The blots were exposed to KODAK Biomax films (Sigma-Adrich) and the signal was quantified by densitometry using Quantity One image software (Bio-Rad).

### 3.5.2D PAGE/SDS PAGE and Western immunoblotting

2DE protein analysis was performed as previously described (Brandi, Dando et al. 2013). Briefly, 80 µg of protein were subjected to IEF with 17 cm immobilized nonlinear pH 3–10 gradient IPG strips using a Protean IEF Cell (Bio-Rad). After IEF, IPG strips were equilibrated and then the proteins were separated using 7-20% SDS-PAGE gels. Confirmation of the proteomic data by immunoblot analysis was performed on an independent sample set as previously described (Polati, Brandi et al. 2015). In particular, 4 different samples chosen among the 4 experimental groups were analysed individually by 1D-WB, or as pool by 2D-WB. We investigated the modulation of fibrinogen γ, γ' and β, serum amyloid component P and complement C3. Briefly, proteins separated by 12%T SDS-PAGE or by IEF pI 3-10, were transferred to a PVDF membrane through electroblotting (60 V for 2 hours at 4°C). After blocking of non-specific sites by TBST-milk

solution, membranes were treated with primary and secondary antibodies at the appropriate dilutions (see Table 1) in 1% non-fat dried milk, 0.05% Tween-20 Tris-buffered saline. The immunocomplexes were detected by ECL (Amersham Biosciences) by a ChemiDoc instrument (Bio-Rad) and the signal was quantified by densitometry using Quantity One image software (Bio-Rad). Equal sample loading was confirmed by Amido black staining.

**Table 1**. List of antibodies used for western blotting validation

| Antibody | Target | Western Blot | Origin | Secondary Antibody |
|---|---|---|---|---|
| **Apo C-III** | Apolipoprotein C-III | 1:10000(IEF) | Abcam | Anti-Rabbit 1:20000 |
| **Apo J** | Apolipoprotein J | 1:1000 | Santa Cruz | Anti-Mouse 1:15000 |
| **C3** | Complement C3 | 1:1000 | Santa Cruz | Anti-Mouse 1:15000 |
| **FGB** | Fibrinogen β (C1C3) | 1:15000 | GeneTex | Anti-Rabbit 1:20000 |
| **FGG** | Fibrinogen γ', CT, clone 2.G2.H9 | 1:1000(2D)<br>1:1000(1D) | Millopore | Anti-Mouse<br>1:15000(2D)<br>1:10000(1D) |
| **FGG** | Fibrinogen γ | 1:1500(2D)<br>1:5000(1D) | GeneTex | Anti-Rabbit<br>1:20000(2D)<br>1:10000(1D) |
| **SAP** | Serum Amyloid P | 1:4000 | GeneTex | Anti-Rabbit 1:15000 |

### 3.6.Isoforms identification and quantification by top down-shotgun mass spectrometry analysis

We set up a reliable Mass Spectrometry based method to quantify Apo C-III isoforms (Apo C-III with 1, 2 or 0 sialic acid molecules) in a group of CAD patients, characterized by low or high levels of total Apo C-III plasma concentration and by low or high level of PUFA, on chronic treatment with statins.

#### Sample Preparation.

The plasma samples were processed using HybridSPE(R)-Phospolipid (Nicolardi, van der Burgt et al. 2013) (SUPELCO). Briefly, an aliquot of 25 μL of plasma sample was fortified with 150 μL of

freshly prepared 0.05% formic acid (Sigma) in water at room temperature for 15 minutes and then loaded onto the Solid Phase Extraction (SPE) cartridge. The SPE was preconditioned with 0.9 mL of 95% Acetonitrile (Sigma) and 0.05% formic acid and 0.9 mL of 0.05% tri-fluoro acetic acid (Sigma) in water. After loading, the SPE was washed three times with 0.9 mL of tri-fluoro acetic acid in water. The absorbed proteins were eluted with 200 µL of acetonitrile at 5%, 10%, 20% and 30%. The four aliquots at 5%, 10%, 20% and 30% of acetonitrile were merged and the eluent was evaporated in a speedvac for further analysis (**Figure 2**).

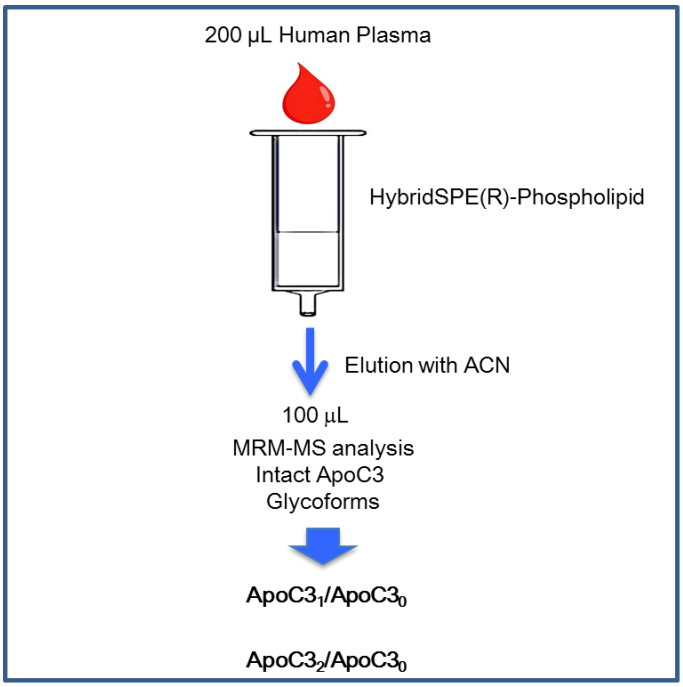


**Figure 2.** Sample preparation workflow: 200 µL of plasma sample was loaded onto an HybridSPE(R)-Phospolipid and the protein adsorbed were eluted with acetonitrile and undergone to top-down analysis

### Intact protein analysis

The sample aliquot was used for a "top-down" analysis of Apo C-III protein and relative quantitation of its different glycoforms as described by Jiang et al. (Jian, Edom et al. 2013) using a full scan high-resolution MS approach. The sample was reconstituted with 20 µL 0.1% formic acid

in water and 2 µL of internal standard (IS) working solution (2.3 pg/mL of the peptide DPEVRPTSAVAA dissolved 0.1% formic acid in water) (Cellmano Biotech Limited). LC-MS/MS analyses were performed by a micro-LC Eksigent Technologies system with as stationary phase an Halo Fused C18 column (0.5 x 100 mm, 2.7 µm; Eksigent Technologies). The injection volume was 4.0 µL and the oven temperature was set at 40 °C. The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow-rate of 15.0 mL $min^{-1}$ and at an increasing concentration of solvent B from 2% to 88% in 5 minutes. The LC system was interfaced with a 5600+ TripleTOF system (AB Sciex) equipped with DuoSpray Ion Source and CDS (Calibrant Delivery System). The ion source parameters in positive turbo ionspray mode were as follows: curtain gas 40 psi, GAS1 40 psi, GAS2 50 psi, ionspray voltage 5500 V and source temperature 500 °C. The declustering potential (DP) and collision energy were 165 and 10 V, respectively. The TOF mass range was set to 600−2000 m/z, and the accumulation time was 0.25 s. Peak integration was conducted using Skyline software. The multiple reaction monitoring (MRM) transitions were 1095.9 > 1095.9 (C3-0, 8+), 974.3 > 974.3 (C3-0, 9+), 1177.9 > 1177.9 (C3-1, 8+), 1047.2 > 1047.2 (C3-1, 9+), 1214.3 > 1214.3 (C3-2, 8+), 1079.5 > 1079.5 (C3-2, 9+), 609.8 > 609.8 (IS, 2+).

**Method Evaluation**

Intra-day and inter-day precision was evaluated for the glycoforms analysis. A pool of the same human plasma was analyzed in triplicate for three runs on separate days. The mean peak area ratios of Apo C-III1/Apo C-III0 and Apo C-III2/Apo C-III0 and their percentages coefficients of variation (% CVs) were calculated for the intra-day 3.68% and 5.95% and inter-day 5.96% and 7.52% analysis. The developed method is reliable and accurate upon repeated analysis as its repeatability is highly satisfactory for the clinical investigation.

### 3.7.Lipoprotein activity assay

The LPL activity was measured by an LPL Activity Assay Kit (Sigma-Aldrich) on citrate plasma samples frozen at -80˚C until use. The evaluation of the enzymatic activity is obtained by measuring the fluorescence emitted by the substrate as a result of its hydrolysis by the LPL present in the samples analyzed. In a 96-well black plate, 100 µL of Master Reaction Mix containing 99 µL of LPL Assay Buffer and 0,5 µL of LPL non-fluorescent Substrate Emulsion were dispensed into each well (dilution 1:200). Subsequently 1 µL of sample, in 100µL of LPL Assay Buffer, were added to each well, to reach the total volume of 200µL. The reaction was conducted at 37˚C for 60 minutes. The measurement of florescence emitted from the hydrolyzed substrate was obtained by the microplate reader Fluoroskan (Ascent), with λ excitation = 370nm and λ emission = 450nm. The corrected fluorescence of the samples was obtained by subtracting the blank value. The calibration curve was prepared by adding 1µL of LPL Standard (represented by the hydrolyzed substrate) in 2.5 mL of LPL Assay Buffer; serial dilutions were then prepared, each containing decreasing concentrations of LPL Standard, 200µL were pipetted into each well. The standard curve was obtained by plotting the Fluorescence Intensity Unit (FIU) as a function of µmoles of fluorescent substrate. The LPL activity was expressed as mol of substrate / h / ml of plasma.

### 3.8.SWATH analysis of proteomic profile

#### Sample preparation procedure

The plasma samples were processed using the Hybrid SPE(R)-Phospolipid-RP-C4 cartridge for solid phase extraction. Briefly, an aliquot of 25 µL of plasma was fortified with 150 µL of freshly prepared 0.05% formic acid in water at room temperature for 15 minutes and then loaded onto the SPE. The SPE was prewashed with 0.9 mL of 95% acetonitrile/4.95% water/0.05% formic acid and preconditioned with 0.9 mL of 0.05% tri-fluoro acetic acid in water. After the sample loading, the SPE was washed three times with 0.9 mL of a tri-fluoro acetic acid solution. Plasma proteins were fractionated using the RP-C4 SPE: the absorbed proteins were eluted with 200 µL of acetonitrile at

5%, 10%, 20% and 30%. The eluted sample were merged and 250 µL of Ambic was added (pH 8.5) that were then digested with chymotrypsin at 1:20 (w/w) enzyme to protein ratio for 18 h at 27 °C. After the digestion, the sample was evaporated for the LC-MS/MS analysis (**Figure 3**). As Table- isotope-labeled peptide standard (DPEVRPTSAVAA, Val- $^{13}C_5{}^{15}N_1$ at V10, Cellmano Biotech Limited) was spiked into the samples before the LC-MS/MS analysis and used for instrument quality control.

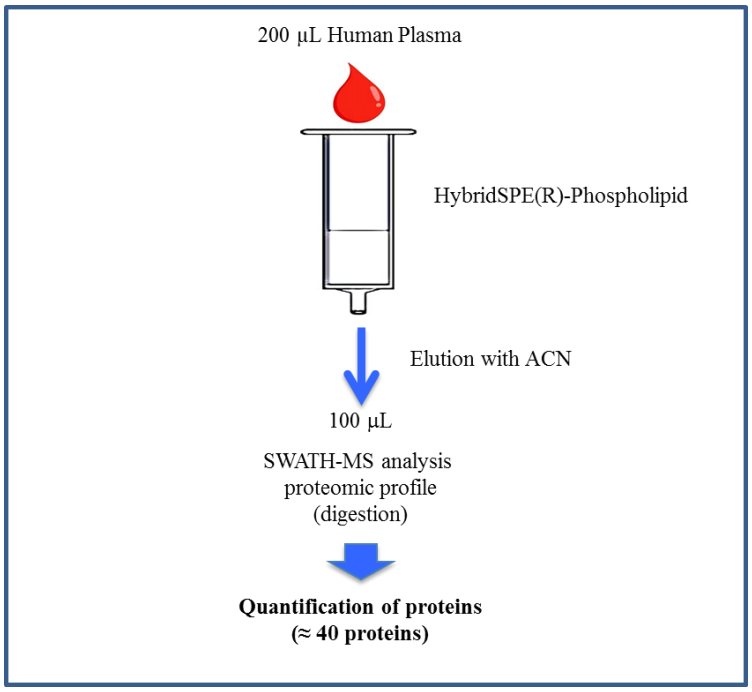


**Figure 3.** Sample preparation workflow: 200 µL of plasma sample was loaded onto an HybridSPE(R)-Phospolipid and the protein adsorbed were eluted with acetonitrile for the qualitative and quantitative proteomic profile of the sample

### Data acquisition

The digested plasma samples were analyzed on a micro-LC Eksigent Technologies interfaced to a 5600+ TripleTOF mass spectrometer system (AB Sciex) equipped with a DuoSpray Ion Source and a CDS (Calibrant Delivery System). The LC column was a Halo Fused C18 with a pre-column

ProteCol C18G. The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow-rate of 15.0 μL $min^{-1}$ at an increasing concentration of solvent B from 2% to 86% in 17 min. The injection volume was 4.0 μL and the oven temperature was set at 40 °C. For identification purposes the mass spectrometer analysis was performed using a mass range of 100–1500 Da (TOF scan with an accumulation time of 0.25 s), followed by a MS/MS product ion scan from 200 to 1250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps (35 candidate ions can be monitored during every cycle). The ion source parameters in electrospray positive mode were set as follows: curtain gas (N2) at 25 psig, nebulizer gas GAS1 at 25 psig, and GAS2 at 20 psig, ionspray floating voltage (ISFV) at 5000 V, source temperature at 450 C and declustering potential at 25 V. For the quantification the samples were subjected to cyclic data independent analysis (Stancu, Plesea et al.) of the mass spectra, using a 25-Da window: the mass spectrometer was operated such that a 50-ms survey scan (TOF-MS) was performed and subsequent MS/MS experiments were performed on all precursors. These MS/MS experiments were performed in a cyclic manner using an accumulation time of 40 ms per 25-Da swath (36 swaths in total) for a total cycle time of 1.5408 s (**Figure 4**). The ions were fragmented for each MS/MS experiment in the collision cell using the rolling collision energy. The MS data were acquired with Analyst TF 1.7 (AB SCIEX, Concord, Canada). One data dependent acquisition (DDA) and three DIA acquisitions were performed.

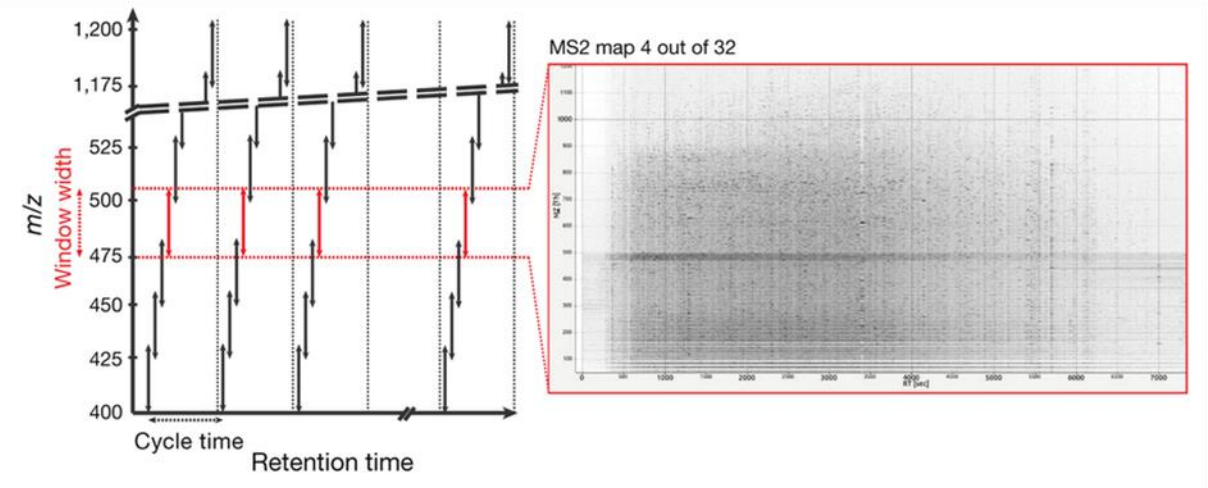


**Figure 4.** The SWATH-MS method consists of sequential acquisition of fragment-ion spectra with overlapping precursor isolation windows. Here, a swath window width of 25 m/z is depicted which allows stepping through a mass range of 400–1200 m/z in 32 individual steps. If all fragment-ion spectra of the same isolation window are aligned, an MS2 map (so-called swath) is obtained (right side, swath 4 out of 32 is schematically shown). SWATH-MS data reach the size of terabytes.

### Protein database search

The DDA files were searched using Protein Pilot software v. 4.2 (AB SCIEX) and Mascot v. 2.4 (Matrix Science Inc.). The DIA files were converted to pseudo-MS/MS spectra with DIA-Umpire software and were searched as DDA files (Tsou, Avtonomov et al. 2015, Tsou, Tsai et al. 2016). Chymotrypsin as digestion enzyme was specified for both the software. For Mascot we used 2 missed cleavages, the instrument was set to ESI-QUAD-TOF and the following modifications were specified for the search: carbamidomethyl cysteins as fixed modification and oxidized methionine as variable modification. A search tolerance of 0.08 Da was specified for the peptide mass tolerance, and 10 ppm for the MS/MS tolerance. The charges of the peptides to search for were set to 2 +, 3 + and 4 +, and the search was set on monoisotopic mass. The UniProt Swiss-Prot reviewed database containing human proteins (version 2015.07.07, containing 42131 sequence entries) was used and a target-decoy database search was performed. False Discovery Rate (FDR) was fixed at 1%.

### Protein quantification

The quantification was performed by integrating the extracted ion chromatogram of all the unique ions for a given peptide. The quantification was carried out with PeakView 2.0 and MarkerView 1.2. (AB SCIEX). The result file from the DDA acquisitions were used for the library generation using a protein FDR threshold of 1% (Wu, Song et al. 2016). Six peptides per protein and six transitions per peptide were extracted from the SWATH files. Shared peptides were excluded as well as peptides with modifications. Peptides with FDR lower than 1.0% were exported in MarkerView for the t-test.

### Multivariate Data Analysis and Model Development

Partial Least Square (PLS) (Massart DL 1988, Vandeginste B.G.M. 1988). is a multivariate regression method establishing a relationship between one or more dependent variables (Y) and a group of descriptors (X). X and Y variables are modeled simultaneously, to find the latent variables (LVs) in X that will predict the LVs in Y. Here, a backward elimination (BE) strategy was also applied, eliminating one variable at a time, according to the minimum error in cross-validation. PLS was originally set up to model continuous responses but it can be applied even for classification purposes by establishing an appropriate Y related to the association of each sample to a class. In this case, where two classes are present, a binary Y variable was added, coded so that −1 is attributed to patients characterized by a low Apo C-III level (< 10 mg/dL) and +1 to patients characterized by high Apo C-III levels (> 10 mg/dL). The regression is then carried out between X-block variables (protein counts) and the Y just established. This application for classification purposes is called PLS-DA(Marengo, Robotti et al. 2008, Robotti and Marengo 2016).

The performance of a model, and thus the ability of the significant variables to describing a defined group or in this case the “Apo C-III CAD state” can be evaluated analyzing the accuracy (the ratio of correctly assigned samples), the precision (the capability to not include samples of other classes in the considered class), the sensitivity (it describes the model ability to correctly recognize samples

belonging to the a class), the specificity (it characterizes the ability of a class to reject the samples of all the other classes) and the non-error-rate or NER% (is the average of the class sensitivities).

### 3.9. Lipidomic Approach

#### Plasmatic fatty acids extraction

To 250µl of plasma were added 2750µl of isopropanol+ BHT (butyl-hydroxy-toluene 50 mg/L) and vortexed. After an incubation of 1h at RT 1750µl of chloroform were added, the sample was vortexed and incubate at RT for 1h.the sample was then centrifuged at 3500rpm for 15 minutes. The supernatant was collected and dried at 37˚C by UniVapo (GeneVac); the dried pellet was solubilized in 500 µl of methanol and transferred into LC-chromatography MS vials.

#### Reverse Phase Liquid Chromatography-Electrospray Ionization- Mass Spectrometry Analyses (RPLC-ESI -MS)

Liquid chromatography-electrospray ionization-mass spectrometry allows separation of a wide variety of intact lipid molecular species and gives detailed structural information about lipid head groups and the FAC regiochemical distribution (Welti, Li et al. 2002, Guella, Frassanito et al. 2003, Welti, Shah et al. 2007, Anesi and Guella 2015). To analyze crude lipid extracts we used a Hewlett-Packard Model 1100 Series liquid chromatograph (Hewlett-Packard Development Company) coupled both to a Bruker Esquire-LC quadrupole IT-MS equipped with an ESI source (Bruker Optik GmbH).

Chromatographic separation of lipids was carried out on a Kinetex-C18column (100 x 2.1 mm i.d., poresize100Å, particlesize 2.6 µm) (Phenomenex) with a linear gradient of solvent A (methanol:water 7:3, containing 12mM ammonium acetate) and solvent B (methanol containing 12mM ammonium acetate) from 70%A/30%B to 100% B in 40 min, at a constant flow rate of 0.3ml/min. Final conditions were kept for at least 30 min to ensure the complete elution of non-polar lipids. Aliquots of 10 mL of crude extract in methanol.

Each crude extract was separately analyzed in positive ionization mode in the range 50–1200 m/z with a scan range of 13000 unit $s^{-1}$. For the analysis, high purity nitrogen was used at a pressure of 35 psi, at a temperature of 300 ˚C and a flow rate of 7 L $min^{-1}$. The high voltage capillary was set at 4000 V for positive ionization mode.

**Relative Quantification of Lipids and Data Analysis**

Raw data were analyzed by Data Analysis 3.0 software (Bruker Daltonik). Each lipid molecular species was quantified with respect to the total area of all lipid species belonging to the same class (e.g., relative quantification of phosphatidylcholine (PC) was performed with respect to total area of PC).

For statistical analyses, the unsaturation index (UI) and the average chain length (ACL) were calculated for each lipid class, using the formulas

$$UI_{classy} = \Sigma(\text{relative area lipid}_x * \text{double bond number of lipid}_x)$$

and

$$ACL_{classy} = \Sigma(\text{relative area lipid}_x * \text{acyl chain length of lipid}_x)$$

where $lipid_x$ represents each single molecular species belonging to the y lipid class, respectively.

Data were log(x+1) transformed and scaled using the Pareto method. At first, we applied a principal component analysis (PCA) (a unsupervised method) to define homogeneous clusters of taxa based on % area of single molecular species, UI and ACL. Then, we used the identified clusters as dependent variables first in partial least square – discriminant analysis (PLS-DA) followed by orthogonal partial least square (OPLS-DA), both supervised methods. Significance of PLS-DA was determined with permutation tests (200 permutations). PCA and DA were performed with Simca- P 13.0 software (UmetricsAB). We furthermore analyzed the correlation loading plots of OPLS-DA analysis to determine which metabolites contributed to the separation of clusters by setting a correlation coefficient p(corr) threshold of 0.75; a two-tailed Welchtest for single candidate markers

was carried out to investigate their status as markers; unequal variance between groups was considered.

### 3.10.Statistical Analysis

Statistical analysis was performed with SPSS 20.0 software (IBM Corporation). Continuous variables are expressed as mean ± standard deviation. Statistical analysis were performed on log-transformed values for the variables having a skewed distribution (e.g. TG, Apo E), but for sake of clarity they were also expressed as mean ± standard deviation. Continuous variables were analyzed by t-test and ANOVA, with polynomial contrasts for linear trend when indicated. Correlations between continuous variables were assessed by means of Pearson's R test, as well as by linear regression models estimating standardized beta coefficients. Possible interactions between Apo C-III and PUFA in determining TG plasma levels were assessed by means of general linear models. Categorical variables were compared using the $\chi 2$ test, with $\chi 2$ for linear trend when indicated. A P-value $<0.05$ was considered significant.

Principal Component Analysis (PCA) (Massart DL 1988, Massart DL 1998) was then applied to provide a general overview of the correlations existing between the variables and the existence of groups of samples. PCA and graphical representations were carried out by Statistica v. 7.1 (StatSoft Inc). PCA (Massart DL 1988, Massart DL 1998) is a multivariate pattern recognition method that allows the representation of the original dataset in a new reference system defined by new variables called principal components (PCs). PCs are linear combinations of the original variables and are orthogonal to each other, so that they explain independent sources of information. They are calculated hierarchically in order of decreasing percentage of explained variance; this feature allows an effective dimensionality reduction: by the use of a restricted number of significant PCs experimental noise and random variations, accounted for by the last PCs, can be eliminated. The coordinates of the samples in the space given by the PCs are called scores, while the coefficients of the linear combination describing each PC are called loadings and represent the weights of the

original variables on each PC. From the analysis of the scores it is possible to identify groups of samples; the reasons for their clustering can be instead identified in the corresponding loadings.

# 4. RESULTS

### 4.1. Analysis of Apolipoprotein C-III glycoforms by IEF

We selected a pool of CAD patients (n=26) subdivided according to Apo C-III and PUFA levels (**Table 2**). Briefly The samples were classified on the basis of plasmatic levels of Apo C-III as "low" (< 9.2 mg/dL) or "high" (≥ 12.6 mg/dL); and on the basis of fatty acids as "profile A" (> 40% PUFA and < 25% MUFA) and "profile B" (< 40% PUFA and > 25% MUFA). As reported in **Table 2**, four groups of samples were obtained: low Apo C-III /profile A (group 1; n=7); low Apo C-III /profile B (group 2; n=5); high Apo C-III /profile A (group 3; n=7); high Apo C-III /profile B (group 4; n=7). The general characteristics of the study population are summarized in **Table 3**

**Table 2.** Groups of CAD patients observed, subdivided according to Apo C-III levels and fatty acids profile**.**

| | **Apo C-III plasma levels (mg/dl)** | **MUFA (%)** **Cut off 25%** | **PUFA (%)** **cut off 40%** |
|---|---|---|---|
| **Group 1 (n=7)** | 7.19±1.71 | 23.27 (A) | 39.25 (A) |
| **Group 2 (n=5)** | 7.25±1.35 | 27.18 (B) | 36.54 (B) |
| **Group 3 (n=7)** | 13.78±1.53 | 24.20 (A) | 41.52 (A) |
| **Group 4 (n=7)** | 20.83±1.73 | 33.12 (B) | 29.98 (B) |

**Table 3.** Clinical and laboratory characteristics of the considered patients**.**

| | Group 1 (n=7) | Group 2 (n=5) | Group 3 (n=7) | Group 4 (n=7) | p* |
|---|---|---|---|---|---|
| **Male (%)** | 100 | 100 | 100 | 100 | |
| **Age** | 47.25±11.93 | 56±8.69 | 64.71±8.85 | 53.14±9.48 | 0.05 |
| **Smoking(%)** | 57.14 | 75 | 57.14 | 83.33 | <0.001 |
| **MI (%)** | 0 | 0 | 0 | 50 | <0.001 |
| **Hypertension (%)** | 57.14 | 40 | 14.29 | 42.86 | <0.001 |
| **Diabetes (%)** | 0 | 20 | 0 | 42.86 | <0.001 |
| **Triglycerides (mmol/L)** | 1.22±0.44 | 1.19±0.62 | 1.49±0.68 | 4.21±1.81 | 0.01 |
| **Total Cholesterol (mmol/L)** | 4.32±0.96 | 5.11±0.96 | 6.07±0.83 | 6.59±0.95 | <0.001 |
| **HDL-Cholesterol (mmol/L)** | 1.01±0.23 | 1.25±0.38 | 1.81±0.55 | 1.01±0.26 | NS |
| **LDL-Cholesterol (mmol/L)** | 2.86±0.83 | 3.42±0.73 | 3.71±0.77 | 4.29±0.97 | 0.02 |
| **Apo A1 (g/L)** | 1.15±0.16 | 1.21±0.20 | 1.64±0.39 | 1.25±0.16 | 0.02 |
| **Apo B (g/L)** | 0.83±0.25 | 0.94±0.14 | 1.10±0.26 | 1.51±0.33 | <0.001 |
| **Apo E (g/L)** | 0.03±0.01 | 0.04±0.01 | 0.04±0.01 | 0.06±0.04 | 0.04 |
| **PUFA** | 39.25 | 36.54 | 41.52 | 29.98 | NS |
| **MUFA** | 23.27 | 27.18 | 24.20 | 33.12 | NS |

NS: not significant

To determine the frequency of different glycoforms of Apo C-III in the four analyzed groups , we performed a screening of pooled plasma samples by the use of IEF and subsequent immunoblotting (**Figure 5**). In all the analyzed samples it was possible to distinguish the two glycoforms of Apo C-III (Apo C-III1, Apo C-III2); while the third glycoform (Apo C-III0) was detected albeit slightly,

only in the samples of group 3. The data obtained suggest that in all the CAD patients Apo C-III1 is more abundant than Apo C-III2 isoform.

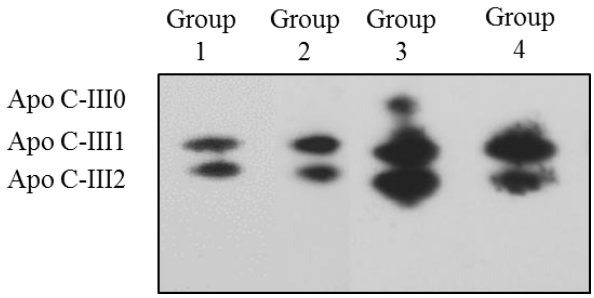


**Figure 5.** Apo C-III isofocusing and subsequent immunoblotting representative of the analyzed samples. Pooled plasma samples from patients with low Apo C-III and high PUFA (Group 1), low Apo C-III and low PUFA (Group 2), high Apo C-III and high PUFA (Group 3), high Apo C-III and low PUFA (Group 4), are illustrated.

The signals obtained by technical replicates of Apo C-III isofocusing were quantified by densitometry using Quantity One image software (Bio-Rad). The OD of each glycoform ($OD_{apoC3\text{-}X}$) was converted into approximate concentration (mg/dl apoC3-x) on the basis of total Apo C-III (mg/dl $apoC3_{TOT}$) and total OD of the respective lane ($OD_{TOT}$), by applying the formula: mg/dl apoC3-x = [$OD_{apoC3\text{-}X}$ * mg/dl apoC3]: $OD_{TOT}$. The approximate quantification of each glycoform in each samples is reported in **Table 4**.

**Table 4.** Approximate concentration of Apo C-III glycoforms in the four groups of patients.

| | Triglycerides mmol/l | Apo C-III 0 mg/dl | Apo C-III 1 mg/dl | Apo C-III 2 mg/dl |
|---|---|---|---|---|
| **Group 1** | 1.16 | | 4.9 ± 1.0 | 3.4 ± 1.3 |
| **Group 2** | 1.06 | | 3.3 ± 1.1 | 3.6 ± 0.8 |
| **Group 3** | 1.48 | 1.7 ± 1.1 | 6.8 ± 0.7 | 7.4 ± 1.1 |
| **Group 4** | 4.21 | | 12.9 ± 1.2 | 8.4 ± 1.8 |

### 4.2. Proteomic analysis of CAD patients stratified according to different levels of Apo C-III and fatty acids

Previous comparative proteomic analysis results underlined a total of 43 differentially expressed proteins which are mainly involved in coagulation, complement activation, inflammatory immune response and lipid metabolism. This is the reason why we have decided to investigate, through western immunoblotting, in our four groups of survey, how the Apo C-III can affect the expression of a selected group of protein.

#### Western blot validation of deregulated protein species

We measured the level of Serum Amyloid Protein Complement C3, Fibrinogen γ, Fibrinogen γ' and Fibrinogen β by Western Blotting (Do, Willer et al.) using samples belonging to the four groups (**Table 2**). The 1D (**Figure. 6**) and 2D-WB (**Figure. 7**) confirmed the proteomics findings and suggested a trend of up-regulation of fibrinogen γ in the comparison between group 1 vs group 2, as well as, in the comparison between samples with high Apo C-III (groups 3+4) and with low Apo C-III (groups 1+2). The 2D immunodetected profiles confirmed a strong up-regulation of complement

C3 in the comparison between group 1 vs group 2. As concerning the fibrinogen β, the data obtained confirmed the down-regulation of this protein in the comparison between group 3 and group 4; and also in samples with high Apo C-III (groups 3+4) as compared to samples with low Apo C-III (groups 1+2). Unfortunately, the 2D immunodetected profiles do not allowed to confirm lower amount of serum amyloid component P in plasma samples with high Apo C-III (i.e groups 3 and 4). It should however be noted that 2D-WB suggest a prevalence of serum amyloid in the groups 1 and 3, that are characterized by a greater presence of PUFAs (**Figure 7**).

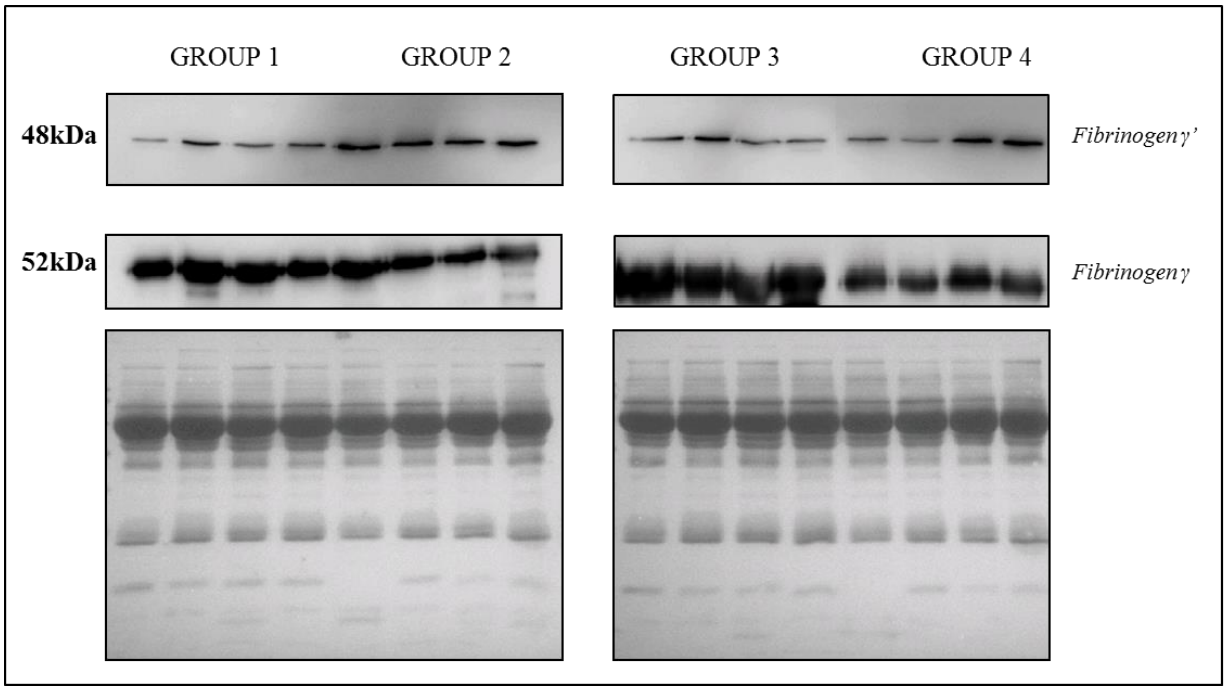


**Figure 6.** Monodimensional western blot analysis of fibrinogen γ' and γ in plasma samples belonging to the four groups of CAD patients. Amido black was used as loading control.

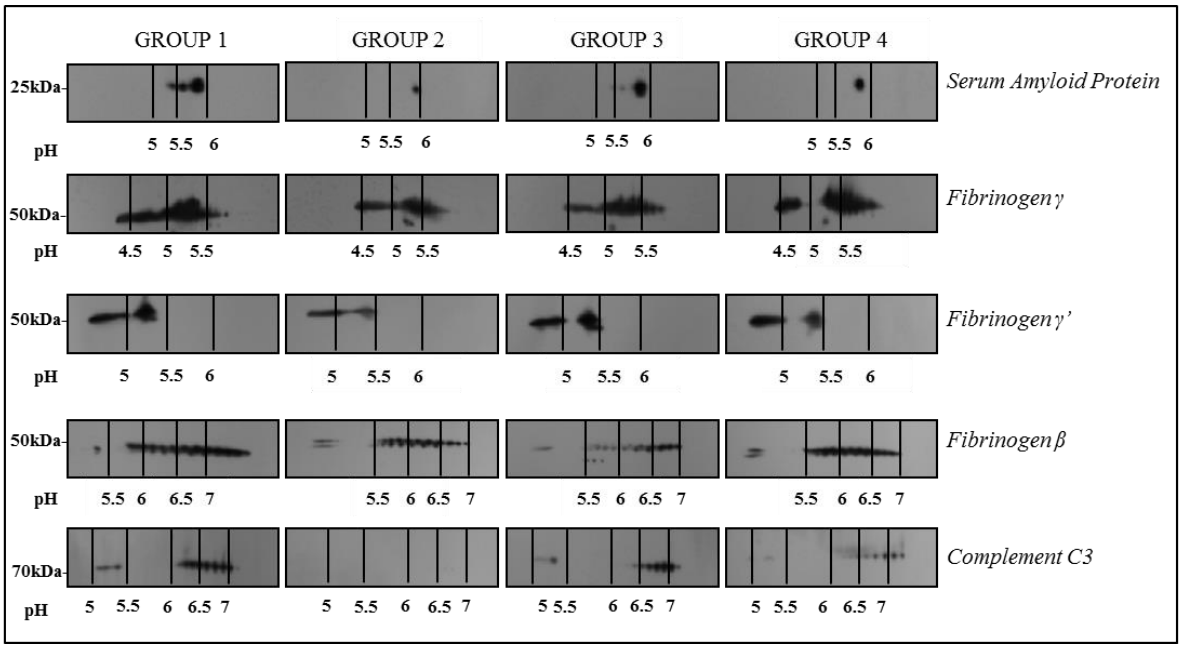


**Figure 7.** 2D western blot analysis of serum amyloid protein, fibrinogen γ , γ', β and complement C3 in plasma samples belonging to the four groups of CAD patients. Amido black was used as a loading control.

### 4.3. Glicoforms quantification in CAD patients by Shotgun-Topdown MS analysis

After the first analysis of Apo C-III isoforms by IEF as illustrated in the previous paragraphs we moved to a more powerful methodology. We in fact applied high resolution mass spectrometry to the analysis of Apo C-III glycoforms in a different set of plasma samples. By applying this technology we were able to detect and quantify Apo C-III non sialylated (Apo C-III0), monosialylated (Apo C-III1)and disialylated (Apo C-III2) .

### Apolipoprotein C-III plasma concentration and glycoforms: relationship with traditional plasma lipid profile and apolipoproteins

The general characteristics of the 51 patients study population are summarized in **Table 5.** Plasma lipids parameters, as well as demographic data, are shown in **Table 6** stratified on the basis of quartile distribution of Apo C-III plasma concentration. As expected, subjects within the highest Apo C-III quartile were characterized by an unfavorable lipid profile with higher levels of

triglycerides, total and LDL cholesterol, as well as higher Apo B and Apo E plasma concentrations. Such results were confirmed by Pearson's correlation analysis (**Table 7**). Considering the relative proportion of Apo C-III glycoforms, heterogeneous trends of correlations were observed (**Table 7**). Apo C-III0 had an inverse correlation (R= -0.351; P=0.009), Apo C-III1 a direct correlation (R=0.382; P=0.004), while Apo C-III2 did not have any significant correlation with total Apo C-III plasma concentrations, as shown in the scatter plot (**Figure 8**) and in the histogram depiction (**Figure 9**).

**Table 5.** Clinical and laboratory characteristics of the cohort of patients with coronary artery disease(CAD)

| | CAD patients (n=51) |
|---|---|
| Age (years) | 59.6±8.1 |
| Male sex (%) | 90.2 |
| Myocardial infarction history (%) | 56.9 |
| Smoking history (%) | 72.5 |
| Hypertension (%) | 68.6 |
| Diabetes (%) | 15.7 |
| Total cholesterol (mM/L) | 4.41±0.81 |
| LDL cholesterol (mM/L) | 2.94±0.75 |
| HDL cholesterol (mM/L) | 1.10±0.28 |
| Triglyceride (mM/L) | 1.87±0.96 |
| Apolipoprotein A (g/L) | 1.48±0.21 |
| Apolipoprotein B (g/L) | 0.78±0.24 |
| Apolipoprotein E (g/L) | 0.039±0.014 |
| Apolipoprotein C-III (mg/dl) | 10.21±1.01 |
| Lipoprotein Lipase activity | 1.56±0.59 |

**Table 6.** Plasma lipid profile parameters according to apolipoprotein C-III (Apo C-III) quartiles distribution**.**

| | Apo C-III quartiles | | | | |
|---|---|---|---|---|---|
| | **<8.7 (mg/dl)** | **8.7-11.6 (mg/dl)** | **11.7-13.8 (mg/dl)** | **>13.8 (mg/dl)** | **P*** |
| **Age (years)** | 60.6±7.7 | 57.9±8.0 | 62.0±4.1 | 58.1±10.9 | NS |
| **Male sex (%)** | 100 | 92.3 | 91.7 | 76.9 | NS |
| **Chol tot (mM/L)** | 3.90±0.36 | 4.20±0.46 | 4.56±0.84 | 5.00±1.02 | <0.001 |
| **LDL (mM/L)** | 2.55±0.31 | 2.75±0.48 | 3.08±0.81 | 3.40±0.97 | 0.001 |
| **HDL (mM/L)** | 1.15±0.27 | 1.11±0.31 | 1.07±0.28 | 1.05±0.27 | NS |
| **TG (mM/L)** | 1.01±0.25 | 1.64±0.76 | 2.10±0.58 | 2.73±1.08 | <0.001 |
| **Apo A (g/L)** | 1.41±0.23 | 1.47±0.16 | 1.51±0.24 | 1.52±0.19 | NS |
| **Apo B (g/L)** | 0.64±0.13 | 0.73±0.15 | 0.79±0.29 | 0.95±0.27 | 0.001 |
| **Apo E (g/L)** | 0.032±0.010 | 0.036±0.012 | 0.038±0.009 | 0.052±0.017 | <0.001 |
| **Apo C-III (mg/dl)** | 6.88±1.56 | 10.21±1.01 | 12.88±0.57 | 16.60±1.96 | <0.001 |
| **LPL activity (mol/h/ml)** | 1.84±0.59 | 1.62±0.66 | 1.48±0.59 | 1.36±0.46 | 0.035 |

* by ANOVA with polynomial contrasts for linear trend or by $\chi 2$-test for linear trend.

NS: no significant

**Table 7**. Correlations of apolipoprotein C-III total plasma concentration and glycoforms proportion with traditional plasma lipid profile**.**

| | | Apo C-III | Chol | HDL | LDL | TG | Apo A | Apo B | Apo E |
|---|---|---|---|---|---|---|---|---|---|
| Total plasma concentration | Apo C-III | - | **r= 0.618** | r=-0.153 | **r= 0.545** | **r= 0.720** | r= 0.111 | **r=0.601** | **r=0.536** |
| | | | **p< 0.001** | p=0.280 | **p= 0.001** | **p< 0.001** | p= 0.437 | **p< 0.001** | **p<0.001** |
| Glycoforms proportion | Apo C-III0 | **r= -0.343** | **r= -0.304** | r= 0.224 | **r= -0.314** | **r= -0.390** | r= 0.222 | **r= -0.299** | **r= -0.370** |
| | | **p= 0.014** | **p= 0.030** | p= 0.114 | **p= 0.025** | **p= 0.005** | p= 0.117 | **p= 0.033** | **p= 0.007** |
| | Apo C-III1 | **r= 0.395** | **r= 0.330** | **r= -0.356** | **r= 0.388** | **r= 0.398** | r=-0.137 | **r= 0.334** | **r= 0.395** |
| | | **p= 0.004** | **p= 0.018** | **p= 0.010** | **p= 0.005** | **p= 0.004** | p= 0.337 | **p= 0.017** | **p< 0.004** |
| | Apo C-III2 | r= -0.126 | r= -0.086 | r= 0.202 | r= -0.148 | r= -0.080 | r= 0.064 | r=-0.097 | r= -0.097 |
| | | p= 0.380 | p= 0.548 | p= 0.154 | p= 0.299 | p= 0.576 | p= 0.655 | p= 0.499 | p= 0.500 |
| ApoC-III1/ApoC-III0 Ratio | | **r= 0.431** | **r= 0.382** | **r= -0.305** | **r= 0.416** | **r= 0.440** | r= -0.165 | **r= 0.376** | **r= 0.431** |
| | | **p= 0.002** | **p= 0.006** | **p= 0.030** | **p= 0.002** | **p= 0.001** | p= 0.247 | **p= 0.007** | **p= 0.002** |

Significant correlations are reported in bold type

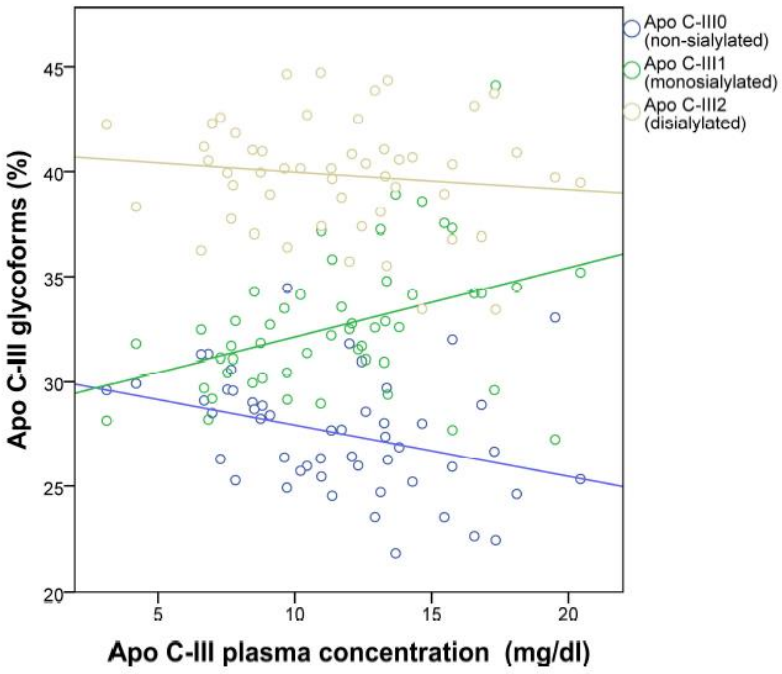


**Figure 8.** Scatter plot of correlations between apolipoprotein C-III glycoforms and apolipoprotein C-III total plasma concentration**.**

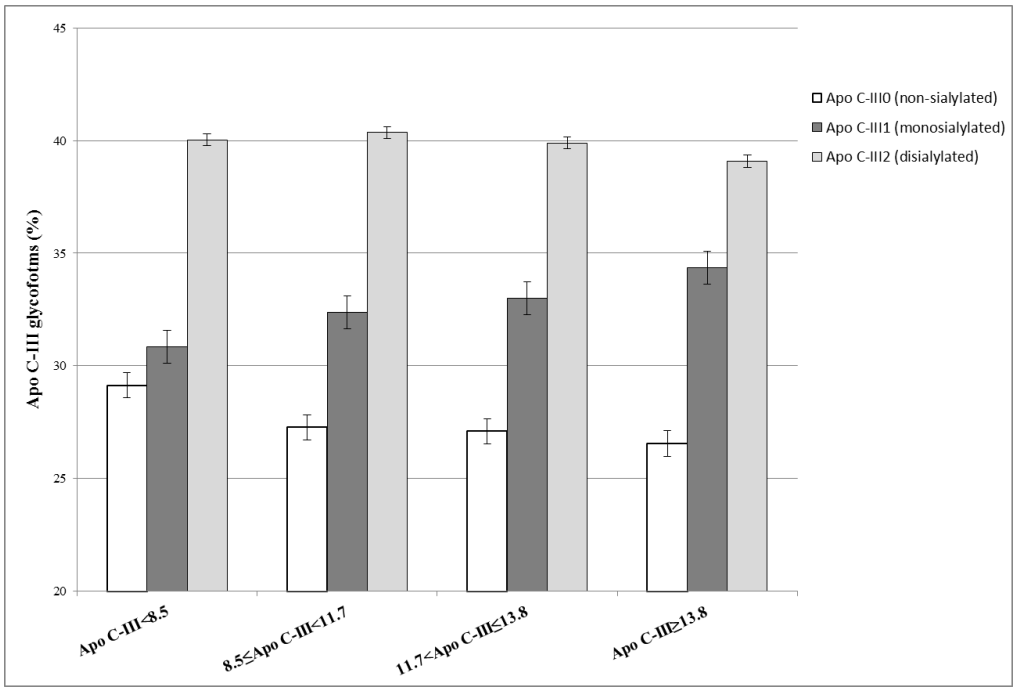


**Figure 9.** Apolipoprotein C-III glycoforms distribution according to total plasma Apolipoprotein C-III concentration stratification in quartiles.

The Apo C-III glycoforms, considered as relative proportion, showed different correlations with the other plasma lipids parameters (**Table 7**). Apo C-III0 was inversely correlated with total and LDL cholesterol, TG, Apo B, and Apo E. In contrast, Apo C-III1 was directly correlated with total and LDL cholesterol, TG, Apo B, Apo E, and inversely with Apo A. No significant correlation was found for Apo C-III2. On the basis of such observations, to enhance the statistical associations related to Apo C-III glycoforms, the ratio of Apo C-III1 on Apo C-III0 (Apo C-III1/Apo C-III0) was also calculated. Apo C-III1/Apo C-III0 correlated directly with total and LDL cholesterol, TG, Apo B, Apo C-III, Apo E, and inversely with HDL, thus reflecting an unfavorable lipid profile (**Table 7**). Most of these correlations were no more statistical significant after adjustment for total Apo C-III concentrations, with the exception of that with TG. Apo C-III1/Apo C-III0 remained a significant predictor of TG levels (standardized β coefficient=0.205; P=0.028) in a linear regression model adjusted for sex, age, and total Apo C-III concentrations, while the association with Apo E was of borderline significance (standardized β coefficient=0.252; P=0.059 by adjusted linear regression model).

**Apo C-III plasma concentration and glycoforms: relationships with plasma fatty acids**

Plasma fatty acid data are summarized in **Table 8**. Analyzing the possible correlations between Apo C-III (considered both as total plasma concentration and as proportion of glycoforms) and plasma fatty acid profile (expressed as relative percentage), several significant associations were found by Pearson's test (**Table 9**). Total Apo C-III plasma concentration correlated directly with C14:0, MUFA, C16:1, and inversely with C20:0, PUFA, C18:4ω3, PUFA ω6, C18:2ω6. Apo C-III0 correlated directly with C20:0, C24:0, C26:0, C20:4ω6 and inversely with C14:0. Apo C-III1 correlated directly with C14:0 and inversely with C20:0. Apo C-III2 correlated inversely with C26:0, C18:3ω3, and C20:4ω6. Apo C-III1/Apo C-III0 correlated directly with C14:0 and inversely with C20:0 and C24:0 (**Table 9**). Including all the significant correlations in adjusted linear regression models C20:0, C18:4ω3, and C18:2ω6 remained significant predictors of Apo C-III plasma concentration variability, C14:0 and C26:0 of Apo C-III0 variability, C14:0 of Apo C-III1

variability, C18:4ω3 of Apo C-III2 variability, C14:0 and C20:0 of Apo C-III1/Apo C-III0 ratio (**Table 10**). These results were confirmed also by principal component analysis (PCA) (**Figure 10**).

**Table 8.** Plasma Fatty Acids levels in the cohort of patients with coronary artery disease (CAD).

| | **CAD patients** |
|---|---|
| SFA (g/100g) | 31.3±1.8 |
| C14:0 | 1.3±0.422 |
| C16:0 | 21.1±1.4 |
| C18:0 | 7.3±0.7 |
| C20:0 | 0.3±0.05 |
| C22:0 | 0.6±0.14 |
| C24:0 | 0.44±0.1 |
| C26:0 | 0.11±0.05 |
| MUFA (g/100g) | 29.5±3.3 |
| C16:1 | 1.96±0.8 |
| C18:1 | 27.3±3.1 |
| C20:1 | 0.2±0.05 |
| PUFA (g/100g) | 39.2±3.8 |
| ω3PUFA (g/100g) | 3.12±0.8 |
| C18:3ω3 | 0.35±0.2 |
| C18:4ω3 | 0.15±0.07 |
| C20:5ω3 | 0.65±0.4 |
| C22:6ω3 | 1.97±0.6 |
| ω6PUFA (g/100g) | 36.1±3.8 |
| C18:2ω6 | 26.4±3.9 |
| C20:2ω6 | 0.3±0.6 |
| C20:4ω6 | 9.42±1.7 |

**Table 9.** Correlations of Apo C-III total plasma concentration and glycoforms proportions with fatty acids plasma levels.

| | SFA | C14:0 | C16:0 | C18:0 | C20:0 | C:22 | C24:0 | C26:0 | MUFA | C16:1 | C18:1 | C20:1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Apo C-III tot** | r= 0.196 | **r= 0.405** | r= 0.183 | r= 0.007 | **r= -0.518** | r=-0.228 | **r= -0.294** | r=-0.160 | **r= 0.507** | **r= 0.467** | **r= 0.434** | r= 0.207 |
| | p= 0.152 | **p= 0.002** | p= 0.182 | p= 0.957 | **p< 0.001** | p= 0.095 | **p= 0.029** | p= 0.245 | **p< 0.001** | **p< 0.001** | **p= 0.001** | p= 0.130 |
| **Apo C-III0** | r= -0.036 | **r= -0.274** | r= -0.057 | r= 0.075 | **r= 0.293** | r= 0.218 | **r= 0.332** | **r= 0.411** | r= -0.256 | r= -0.192 | r= -0.230 | r= -0.033 |
| | p= 0.796 | **p= 0.043** | p= 0.678 | p= 0.584 | **p= 0.030** | p= 0.110 | **p= 0.013** | **p= 0.002** | p= 0.059 | p= 0.159 | p= 0.091 | p= 0.811 |
| **Apo C-III1** | r= 0.246 | **r= 0.396** | r= 0.189 | r= 0.059 | **r= -0.328** | r=-0.143 | r= -0.196 | r=-0.060 | r= 0.119 | r= 0.163 | r= 0.089 | r= 0.021 |
| | p= 0.070 | **p= 0.003** | p= 0.167 | p= 0.670 | **p= 0.014** | p= 0.296 | p= 0.151 | p= 0.662 | p= 0.387 | p= 0.234 | p= 0.517 | p= 0.878 |
| **Apo C-III2** | r= -0.254 | r= -0.187 | r= -0.165 | r= -0.147 | r= 0.088 | r=-0.054 | r= -0.108 | **r=-0.349** | r= 0.122 | r= 0.005 | r= 0.130 | r= 0.009 |
| | p= 0.061 | p= 0.172 | p= 0.230 | p= 0.285 | p= 0.524 | p= 0.697 | p= 0.432 | **p= 0.009** | p= 0.376 | p= 0.974 | p= 0.343 | p= 0.950 |
| **Apo C-III1/Apo C-III0** | r= 0.173 | **r= 0.376** | r= 0.151 | r= -0.003 | **r= -0.399** | r=-0.187 | **r= -0.286** | r=-0.239 | r= 0.201 | r= 0.203 | r= 0.169 | r= 0.016 |
| **Ratio** | p= 0.207 | **p= 0.005** | p= 0.273 | p= 0.982 | **p= 0.011** | p= 0.172 | **p= 0.035** | p= 0.079 | p= 0.140 | p= 0.137 | p= 0.217 | p= 0.906 |

| | PUFA | PUFAω3 | C18:3ω3 | C18:4ω3 | C20:5ω3 | C22:6ω3 | PUFAω6 | C18:2ω6 | C20:2ω6 | C20:4ω6 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Apo C-III tot** | **r= -0.530** | r= 0.015 | r= 0.071 | **r= -0.269** | r= 0.068 | r= -0.116 | **r= -0.522** | **r= -0.501** | r= 0.016 | r= -0.018 |
| | **p< 0.001** | p= 0.915 | p= 0.608 | **p= 0.047** | p= 0.624 | p= 0.401 | **p< 0.001** | **p< 0.001** | p= 0.908 | p= 0.896 |
| **Apo C-III0** | r= 0.239 | r= 0.144 | r= -0.141 | r= 0.232 | r= 0.258 | r= 0.131 | r= 0.203 | r= 0.067 | r= 0.060 | **r= 0.308** |
| | p= 0.079 | p= 0.294 | p= 0.305 | p= 0.088 | p= 0.059 | p= 0.339 | p= 0.136 | p= 0.628 | p= 0.667 | **p= 0.022** |
| **Apo C-III1** | r= -0.216 | r= -0.135 | r= 0.107 | r= 0.093 | r= -0.183 | r= -0.168 | r= -0.182 | r= -0.168 | r= 0.064 | r= -0.033 |
| | p= 0.113 | p= 0.326 | p= 0.438 | p= 0.498 | p= 0.186 | p= 0.221 | p= 0.184 | p= 0.221 | p= 0.648 | p= 0.813 |
| **Apo C-III2** | r= 0.011 | r= 0.012 | r= 0.018 | **r= -0.348** | r= -0.048 | r= 0.063 | r= 0.006 | r= 0.129 | r= -0.132 | **r= -0.277** |
| | p= 0.936 | p= 0.933 | p= 0.896 | **p= 0.009** | p= 0.730 | p= 0.646 | p= 0.964 | p= 0.346 | p= 0.345 | **p= 0.041** |
| **Apo C-III1/Apo C-III0** | r= -0.254 | r= -0.134 | r= 0.129 | r= -0.069 | r= -0.216 | r= -0.150 | r= -0.220 | r= -0.139 | r= -0.002 | r= -0.182 |
| **Ratio** | p= 0.061 | p= 0.329 | p= 0.349 | p= 0.615 | p= 0.117 | p= 0.275 | p= 0.106 | p= 0.313 | p= 0.990 | p= 0.184 |

Significant correlations are reported in bold type

**Table 10.** Fatty acid plasma levels as determinants of Apo C-III plasma concentration (A) and glycoforms proportion (B.C.D.E)

**A) Apo C-III plasma concentration**

| | Standardized β-coefficient | P |
|---|---|---|
| **C20:0** | -0.420 | <0.001 |
| **C18:4 ω-3** | -0.275 | 0.007 |
| **C18:2 ω-6** | -0.449 | <0.001 |

**$R^2$= 0.516**

**B) Apo C-III 0 glycoform proportion**

| | Standardized β-coefficient | P |
|---|---|---|
| **C14:0** | -0.250 | 0.046 |
| **C26:0** | 0.395 | 0.002 |

**$R^2$= 0.231**

**C) Apo C-III 1 glycoform proportion**

| | Standardized β-coefficient | P |
|---|---|---|
| **C14:0** | 0.396 | 0.003 |

**$R^2$= 0.157**

**D) Apo C-IIApo C-III 2 glycoform proportion**

| | Standardized β-coefficient | P |
|---|---|---|
| **C26:0** | -0.241 | 0.063 |
| **C18:4 ω-3** | -0.290 | 0.024 |
| **C20:4 ω-6** | -0.236 | 0.060 |

**$R^2$= 0.250**

**E) Apo C-III 1 / Apo C-III 0 ratio**

| | Standardized β-coefficient | P |
|---|---|---|
| **C14:0** | 0.289 | 0.037 |
| **C20:0** | -0.230 | 0.094 |

**$R^2$= 0.187**

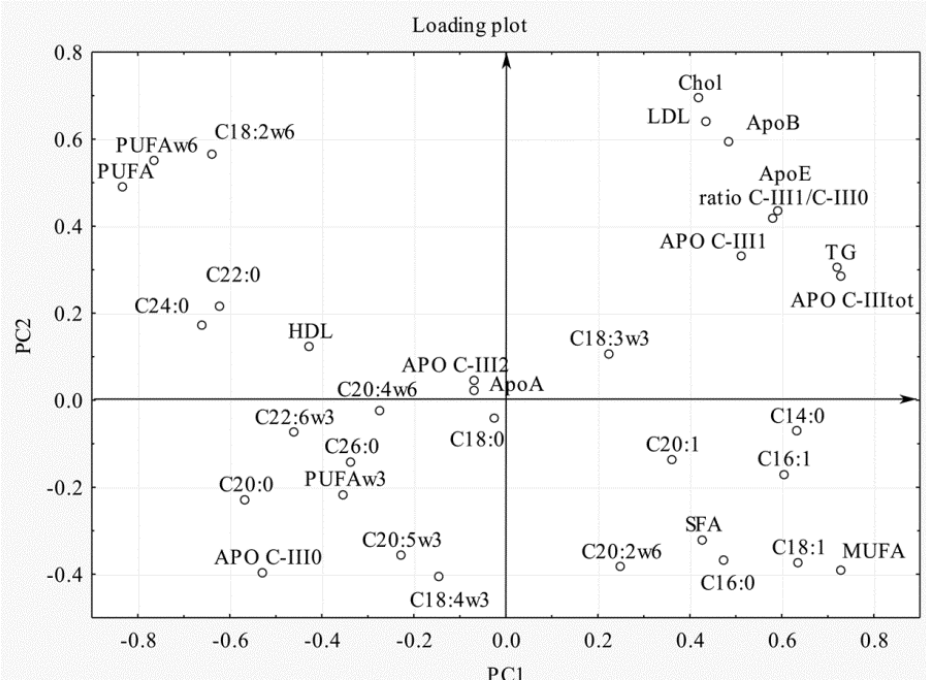


**Figure 10.** PCA results: score plot of the first two PCs on Apo C-III glycoforms, Apo C-III1/Apo C-III1 ratio and Fatty Acids

**Interaction between Apo C-III and PUFA in determining TG levels**.

As expected by earlier studies (Mensink, Zock et al. 2003, Bays, Tighe et al. 2008, Furtado, Campos et al. 2008) the results showed an inverse correlation of PUFA with TRL-related parameters, i.e. TG (R= -0.572; P<0.001) and Apo C-III (R= -0.521; P<0.001). We hypothesized that such associations could be influenced by Apo C-III levels and performed a new analysis stratifying the study population on the basis of Apo C-III median value (11.7 mg/dl). In subject with low Apo C-III plasma concentration (<11.7 mg/dl), PUFA correlated inversely with TG and Apo C-III, as well as there was a positive correlation with Apo C-III0 and negative correlations with Apo C-III1 and Apo C-III1/Apo C-III0 (**Table 11A**). In contrast, for the group of subjects with high Apo C-III plasma concentration (≥11.7 mg/dl), no significant correlation was found (**Table 11B**). There was a significant interaction between Apo C-III and PUFA levels in determining TG plasma concentration (F=5.483; P for interaction=0.023 by general linear model – **Figure 11**).

**Table 11.** Correlations of PUFA levels with triglyceride and apolipoprotein C-III plasma concentration and glycoforms stratified according apolipoprotein C-III plasma concentration median value (11.7mg/dl)

**A) Apo C-III< 11.7mg/dl**

| | TG | Apo C-III tot | Apo C-III0 | Apo C-III1 | Apo C-III2 | Apo C-III1/ ApoC-III0 |
|---|---|---|---|---|---|---|
| **PUFA** | **r= -0.568** | **r= -0.374** | **r=0.390** | **r= -0.403** | r= -0.004 | **r= -0.453** |
| | **p= 0.001** | **p= 0.046** | **p= 0.037** | **p= 0.030** | p= 0.982 | **p= 0.014** |

**B) Apo C-III≥11.7mg/dl**

| | TG | Apo C-III tot | Apo C-III0 | Apo C-III1 | Apo C-III2 | Apo C-III1/ ApoC-III0 |
|---|---|---|---|---|---|---|
| **PUFA** | r= 0.037 | r= 0.091 | r=-0.122 | r= 0.196 | r=-0.116 | r= 0.169 |
| | p= 0.858 | p= 0.658 | p= 0.554 | p= 0.337 | p= 0.571 | p= 0.409 |

Significant correlations are reported in bold type

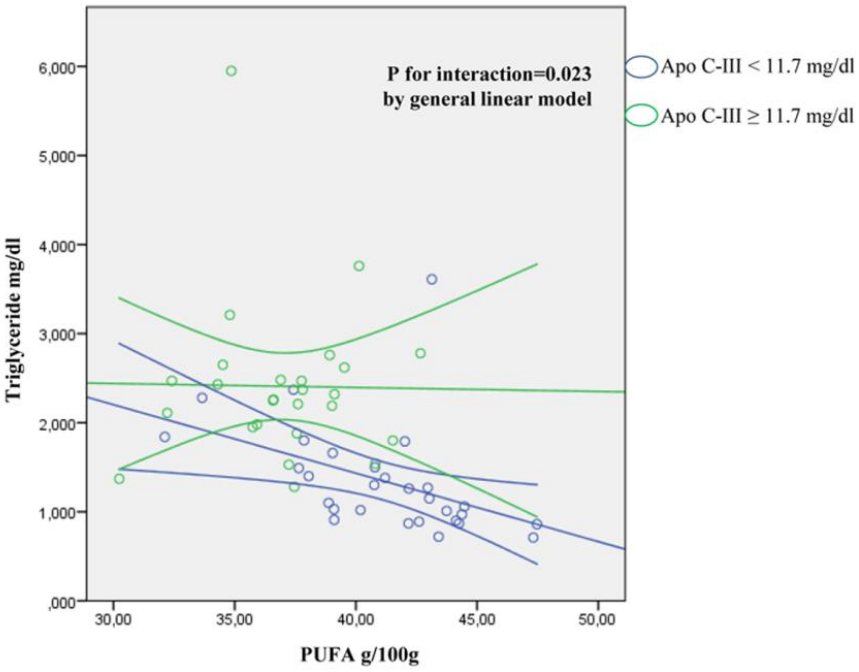


**Figure 11.** Interaction between Apo C-III and PUFA levels in determining TG plasma concentration. The study population was subdivided according to Apolipoprotein C-III plasma median value (11.7 mg/dl).

**Apolipoprotein C-III plasma levels correlation with fibrinogen chains plasma concentration**

On the basis of the validation results obtained in the previously analyzed cohort we further focalized our attention on fibrinogen γ' chain, because several studies show that this isoform levels increased in CAD patients (Lovely, Falls et al. 2002, Lovely, Kazmierczak et al. 2010).This isoform has also thrombogenic effects in arterial site and anti-thrombogenic effect in venous site (Uitte de Willige, de Visser et al. 2005, Lovely, Boshkov et al. 2007). It could be supposed that these latter functions of Fibrinogen γ' could be associated with Apo C-III levels so we analyzed the trend of Fibrinogen γ'chain in the same 51 CAD patients population of MS analysis (**Figure 12**) to found any possible correlation between Apo C-III and Fibrinogen γ' levels.

The analysis between Fibrinogen γ, Fibrinogen γ' and Fibrinogen γ/Fibrinogen γ' ratio and all the lipid profile data didn't show any statistically significant correlation with Apo C-III levels, but a statististically significant correlation with LDL and Apo B levels ($p<0,05$ for both analyses) was found.


**Figure 12**. Western blots analysis representative of the data obtained with antibodies against fibrinogen gamma and gamma' chain in the selected CAD patients.

### 4.4. Lipoprotein lipase analysis

An endpoint analysis was performed in order to assess the LPL activity and analyze it according to both total plasma concentration of Apo C-III and Apo C-III glycoforms.

Evaluation of LPL activity on the 51 CAD patient, previously analyzed for the quantification of Apo C-III glycoforms by topdown analysis, was performed by mean of fluorescence assay. A standard curve was obtained and used for the extrapolation of the enzymatic activity (**Figure 13**).

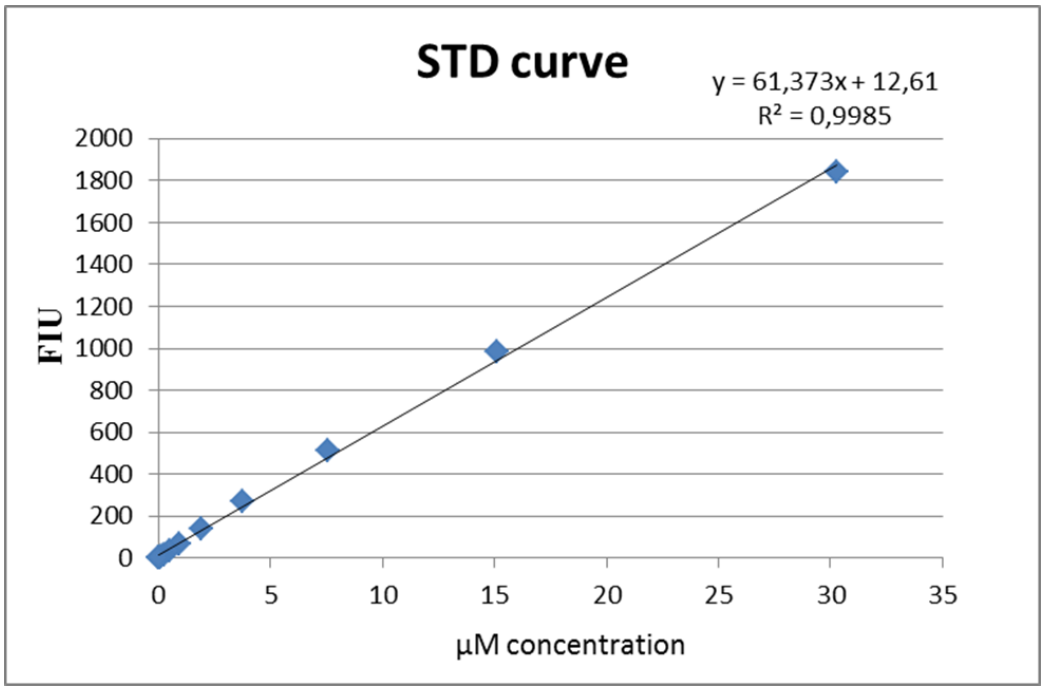


**Figure 13.** LPL assay standard curve.

After statistical evaluation of the results we observed that, as expected, LPL activity decreased progressively across Apo C-III quartiles, from the lowest to the highest (**Figure 14**). On the other hand, no association was found between LPL activity and the relative proportion of Apo C-III glycoforms (**Table 12**). Considering the absolute plasma concentration of Apo C-III glycoforms, all the three isoforms have a similar trend of association with LPL activity with a progressive decrease of enzyme activity from the lowest to the highest plasma concentration (**Figure 15**)

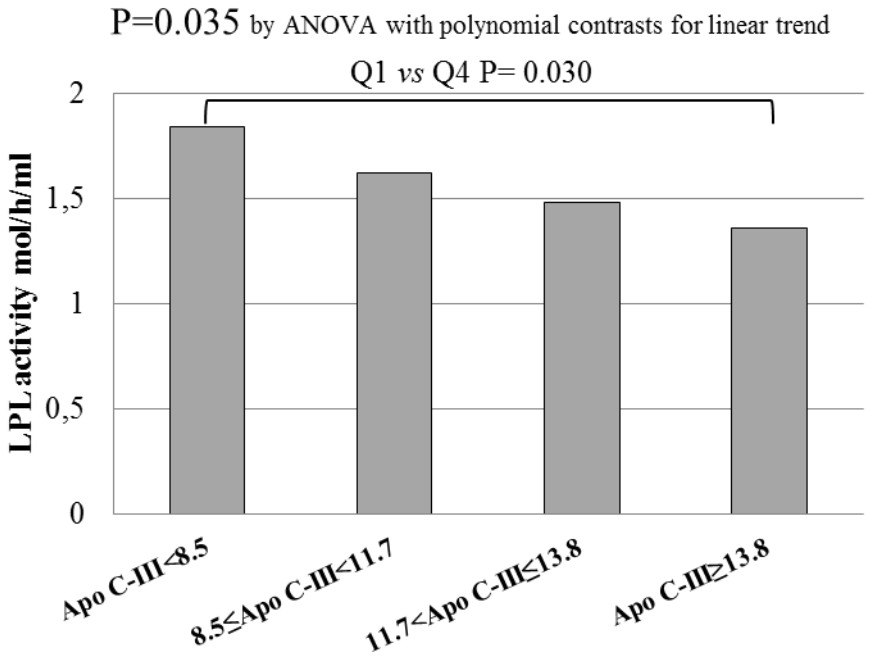


**Figure 14.** Distribution of LPL activity according to total plasma Apolipoprotein C-III concentration stratification in quartiles.

**Table 12**. LPL activity correlation with Apo C-III glycoforms

| | Apo C-III0 | Apo C-III1 | Apo C-III2 |
|---|---|---|---|
| LPL activity | r= 0.022 | r= -0.052 | r= 0.040 |
| | p= 0.880 | p= 0.723 | p= 0.782 |

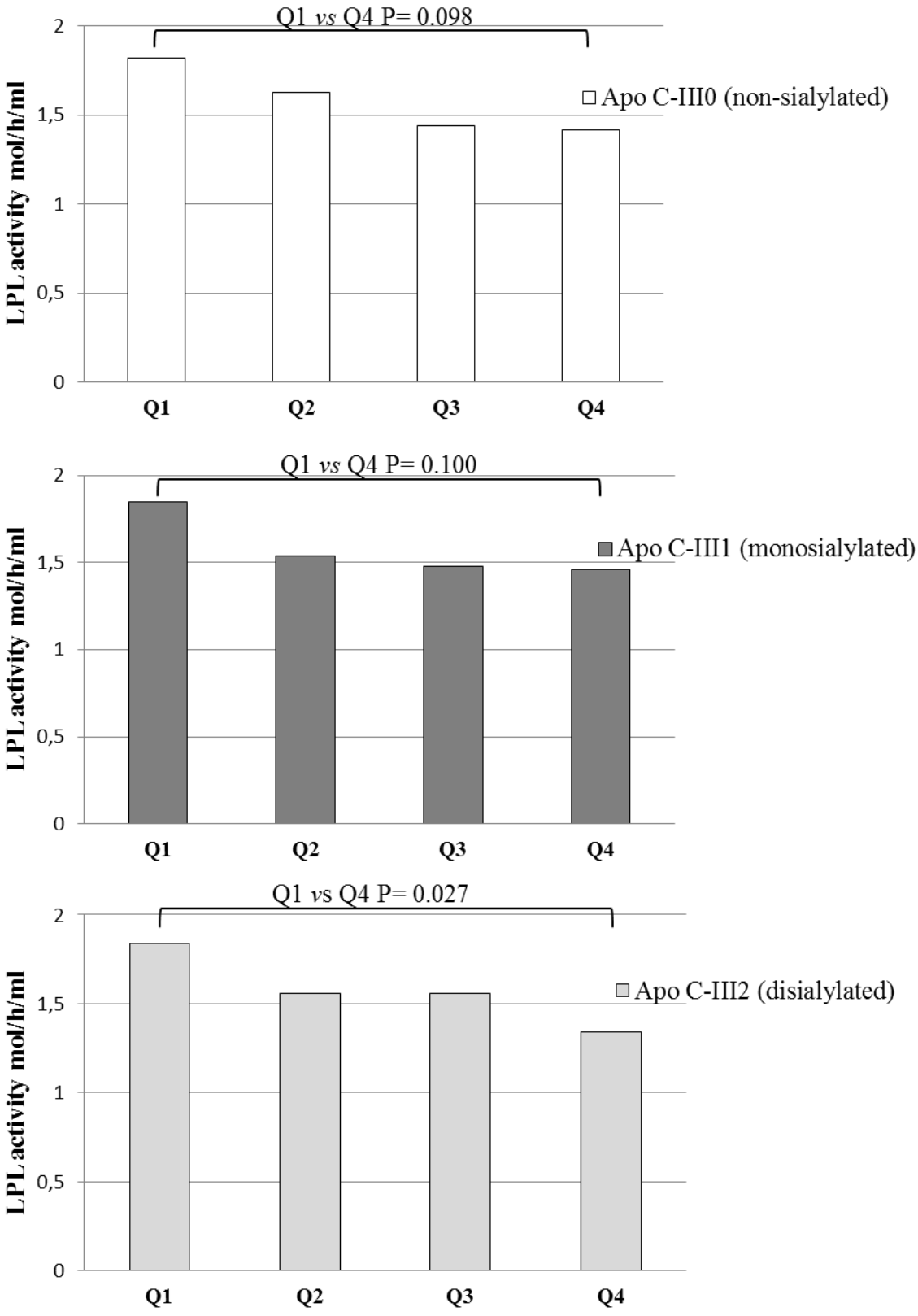


**Figure 15.** Association between LPL activity and Apo C-III glycoforms distribution according to total plasma Apolipoprotein C-III concentration stratification in quartiles: A) Apo C-III0; B)Apo C-III1; C)Apo C-III2.

### 4.5. Plasma Proteome profiles of CAD patients

The plasma proteomic profile of the same set of 51 CAD patients with high and low total Apo C-III was obtained using shotgun LC-MS analysis. The LC-MS analysis allowed the identification of 289 proteins, 180 of them were quantified. **Figure 16** shows the histogram of the log 10 Fold-Change (FC) of the CAD plasma samples divided into two classes: Apo C-III > 10 mg/dL Vs Apo C-III < 10 mg/dL. The up and down regulated proteins were selected using p value <0.05 and fold change >1.3. A total of 21 proteins were regulated, including 7 upregulated and 4 downregulated with FC ≥1.5 (see **Table 13)**

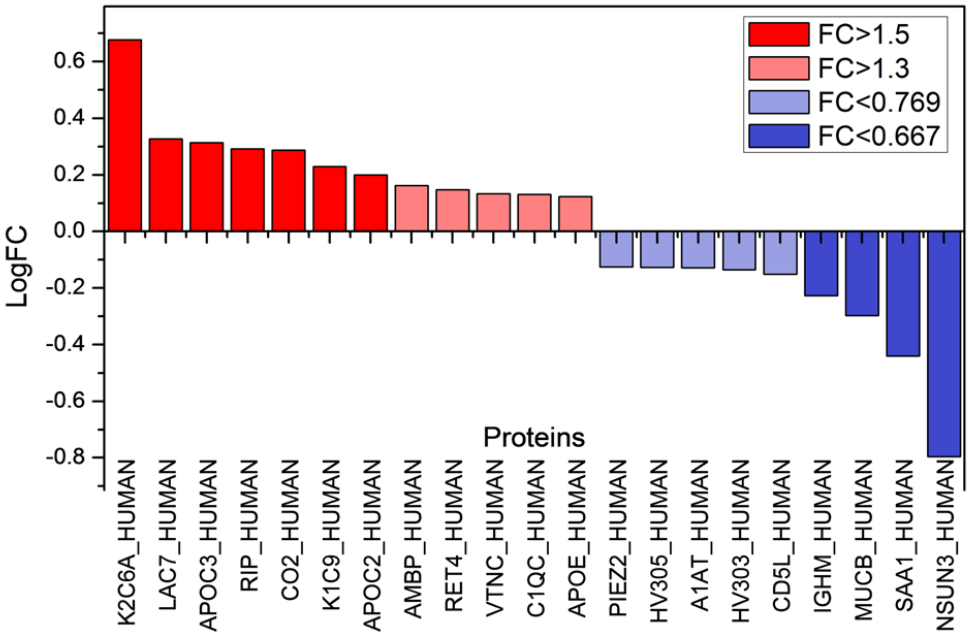


**Figure 16.** log 10 fold-Change of the CAD plasma samples: CAD patients with Apo C-III >10 mg/dL and with Apo C-III < 10 mg/dL were employed (high Apo C-III *vs* low Apo C-III).

**Table 13**. Proteins names, protein accession number, fold change, gene, biological process and molecular function classifications of modulated proteins in CAD patients (protein associated with high Apo C-III levels (Apo C-III> 10: FC>1.5).

| Protein names | Protein accession | FC up vs down | Gene | GO biological process | GO molecular function |
|---|---|---|---|---|---|
| Keratin, type II cytoskeletal 6A | K2C6A_HUMAN | **4.76** | KRT6A | Cell growth and/or maintenance | Structural constituent of cytoskeleton |
| Ig lambda-7 chain C region | LAC7_HUMAN | **2.12** | IGLL5 | Complement activation | Serine-type endopeptidase activity |
| RPA-interacting protein (hRIP) | RIP_HUMAN | **1.96** | RPAIN | DNA repair | Protein complex binding |
| Complement C2 | CO2_HUMAN | **1.93** | C2 | Complement activation | Serine-type endopeptidase activity |
| Keratin, type I cytoskeletal 9 | K1C9_HUMAN | **1.70** | KRT9 | Cell growth and/or maintenance | Structural constituent of cytoskeleton |
| Apolipoprotein C-II | APOC2_HUMAN | **1.59** | APOC2 | Lipoprotein metabolic process | Lipid binding |
| Protein AMBP | AMBP_HUMAN | 1.45 | AMBP | Immune response | Serine-type endopeptidase inhibitor activity |
| Retinol-binding protein 4 | RET4_HUMAN | 1.41 | RBP4 | Cardiac muscle tissue development | Transporter activity |
| Vitronectin (VN) | VTNC_HUMAN | 1.36 | VTN | Immune response negative regulation of blood coagulation | Extracellular matrix binding |
| Complement C1q subcomponent subunit C | C1QC_HUMAN | 1.35 | C1QC | Complement activation | Serine-type endopeptidase activity |
| Apolipoprotein E | APOE_HUMAN | 1.33 | APOE | Lipoprotein metabolic process negative regulation of blood coagulation | Low-density lipoprotein particle receptor binding |
| Piezo-type mechanosensitive ion channel component 2 | PIEZ2_HUMAN | 0.75 | PIEZO2 | Cation transport | Mechanically-gated ion channel activit |
| Immunoglobulin heavy variable 3-13 | HV305_HUMAN | 0.75 | IGHV3-13 | Complement activation | Serine-type endopeptidase activity |
| Alpha-1-antitrypsin | A1AT_HUMAN | 0.74 | SERPINA1 | Acute-phase response blood coagulation | Serine-type endopeptidase inhibitor activity |
| Immunoglobulin heavy variable 3-23 | HV303_HUMAN | 0.73 | IGHV3-23 | Complement activation | Serine-type endopeptidase activity |
| CD5 antigen-like | CD5L_HUMAN | 0.71 | CD5L | Immune response | Defense/immunity protein activity |
| Ig mu chain C region | IGHM_HUMAN | **0.59** | IGHM | Immune response | Antigen binding |
| Ig mu heavy chain disease protein (BOT) | MUCB_HUMAN | **0.50** | | Immune response | Antigen binding |
| Serum amyloid A-1 protein | SAA1_HUMAN | **0.36** | SAA1 | Innate immune response | Antigen binding |
| Putative methyltransferase NSUN3 | NSUN3_HUMAN | **0.16** | NSUN3 | rRNA methylation | Methyltransferase activity |

The 21 regulated proteins were, then, submitted to the web-based tool Cytoscape to visualize the non-redundant GO terms and pathways in functionally organized networks: the resulted image

(**Figure 17**) reflects the relations between the biological terms based on the similarity of their linked gene/proteins. The analysis revealed the presence of four functional clusters linked to the complement activation, the low-density lipoprotein, the acute phase response and the intermediated filament based process

The Cytoscape environment was subdivided in two protein groups (up-regulated and down-regulated) that were used in order to illustrate their functional differences, as reported in **Figure 17.**

**Figure 17** shows a clear up regulation of lipoprotein metabolism protein (up-regulated in patients with high Apo C-III), the proteins linked to the activity of the complement are both up and down regulated, while the acute phase response group is down-regulated.

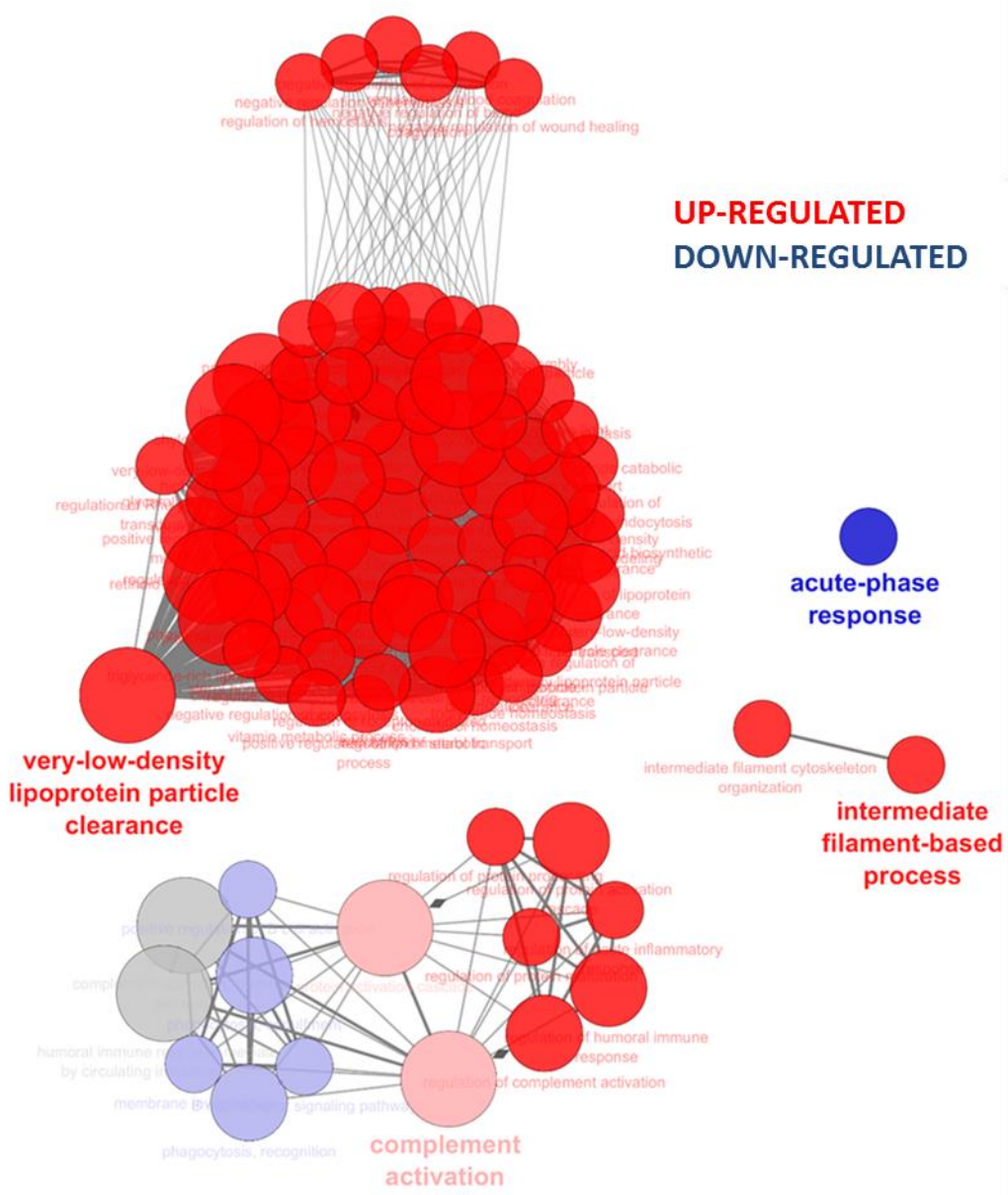


**Figure 17.** Cytoscape representation of functional clusters of regulated proteins in patients with high Apo C-III levels. Lipoprotein metabolism proteins are up-regulated, the proteins linked to the activity of the complement are both up and down regulated, while the acute phase response group is down-regulated

A protein-protein interaction analysis of the significant proteins was performed using STRING (Search Tool for the Retrieval of Interacting Genes) (**Figure 18**). STRING is a freely available database that relies on known and predicted protein interactions and that quantitatively integrates

interaction data from high-throughput experiments, genomic context, co-expression and other literature.

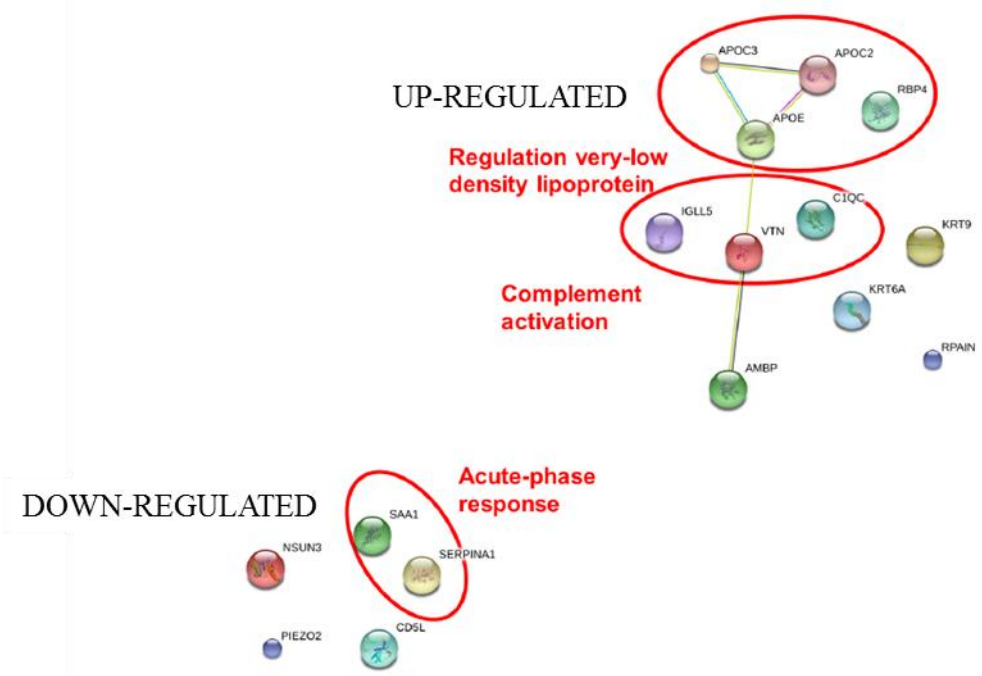


**Figure 18.** STRING network analysis of up regulated and down regulated proteins for the CAD patients with high Apo C-III levels.

The network related to lipoprotein metabolism, already highlighted by Cytoscape, shows that *APOC3* and *APOC2* have a co-expression connection while *APOC3* - *APOE* and *APOC2* - *APOE* are characterized by an interaction linked to literature works.

### Multivariate statistical analysis

In the case where a large number of descriptors (X variables) are present or a large experimental error is expected, it can be quite difficult to obtain a final model described by representative variables with a suitable predictive ability. In these cases, techniques for variable selection such as partial least square – discriminant analysis (PLS-DA) are usually exploited.

The PLS-DA was performed on all the samples in order to identify clusters and protein correlations between the two groups of CAD patients. The dataset was arranged in a matrix of 153 samples (51 patients x 3 instrumental replications, divided in two groups: patients with Apo C-III plasma levels < 10 mg/dL and patients with Apo C-III plasma levels > 10 mg/dL) and 180 variables (the signal of the quantified proteins). Data were first row-scaled to eliminate variations due to small differences in the total protein content of the initial sample; then, autoscaling was applied by the following equation:

$$x'_{ij} = \frac{(x_{ij} - \bar{x}_j)}{s_j}$$

where $x'_{ij}$ is the autoscaled value for the i-th sample and the j-th variable, $x_{ij}$ is the row-scaled value for the i-th sample and the j-th variable and $s_j$ is the standard deviation of the j-th variable after row-scaling.

After autoscaling all the variables are characterized by a null average value and unit variance, so that scale effects are eliminated. PLS-DA was then applied with a variable selection procedure in backward elimination: one variable is eliminated at a time according to the lowest error in cross-validation. Leave-more-out cross-validation was applied with 5 cancellation groups, taking out all the three replications of the same sample at a time. The final model contains 42 proteins and 3 latent variables (LVs).

We then performed the partial least square – discriminant analysis (PLS-DA) on all the samples in order to identify clusters and protein correlations between the two groups of CAD patients: Apo C-III < 10 mg/dL (blue dot) and Apo C-III > 10 mg/dL (red dot) (**Figure 19**). The PLS-DA model was carried out in a variable selection with backward elimination in cross-validation.

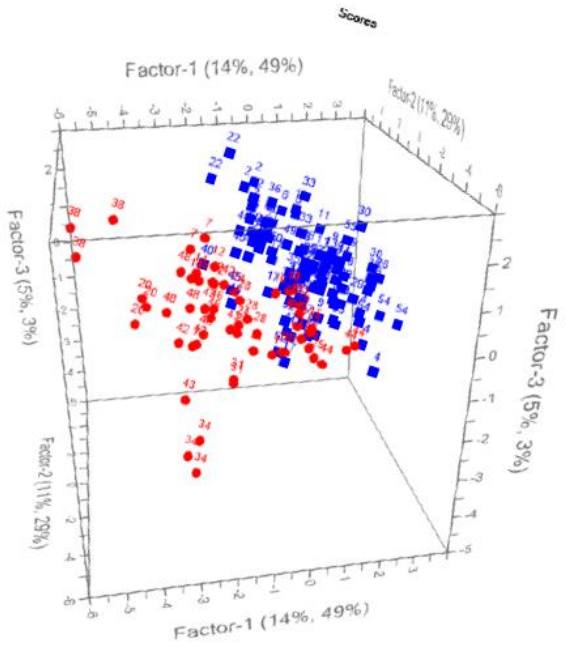


**Figure 19.** Score plot of the first three LVs calculated. Samples are separated according to the corresponding plasma level of Apo C-III (Apo C-III < 10 mg/dL (blue dot) and Apo C-III > 10 mg/dL (red dot).

The model with 3 LVs accounts for about the 80% of the information about class belonging and about the 30% of the information about the proteins.

**Table 14** reports the regression coefficients of the final model: a positive coefficient corresponds to proteins with a larger signal in patients with Apo C-III levels above 10 mg/dL and smaller signals in patients with Apo C-III levels below 10 mg/dL, while variables with a negative coefficient show an opposite behavior. The larger the absolute value of the regression coefficient, the larger the influence of the corresponding protein on the final model. The multivariate model identifies as discriminating a panel of proteins that on their whole increase and/or decrease in the two classes investigated: the regulations have to be identified as the contribution of all the 42 proteins on the final model, rather than observing single markers. In other words, the Backward Elimination variable selection (BE)-PLS-DA model identifies a correlation structure differentiating on its whole patients with low from those with high Apo C-III levels.

42 variables were significant for the two groups of CAD patients, in the **Table 14** are reported the proteins correlated with patients with Apo C-III < 10mg/dL (blue) and with patients with Apo C-III > 10 mg/dL (red).

**Table 14.** Regression coefficients of the proteins included in the final BE-PLS-DA model containing 3 LVs.

| | PROTEIN NAMES | COEFF. |
|---|---|---|
| IGHG4_HUMAN | Ig gamma-4 chain C region | -0.0088 |
| PPC1A_HUMAN | Phospholipid phosphatase 4 | -0.0107 |
| IGHG3_HUMAN | Ig gamma-3 chain C region (HDC) (Heavy chain disease protein) | -0.0109 |
| LV104_HUMAN | Immunoglobulin lambda variable 1-51 | -0.0112 |
| LAC2_HUMAN | Ig lambda-2 chain C regions | -0.0161 |
| PON1_HUMAN | Serum paraoxonase/arylesterase 1 | -0.0260 |
| NRX3A_HUMAN | Neurexin-3 (Neurexin III-alpha) (Neurexin-3-alpha) | -0.0292 |
| SMAG2_HUMAN | Protein Smaug homolog 2 | -0.0351 |
| SLAI2_HUMAN | SLAIN motif-containing protein 2 | -0.0397 |
| GELS_HUMAN | Gelsolin (AGEL) (Actin-depolymerizing factor) | -0.0403 |
| HBB_HUMAN | Hemoglobin subunit beta (Beta-globin) | -0.0407 |
| A1BG_HUMAN | Alpha-1B-glycoprotein (Alpha-1-B glycoprotein) | -0.0450 |
| HBA_HUMAN | Hemoglobin subunit alpha (Alpha-globin) | -0.0488 |
| CO4A_HUMAN | Complement C4-A (Acidic complement C4) | -0.0507 |
| CO9_HUMAN | Complement component C9 | -0.0523 |
| KV103_HUMAN | Immunoglobulin kappa variable 1D-33 | -0.0537 |
| NSUN3_HUMAN | Putative methyltransferase NSUN3 | -0.0550 |
| KV106_HUMAN | Immunoglobulin kappa variable 1-5 | -0.0643 |
| KV306_HUMAN | Immunoglobulin kappa variable 3-15 | -0.0679 |
| KV309_HUMAN | Immunoglobulin kappa variable 3-11 | -0.0686 |
| A1AT_HUMAN | Alpha-1-antitrypsin (Alpha-1 protease inhibitor) | -0.0694 |
| SAA1_HUMAN | Serum amyloid A-1 protein | -0.0699 |
| AACT_HUMAN | Alpha-1-antichymotrypsin (ACT) | -0.0710 |
| HV305_HUMAN | Immunoglobulin heavy variable 3-13 | -0.0734 |
| PIEZ2_HUMAN | Piezo-type mechanosensitive ion channel component 2 | -0.0874 |
| TTHY_HUMAN | Transthyretin (ATTR) (Prealbumin) (TBPA) | 0.0140 |
| IGHA1_HUMAN | Ig alpha-1 chain C region | 0.0217 |
| FCN3_HUMAN | Ficolin-3 (Collagen/fibrinogen domain-containing lectin 3 p35) | 0.0219 |
| ITIH1_HUMAN | Inter-alpha-trypsin inhibitor heavy chain H1 | 0.0287 |
| LBP_HUMAN | Lipopolysaccharide-binding protein (LBP) | 0.0338 |
| LV105_HUMAN | Immunoglobulin lambda variable 1-40 | 0.0404 |
| GRD2I_HUMAN | Delphilin (Glutamate receptor) | 0.0415 |
| C1QC_HUMAN | Complement C1q subcomponent subunit C | 0.0521 |
| LAC7_HUMAN | Ig lambda-7 chain C region | 0.0534 |
| FHR1_HUMAN | Complement factor H-related protein 1 | 0.0609 |
| CPN2_HUMAN | Carboxypeptidase N subunit 2 | 0.0704 |
| APOC2_HUMAN | Apolipoprotein C-II (Apo-CII) | 0.0722 |
| RET4_HUMAN | Retinol-binding protein 4 (Plasma retinol-binding protein) | 0.0739 |
| AMBP_HUMAN | Protein AMBP (Alpha-1 microglycoprotein) | 0.0847 |
| APOH_HUMAN | Beta-2-glycoprotein 1 (Anticardiolipin cofactor) | 0.0976 |
| APOE_HUMAN | Apolipoprotein E (Apo-E) | 0.1001 |
| APOC3_HUMAN | Apolipoprotein C-III (Apo-CIII) | 0.1266 |

**Table 15** reports the classification performances of the PLS-DA model with the backward elimination variable selection (BE-PLS-DA) applied to all the proteins and of the PLS-DA model calculated including all the 21 proteins resulted as statistically significant by the monovariate approach.

**Table 15.** Classification performances of of the PLS-DA model with the BE-PLS-DA calculated including all the 21 proteins resulted as statistically significant by the monovariate approach. Cross-validation results are presented for 51 patients and 5 cancellation groups: in both cases all the replications of the same sample were excluded at a time.

| | BE-PLS-DA (42 variables) | | | PLS-DA (21 variables identified by monovariate statistics) | | |
|---|---|---|---|---|---|---|
| | **Fitting** | **CV (51 canc. groups)** | **CV (5 canc. groups)** | **Fitting** | **CV (51 canc. groups)** | **CV (5 canc. groups)** |
| **Accuracy%** | 96.79 | 93.59 | 83.33 | 80.77 | 75.64 | 72.44 |
| **NER%** | 97.47 | 94.21 | 85.38 | 82.99 | 78.58 | 74.56 |
| **Precision class "low Apo C-III"** | 91.94 | 87.30 | 70.67 | 67.53 | 61.45 | 58.75 |
| **Precision class "high Apo C-III"** | 100 | 97.95 | 95.06 | 93.67 | 91.78 | 86.84 |
| **Sensitivity class "low Apo C-III"** | 100 | 96.49 | 92.98 | 91.23 | 89.47 | 82.46 |
| **Sensitivity class "high Apo C-III"** | 94.95 | 91.92 | 77.78 | 74.75 | 67.68 | 66.67 |
| **Specificity class "low Apo C-III"** | 94.95 | 91.92 | 77.78 | 74.75 | 67.68 | 66.67 |
| **Specificity class "high Apo C-III"** | 100 | 96.49 | 92.98 | 91.23 | 89.47 | 82.46 |

The results show that the panel of proteins identified by the multivariate strategy shows a better classification ability with respect to the model built including exclusively the markers identified by the monovariate approach. The results show good classification performances with quite good accuracy levels.

The 42 significant variables obtained from the multivariate approach were then used to described/characterize the two CAD groups, to identify pathways and biological functions linked to "Apo C-III CAD state". The STRING analysis, in **Figure 20**, of the proteins from the "high Apo C-

III patients" shows the clear connection with the triglyceride catabolic process, the lipid binding and the lipid transport (**Figure 20**).

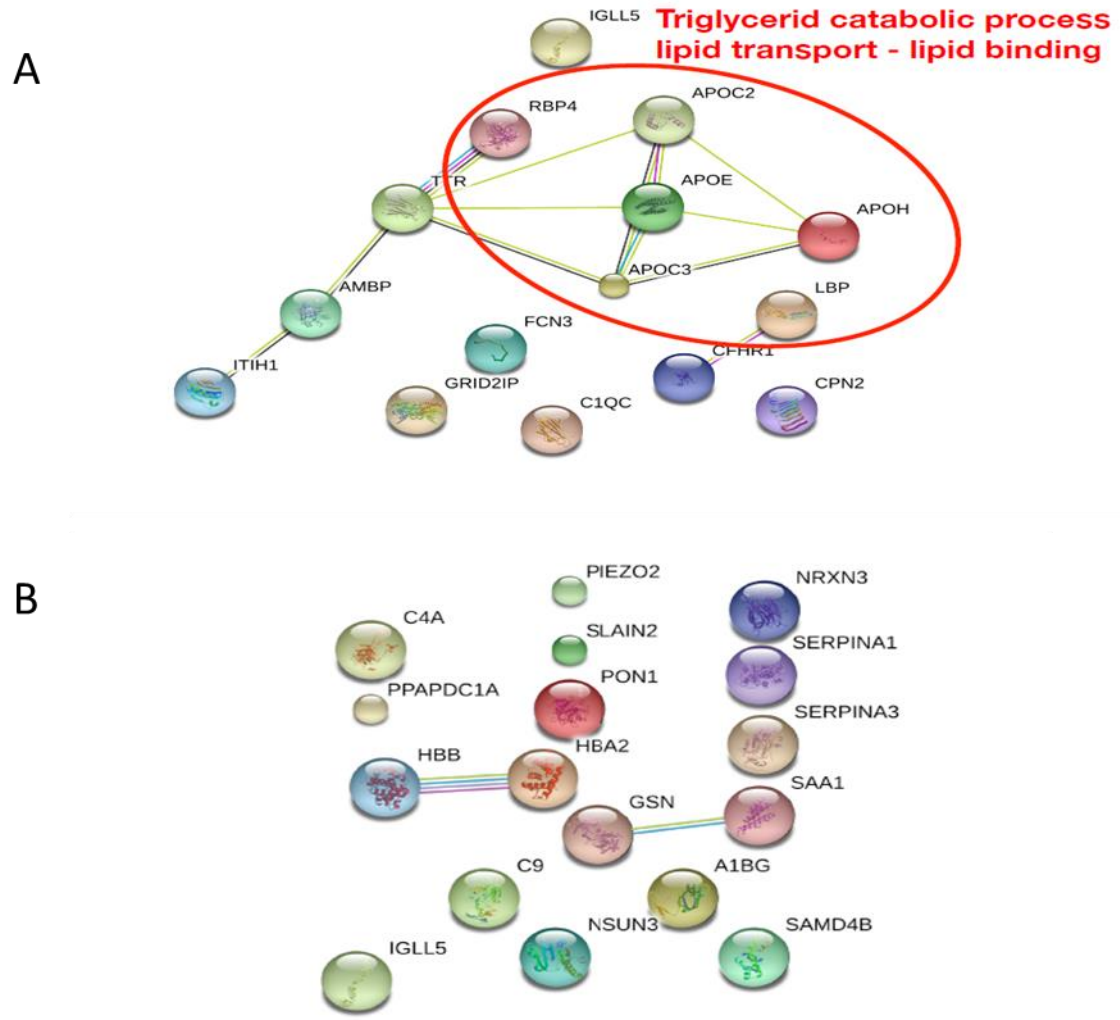


**Figure 20.** STRING analysis of the proteins from the "high Apo C-III patients"(A) and "low Apo C-III patients"(B) group respectively.

### 4.6. Plasma Lipidomic analysis of patients stratified according to Apolipoprotein C-III Apolipoprotein E and Triglycerides

#### Gas-Chromatography analysis versus Liquid Chromatography-Mass Spectrometry

We analyzed a set of 39 plasma samples obtained from CAD and CAD free patients stratified according to Apo C-III, Apo E and TG levels in order to evaluate their lipids profiles get insights into the contribution of the different molecules. The general characteristic of the patients are shown in **Table 16.** We assessed FA by gas chromatography analysis and lipids by LC-MS. Briefly patients were subdivided into 6 groups: group 1 characterized by low levels of TG Apo E and Apo C-III, group 2 characterized by low levels of TG and Apo E and high levels of Apo C-III, group 3 characterized by low levels of TG and Apo C-III and high levels of Apo E, group 4 characterized by low levels of TG and high levels of Apo E and Apo C-III, group 5 characterized by high levels of TG and low levels of Apo E and Apo C-III, group 6 characterized by high levels of TG, Apo E and Apo C-III. The six groups are illustrated in **Table 17.**

The gas-chromatography (GC) analysis showed the presence of different classes of free fatty acids from 14 to 24 atoms of carbon in the plasma of analyzed subjects. The most abundant FAs were palmitic acid (C:16) and stearic acid (C:18). Observed as a whole, all the data gave by GC approach didn't show a peculiar distribution for a possible classification, all of them presented similar profiles. On the other hand, the LC MS analysis was able to give separation profiles as illustrated in **Figure 21**, where molecules are separated according to the number of carbon chains and polarity.

**Table 16.** Clinical and laboratory characteristics of the cohort of CAD and CAD free patients.

| | Patients |
|---|---|
| **Age (years)** | 65.59±7.91 |
| **Male sex (%)** | 48.7% |
| **CAD** | 46.2% |
| **Myocardial infarction history (%)** | 17.9% |
| **Smoking history (%)** | 35.9% |
| **Hypertension (%)** | 56.4% |
| **Diabetes (%)** | 12.8% |
| **Total cholesterol (mM/L)** | 5.3±1.21 |
| **LDL cholesterol (mM/L)** | 3.3±0.95 |
| **HDL cholesterol (mM/L)** | 1.44± |
| **Triglyceride (mM/L)** | 1.4±0.75 |
| **Apolipoprotein A (g/L)** | 1.32±0.34 |
| **Apolipoprotein B (g/L)** | 1.05±0.28 |
| **Apolipoprotein E (g/L)** | 0.043±0.014 |
| **Apolipoprotein C-III (mg/dl)** | 10.68±3.62 |

**Table 17**. Group of survey for lipidomic analysis

| | **Group 1** ↓TG-↓Apo C-III-↓Apo E **(n=5)** | **Group 2** ↓TG-↑Apo C-III-↓Apo E **(n=4)** | **Group 3** ↓TG-↓Apo C-III-↑Apo E **(n=5)** | **Group 4** ↓TG-↑Apo C-III-↑Apo E **(n=5)** | **Group 5** ↑TG-↓Apo C-III-↓Apo E **(n=5)** | **Group 6** ↑TG-↑Apo C-III-↑Apo E **(n=5)** | **P*** |
|---|---|---|---|---|---|---|---|
| **Apo C-III (mg/dL)** | 7.26±0.55 | 13.88±1.77 | 7.89±1.42 | 13.59±2.10 | 7.45±1.36 | 14.94±1.68 | <0.001 |
| **Apo E (g/L)** | 0.029±0.003 | 0.030±0.005 | 0.046±0.002 | 0.059±0.008 | 0.034±0.002 | 0.058±0.009 | <0.001 |
| **TG (mmol/L)** | 1.01±0.11 | 0.94±0.16 | 0.90±0.25 | 0.93±0.09 | 2.45±0.27 | 2.68±0.34 | <0.001 |
| **Age (years)** | 68.8±3.8 | 69.3±2.9 | 62.8±3.6 | 66.6±7.2 | 57.0±4.4 | 63.8±5.5 | 0.013 |
| **Males (n)** | 3/5 | 0/4 | 3/5 | 2/5 | 4/5 | 3/5 | NS |
| **CAD (n)** | 3/5 | 3/4 | 1/5 | 3/5 | 5/5 | 3/5 | NS |

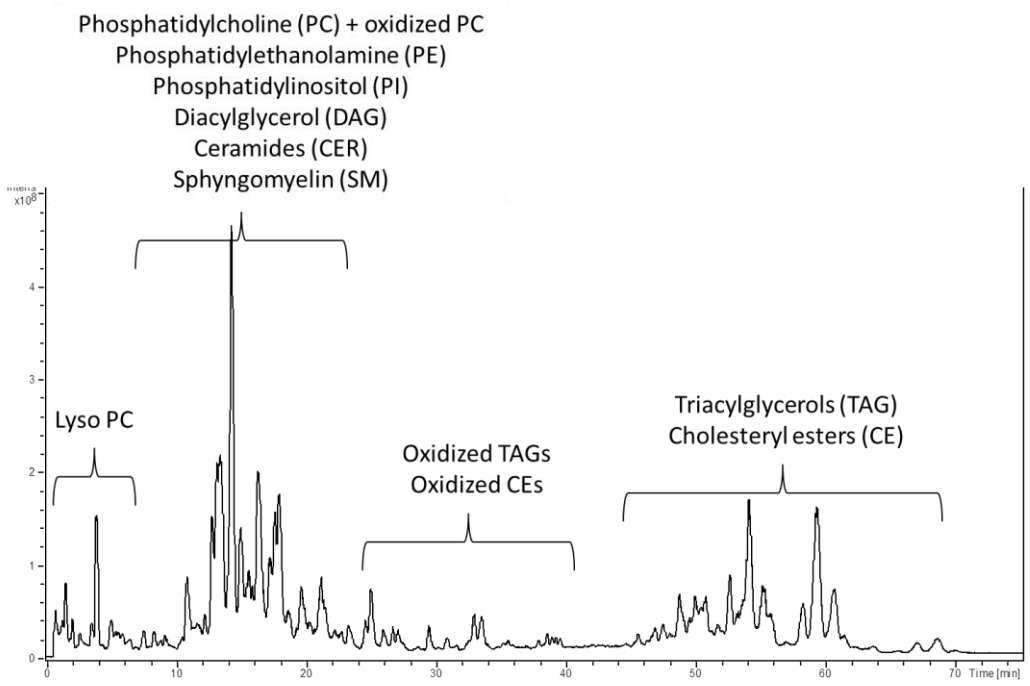


**Figure 21.** Chromatogram representative of the RPLC-ESI -MS analysis performed on plasma samples.

A total of 254 lipid species were identified 34 Cholesteryl Esters (CE) and Cholesteryl Esters oxidized (CE-oxidized); 5 Ceramides; 9 Diacylglycerol (Gangabadage, Zdunek et al.); 9 Lysophosphatidylcholines (Lyso-PC); 64 Phosphatidylcholines (PC); 9 Phosphatidylethanolamine (PE) 1 Lysophosphatidylethanolamine (PE); 9 Phosphatidylinositol (PI); 35 sphingosylphosphorylcholine (SM); 79 triacylglycerol (TAG).

The principal component analysis (PCA), an analysis unsupervised of the data (**Figure 22**) showed a subdivision of the population in 2 groups according to the Apo E levels (high Apo E levels in red, low Apo E in green). More precisely, in the red group were included most of the samples belonging to group 3, 4 and 6 (see **Table 17**) characterized by high levels of Apo E, while in the green group were included most of the sampler with low Apo E levels, i.e. groups 1, 2, and 5. The data were, analyzed by mean of scaling method of Pareto, which allows to reduce the influence of the most intense peaks and emphasizes the less intense metabolites still having a biological value.

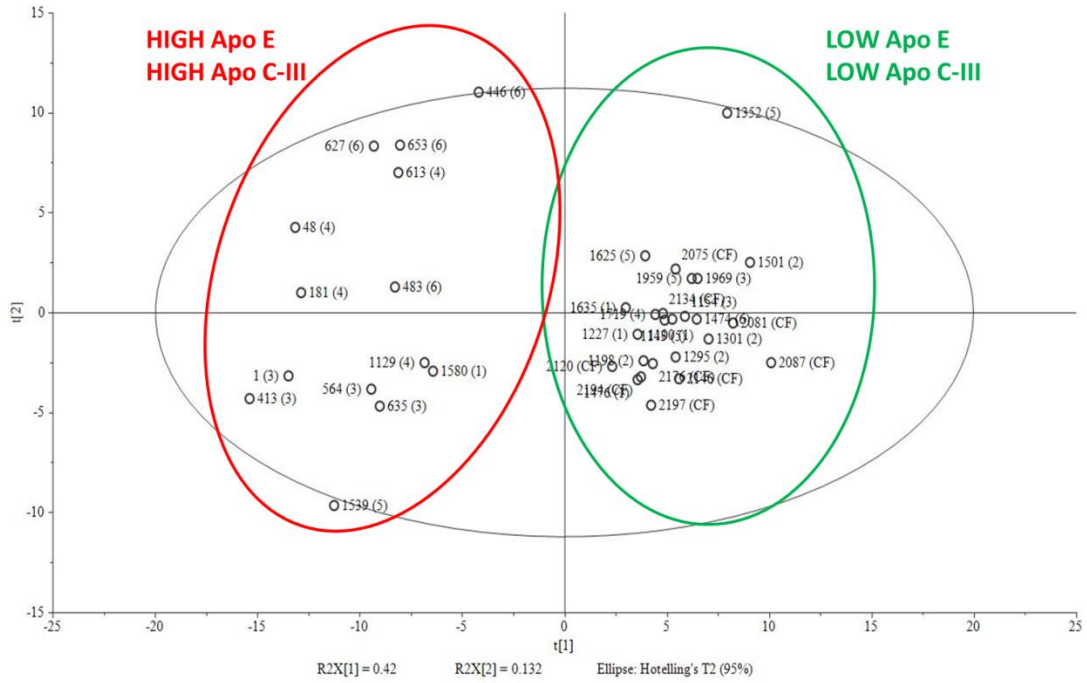


**Figure 22.** Principal component analysis on the LC-MS data– Pareto Scaling – log transformed. PC1 explains 42% of the total information, instead the second component PC2 explains the 13% of total information.

Then the Partial Least Squares- Discriminant Analysis (PLS-DA), a supervised method, was performed to give a validated model through permutation test (200 permutations) changing the samples order, considering statistically significant only model with a Q2> 0.5. According to this condition only two of all the models resulted statistically significant, the subdivision in two and three groups (**Table 18**).

On these models we performed Orthogonal Partial Least Squares- Discriminant Analysis (OPLS-DA) in order to identify the metabolites which contribute to the classes separation whit a cut off of p(corr)≥0.75. In this way it is possible to select the metabolite which will pass the t-test at 99%.

**Table 18.** Partial least square – discriminant analysis (PLS-DA)

| PLS-DA Model | Q2 |
|---|---|
| Subdivision in 6 groups | 0.0864 |
| **Subdivision in 2 groups by PC1** | **0.944** |
| **Subdivision in 3 groups byPC2** | **0.738** |
| Group 1 (↓TG-↓ApoC-III-↓ApoE) vs all | -0.0202 |
| Group 2 (↓TG-↑ApoC-III-↓ApoE) vs all | 0.291 |
| Group 3 (↓TG-↓ApoC-III-↑ApoE) vs all | 0.432 |
| Group 4 (↓TG-↑ApoC-III-↑ApoE) vs all | 0.291 |
| Group 5 (↑TG-↓ApoC-III-↓ApoE) vs all | -0.0385 |
| Group 6 (↑TG-↑ApoC-III-↑ApoE) vs all | 0.406 |

The S-plot in **Figure 23** shows the OPLS-DA on two groups (Q2=0.944-see **Table 18**). In red we can observed the metabolites associated to the groups with high levels of Apo E, instead in green we observe the metabolites associated to the groups with low levels of Apo E. All the metabolites have a p(corr)≥0.75 and are summarized in **Tables 19** and **20**.

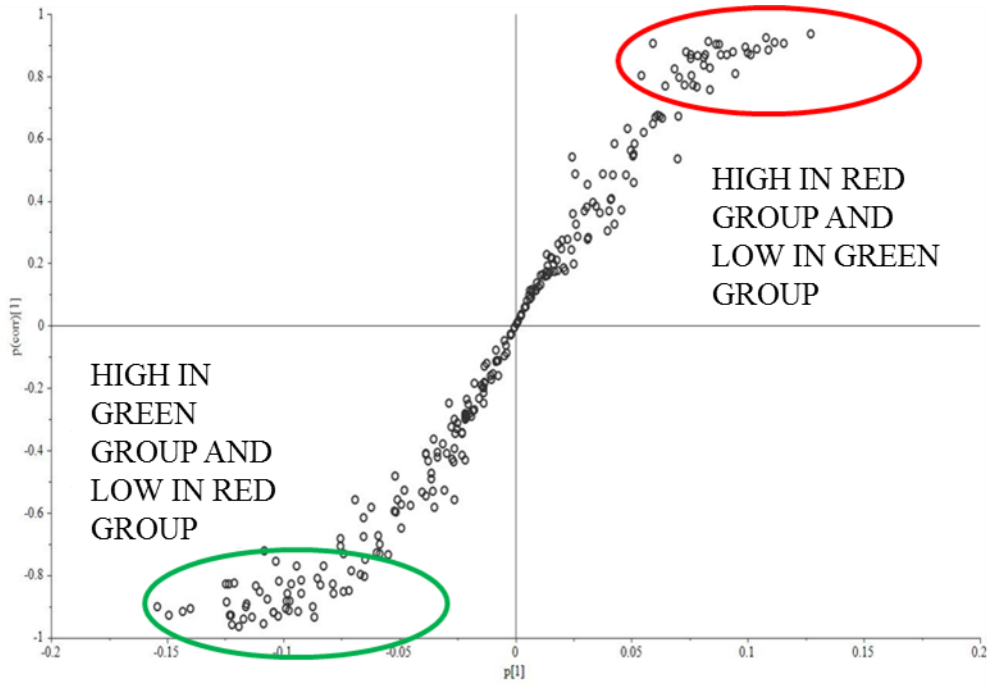


**Figure 23**. S-plot shows the OPLS-DA on two groups (Q2=0.944) subdivided according to Apo E plasma levels. In the red are shown the metabolites that characterize the groups with high levels of Apo E. In the green circle are illustrated the metabolites that characterize groups with low levels of Apo E. Metabolites were selected according to p(corr)>0.75

The OPLS-DA analysis of the patients with high levels of Apo E underlined the presence of 34 lipid species belonging to the family of PC, Lyso PC, TAG and CE (**Table 19**). In particular what is worth of interest is the presence of di-oxidized CE. In the group characterized by low levels of Apo E, instead, 48 lipid species were found, belonging, in particular, to the family of PC and TAG. None CE oxidized were observed (**Table 20**).

**Table 19.** Metabolites in samples with high Apo E levels (p corr)>0.75)

| m/z | RT | class | ID |
|---|---|---|---|
| 756.674 | 4.33621 | PC | PC 34:2-OH -[M+H-18]+ |
| 774.571 | 4.92215 | PC | PC 34:2-OH (16:0/18:2-OH) |
| 790.569 | 3.31241 | PC | PC 34:2-OOH (16:0/18:2-OOH) |
| 772.664 | 5.17473 | PC | PC 34:3-OH (16:0/18:3-OH) |
| 772.654 | 5.5346 | PC | PC 34:3-OH (16:0/18:3-OH) |
| 802.546 | 7.36171 | PC | PC 36:2-OH (18:0/18:2-OH) |
| 800.604 | 7.77743 | PC | PC 36:3-OH (18:0/18:3-OH) |
| 800.553 | 8.23455 | PC | PC 36:3-OH (18:0/18:3-OH) |
| 780.616 | 4.05788 | PC | PC 36:4-OH (18:2/18:2-OH) |
| 496.383 | 1.48833 | LYSO PC | LYSO PC 16:0 |
| 524.35 | 2.05214 | LYSO PC | LYSO PC 18:0 |
| 480.419 | 1.69146 | LYSO PC | LYSO P-PC 16:1 |
| 508.47 | 1.97039 | LYSO PC | LYSO P-PC 18:1 |
| 510.49 | 2.59712 | LYSO PC | LYSO P-PC 18:0 |
| 341.359 | 2.04925 | LYSO PE | LYSO PE 18:0 |
| 867.749 | 34.6308 | TAG | TAG 50:3-OH (14:0/18:1/18:2-OH) - Na ADDUCT |
| 923.793 | 41.3612 | TAG | TAG 54:3 -OH (18:0/18:1/18:2-OH) - Na ADDUCT |
| 923.786 | 41.934 | TAG | TAG 54:3 -OH (18:0/18:1/18:2-OH) - Na ADDUCT |
| 895.705 | 37.8534 | TAG | TAG 52:3-OH - Na ADDUCT |
| 895.756 | 38.3996 | TAG | TAG 52:3-OH - Na ADDUCT |
| 645.562 | 48.8724 | CE | CE 16:1 - Na ADDUCT |
| 703.655 | 26.7012 | CE | CE 18:2 DIOXIDIZED - Na ADDUCT |
| 703.643 | 27.0215 | CE | CE 18:2 DIOXIDIZED - Na ADDUCT |
| 703.652 | 28.8351 | CE | CE 18:2 DIOXIDIZED - Na ADDUCT |
| 687.533 | 35.3774 | CE | CE 18:2-OH - Na ADDUCT |
| 687.629 | 32.3605 | CE | CE 18:2-OH - Na ADDUCT |
| 687.638 | 33.3578 | CE | CE 18:2-OH - Na ADDUCT |
| 701.658 | 29.3099 | CE | CE 18:3 -DIOXIDIZED - Na ADDUCT |
| 701.646 | 31.8689 | CE | CE 18:3 -DIOXIDIZED - Na ADDUCT |
| 685.543 | 35.4172 | CE | CE 18:3-OH - Na ADDUCT |
| 727.613 | 21.8935 | CE | CE 20:4 DIOXIDIZED - Na ADDUCT |
| 727.586 | 22.616 | CE | CE 20:4 DIOXIDIZED - Na ADDUCT |
| 727.544 | 23.4461 | CE | CE 20:4 DIOXIDIZED - Na ADDUCT |
| 727.636 | 25.2206 | CE | CE 20:4 DIOXIDIZED - Na ADDUCT |

**Table 20.** Metabolites in sample with low Apo E levels (p(corr)>0.75)

| m/z | RT | Class | ID |
|---|---|---|---|
| 756.607 | 10.31 | PC | PC 34:3 |
| 756.621 | 11.1355 | PC | PC 34:3 |
| 768.529 | 11.0706 | PC | PC 35:4 |
| 784.611 | 14.7277 | PC | PC 36:3 |
| 782.545 | 12.3839 | PC | PC 36:4 |
| 780.545 | 10.116 | PC | PC 36:5 |
| 780.527 | 10.8735 | PC | PC 36:5 |
| 812.55 | 18.4739 | PC | PC 38:3 |
| 812.596 | 19.3135 | PC | PC 38:3 |
| 810.513 | 16.4369 | PC | PC 38:4 |
| 810.548 | 17.3714 | PC | PC 38:4 |
| 810.475 | 15.6519 | PC | PC 38:4 |
| 808.46 | 13.8714 | PC | PC 38:5 |
| 808.45 | 14.9887 | PC | PC 38:5 |
| 806.439 | 12.0739 | PC | PC 38:6 |
| 806.459 | 12.6321 | PC | PC 38:6 |
| 836.586 | 18.4013 | PC | PC 40:5 |
| 834.625 | 16.9473 | PC | PC 40:6 |
| 768.572 | 15.6778 | PC | P-PC 36:4 (O:16:O/20:4) |
| 766.612 | 14.8734 | PC | P-PC 36:5 (O:16:1/20:4) |
| 796.627 | 20.3791 | PC | P-PC 38:4 (O-18:0/20:4) |
| 794.604 | 16.5194 | PC | P-PC 38:5 (O-18:1/20:4) |
| 629.646 | 12.3116 | PI | PI 38:3 |
| 627.633 | 10.887 | PI | PI 38:4 |
| 627.607 | 14.3786 | PI | PI 38:4 |
| 731.594 | 15.673 | SM | SM 36:1 |
| 873.78 | 44.5087 | TAG | TAG 52:6 - Na ADDUCT |
| 875.819 | 46.2899 | TAG | TAG 52:5 (16:1/18:2/18:2) - Na ADDUCT |
| 899.687 | 45.7328 | TAG | TAG 54:7 (18:2/18:2/18:3) - Na ADDUCT |
| 901.723 | 47.3067 | TAG | TAG 54:6 (18:2/18:2/18:2) - Na ADDUCT |
| 901.712 | 48.9914 | TAG | TAG 54:6 - Na ADDUCT |
| 903.712 | 52.7673 | TAG | TAG 54:5 - Na ADDUCT |
| 903.731 | 50.762 | TAG | TAG 54:5 (18:1/18:2/18:2) - Na ADDUCT |
| 925.71 | 46.7861 | TAG | TAG 56:8 - Na ADDUCT |
| 925.718 | 47.8327 | TAG | TAG 56:8 - Na ADDUCT |
| 927.641 | 49.6071 | TAG | TAG 56:7 - Na ADDUCT |

| | | | |
|---|---|---|---|
| 927.644 | 51.3708 | TAG | TAG 56:7 - Na ADDUCT |
| 929.66 | 53.5527 | TAG | TAG 56:6 - Na ADDUCT |
| 929.656 | 54.4679 | TAG | TAG 56:6 - Na ADDUCT |
| 931.724 | 56.9054 | TAG | TAG 56:5 - Na ADDUCT |
| 931.714 | 58.0723 | TAG | TAG 56:5 - Na ADDUCT |
| 931.722 | 59.576 | TAG | TAG 56:5 (18:0/18:1/20:4) - Na ADDUCT |
| 669.645 | 46.5392 | CE | CE 18:3 - Na ADDUCT |
| 693.633 | 45.3807 | CE | CE 20:5 - Na ADDUCT |
| 695.623 | 48.6954 | CE | CE 20:4 - Na ADDUCT |
| 697.657 | 51.7641 | CE | CE 20:3 - Na ADDUCT |
| 719.626 | 47.1568 | CE | CE 22:6 -Na ADDUCT |
| 711.554 | 33.5169 | CE | CE 20:4-OH - Na ADDUCT |

According to PLS-DA on the subdivision in three groups (Q2=0.738 **Table 18**) PLS-DA (**Figure 24**) confirms the data observed for the analysis for the subdivision in 2 groups but, underlines also the presence of a third category of population (orange) with high levels of Apo E and low levels of Apo C-III (group 3 **Table 17**).

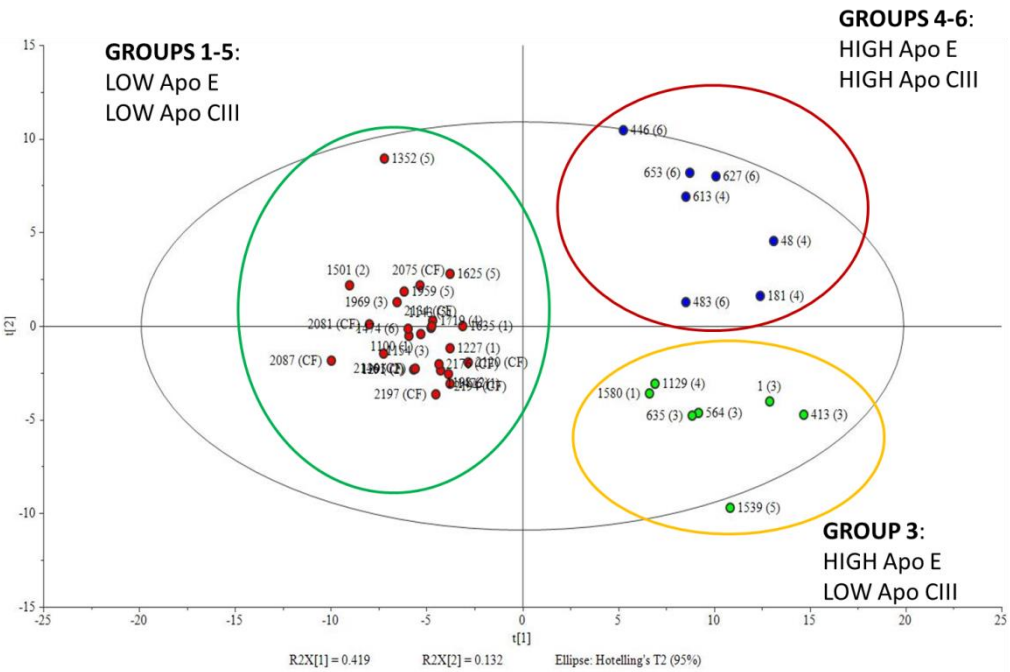


**Figure 24.** PLS-DA on three groups: Q2: 0.738

The OPLS-DA confirmed the data obtained with PLS-DA and the presence of three groups classification but for the third group (orange) none of the metabolites already observed for the other groups were found. This third group didn't present any possible marker ( see **Figure 25**).

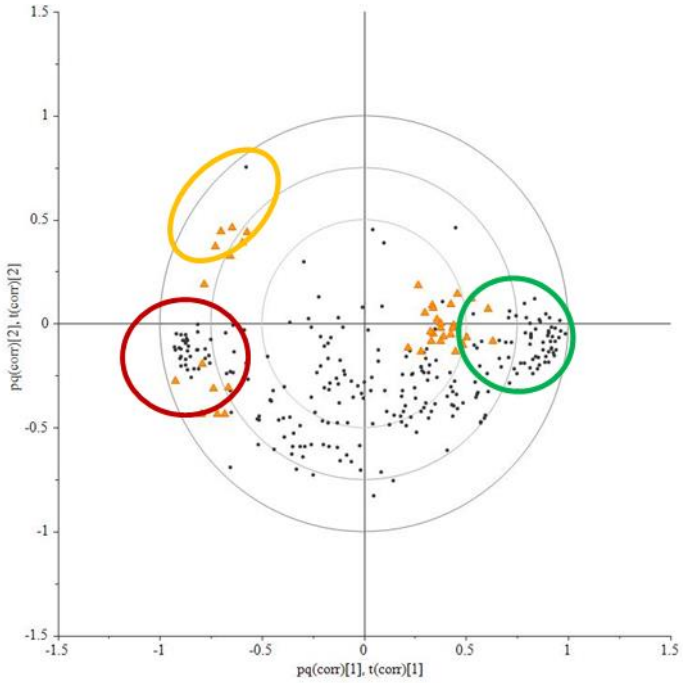


**Figure 25.** OPLS-DA on three groups. The dots indicate the metabolites while the triangles indicate the sample. All the metabolites observed near the sample are the metabolites that characterized the group.

# 5. DISCUSSION

## 5.1.Distribution of glycoforms of Apolipoprotein C-III in coronary patients

Apo C-III is present mainly in 3 isoforms termed Apo C-III0, Apo C-III1, and Apo C-III2, depending on the number of sialic acid molecules (0 to 2) at the oligosaccharide portions of the protein (Nicolardi, van der Burgt et al. 2013). It has been estimated that each glycoform may physiologically contribute, respectively, to approximately 10%(Apo C-III0), 55% (Apo C-III1), and 35% (Apo C-III2) of the total circulating Apo C-III levels (Nicolardi, van der Burgt et al. 2013). We firstly analyzed Apo CIII glycoforms by IEF. Isofocusing is a simple and rapid technique that allows semi-quantitative determination of Apo C-III glycoforms. We analyzed 4 groups of CAD patients stratified according to Apo C-III and PUFA levels.

By the IEF approach for the quantification of the three Apo C-III glycoforms in the four observed groups we found that despite the high levels of total Apo C-III in group 3 (**Table 4** ), a proportion between glycoforms 1 and 2 is maintained probably due to the known positive effect of PUFA. On the contrary, patients of group 4, having low PUFA plasma levels, presented an alteration of the relative proportion between the glycoforms 1 and 2, being Apo C-III1 more abundant. We observed that the group 3, selected for high Apo C-III levels, showed TG mean concentrations lower than expected. The finding suggests that this group might represent a peculiar subset in which the association high Apo C-III-high TG is missing. This apparent anomaly deserves further in-depth study to assess the incidence of cardiovascular events and the overall survival in this specific patient subset.

## 5.2. Deregulated plasmatic proteins in CAD patients with different Apo C-III levels

On the basis of the role of the Apo C-III not only in the lipoprotein metabolism but also in inflammatory phenomena and thrombotic events, worth of interest is to observe how the total plasma Apo C-III concentration may affects the expression of the proteins involved in such

different pathway. Then, exploiting previous comparative analysis results, on the same population of IEF analysis, we decided to select five proteins ( Serum Amyloid Protein, Fibrinogen β, Fibrinogen γ, Fibrinogen γ' and Complement C3) differentially expressed according to Apo C-III concentration and to validate the results by western blotting.

Interestingly, we found that patients with high Apo C-III have a lower plasmatic level of serum amyloid P component. This protein is a member of pentraxins family, that promotes the cholesterol efflux from cells (Song, Cai et al. 2010). It binds the oxidized LDLs, preventing lipids storage in macrophages and their conversion into foam cells that would bring to atherosclerotic damage formation (Stewart, Tseng et al. 2005). The data allow to hypothesize that patients with high Apo C-III could be therefore more prone to the formation of atherosclerotic plaques also as a consequence of reduced plasma levels of serum amyloid P component. Further evidence is however necessary to definitively confirm this hypothesis.

According to a previous study, Apo C-III is involved not only in atherosclerotic plaques formation, but also in the coagulation pathway, since there is an independent association between Apo C-III levels and thrombin generation (Olivieri, Martinelli et al. 2010). So it is worth noting that we observed in patients with high Apo C-III a modulation of different fibrinogen chains. In particular, we found decreased levels of fibrinogen β and increased levels of fibrinogen γ in patients with high Apo C-III. Fibrinogen γ prime (γ') arises from a splice variant of the γ-chain messenger RNA resulting from an alternative polyadenylation signal in intron 9 (Chung and Davie 1984, Fornace, Cummings et al. 1984). The alternative polyadenylation leads to the translation of a unique 20-amino-acid C-terminal extension encoded by intron 9, which substitutes the 4 amino acids of exon 10 (Fornace, Cummings et al. 1984, Drouet, Paolucci et al. 1999, Mannila, Lovely et al. 2007). The association with different thrombotic diseases has been ascribed in part to the effects of γ' on clot structure, on thrombin activity and fibrinolysis (Uitte de Willige, Standeven et al. 2009). Several studies show that fibrinogen γ' levels increased in CAD patients (Lovely, Falls et al. 2002, Lovely, Kazmierczak et al. 2010). Fibrinogen γ' is associated with an opposite trend with both venous (Uitte

de Willige, de Visser et al. 2005, Lovely, Boshkov et al. 2007, Lovely, Kazmierczak et al. 2010) and arterial thrombosis (Drouet, Paolucci et al. 1999, Lovely, Falls et al. 2002, Mannila, Lovely et al. 2007, Cheung, Uitte de Willige et al. 2008, van den Herik, Cheung et al. 2011). This association with different thrombotic disorders has been ascribed in part to the effects of fibrinogen γ' on clot structure, crosslinking by factor XIIIa, thrombin activity, or fibrinolysis (Uitte de Willige, Standeven et al. 2009). In particular it was observed how this protein has thrombogenic effects in arterial site (Uitte de Willige, Standeven et al. 2009) and anti-thrombogenic effect in venous site (Uitte de Willige, de Visser et al. 2005, Lovely, Boshkov et al. 2007).Since it could be supposed that these latter function could be associated with Apo C-III levels, we performed 1D and 2D immunoblottin analyses of fibrinogen γ‘ chain (**Figure. 6** and **Figure.7**). The data confirmed the comparative analysis results. Briefly we observe an down-regulated Fibrinogen β in groups with high levels of Apo C-III compared to groups with low levels of Apo C-III and in groups with high Apo C-III levels an up-regulation of Fibrinogen β in profile B (low PUFA levels). An opposite trend, instead was observed for the Fibrinogen γ and γ' an up-regulation in groups with high levels of Apo C-III compared with low levels of Apo C-III, with also an up- regulation in groups with low levels of Apo C-III profile A (high PUFA).

Finally atherosclerosis is an inflammatory disease and several studies suggest that the complement system, being one of the main components of innate immunity, is involved in its pathogenesis, although its role has not yet been fully elucidated. Growing evidences indicate that complement activation occurs within atherosclerotic plaques, playing a dual effect: it has a protective function removing apoptotic cells and cell debris from atheroma, and it also induces pro-inflammatory events leading to the destabilization of the plaque (Speidl, Kastl et al. 2011). We reported the modulation of several components of complement but in particular, the increase of complement C3 levels suggested that this component is not consumed. The 2D western blotting analysis confirmed the complement C3 trend (**Figure 7**), underlining an upregulation of this protein in patients with low levels of Apo C-III profile A (high PUFA) versus patients with profile B (low PUFA).

### 5.3. Apolipoprotein C-III quantification by Shotgun-Top down MS analysis

We moved to a more powerful technology for the analysis of Apo C-III isoforms, being able to quantify Apo C-III0, 1 and 2, in 51 stable CAD patients, carefully selected.

Apo C-III- and TG- rich particles not only may contain variable amounts of other apolipoproteins (such as Apo B and Apo E) but also may differ in their relative proportions of sialylated isoforms of Apo C-III. A substantially unresolved question concerns the fact whether – in case of pathological increase of Apo C-III - all these isoforms change in parallel, or asymmetric variations for some of them may be recognized. As Apo C-III sialylation appears to be under metabolic control (Yassine, Trenchevska et al. 2015), this latter possibility may associate with metabolic and cardiovascular disorders and therefore be particularly important in patients with high "residual CV risk" such as CAD patients treated with statins but still presenting elevated Apo C-III concentrations.

This study aimed at answering this question through a rather original approach, i.e. by examining two subgroups of CAD patients, all treated with statins but showing a fully divergent concentration (very low vs very high) of total circulating Apo C-III.

By this approach, it was possible not only to compare subjects at very different risk in the setting of the secondary cardiovascular prevention, but also to study the proportional expression of the various isoforms through the entire range of concentrations of Apo C-III. Under this respect, the most important results are graphically summarized in **Figure 8**, where the relative proportions of any single isoform are plotted against the total plasma concentration of Apo C-III. The only glycoform that strictly reflects the trend of the total apolipoprotein concentrations was the monosialylated form, Apo C-III1. In contrast, fully or non- sialylated (Apo C-III2 and Apo C-III0, respectively) did not correlated at all or even presented a negative correlation with the total Apo C-III (**Figure 8**).

The finding of a "neutral" proportion and a quite stable ratio of di-sialylated isoform Apo C-III2 to total Apo C-III is consistent with the results recently presented by Yassine and coll. (Yassine, Trenchevska et al. 2015) but not by others (Koska, Yassine et al. 2016); of note, both in our study and in the Yassine's one, a MS methodology was used, while this was not the case for previous

reports that instead adopted an iso-electrofocusing (IEF) based approach for isoforms evaluation (Wopereis, Grunewald et al. 2003, Wada, Kadoya et al. 2012).

It is therefore plausible that these results are largely dependent on the methodology employed; as MS is generally considered more precise and reliable than IEF, any comparison among reports using methodologies other than MS is likely meaningless.

More surprising and even counterintuitive was the distinct association between relative amounts of Apo C-III0 (non-sialylated) isoform and total Apo C-III, that resulted to be related in a negative way. In other words, CAD patients presenting lower concentrations of total Apo C-III were also the individuals with higher proportion of non-sialylated apolipoprotein, thus suggesting to some extent a protective role for this isoform.

In principle, it is possible to speculate that low levels of total Apo C-III match low levels of its isoforms. The observed paradoxical behavior may be explained by metabolic or pharmacological reasons. Considering that all patients were taking statins, the result could be related to a different pharmacological sensitivity for statins of a subgroup of individuals. Subjects receiving the maximal benefit in terms of reduction of total Apo C-III by statin therapy can have a decrease in their relative amount of sialylated isoforms and, in turn, a proportional apparent increase in Apo C-III0 content. On the contrary, patients with persistently elevated total Apo C-III, i.e. in a "statin-resistant" condition, seemed to be characterized by an increase of monosialylated isoforms. This may be in agreement with a possible inhibitory role of the statins on the apolipoprotein sialylation process. Thus, the relation between pharmacological effects of these drugs and sialylation may open new perspectives that deserve further investigation.

The second question addressed in the present work concerns the relationship between Apo C-III glycoforms and other lipids and lipoproteins, in particular with the "harmful" Apolipoprotein B and the "protective" Apolipoprotein E.

Our results suggest that Apo C-III glycoforms vary in their association with plasma lipids and apolipoproteins and therefore - in turn – with the cardiovascular risk traditionally associated with

their levels. Thus, a measure of total Apo C-III may not strictly reflect the overall risk represented by the single isoform. In a comparable way to the concentration of total Apo C-III, the monosialylated isoform resulted to be statistically correlated with a less favorable lipid profile, including an increase of plasma total and LDL cholesterol, TG, Apo B and Apo E. As a consequence, a relatively elevated amount of this isoform seemed to characterize the same "harmful" lipid situation that was observed when total Apo C-III is elevated. Many previous studies in fact showed not only that total Apo C-III concentrations are associated with circulating Apo B-enriched lipoproteins carrying substantial amount of cholesterol and TG, but also that this represents a condition of elevated risk for CAD patients (Alaupovic, Mack et al. 1997, Lee, Campos et al. 2003, Mendivil, Rimm et al. 2011, Mendivil, Rimm et al. 2013).

The relationship between Apo C-III glycoforms and Apo E has been poorly investigated. Particles rich in Apo C-III are also abundant in Apo E (Zheng, Khoo et al. 2007, Mendivil, Zheng et al. 2010, Zheng, Khoo et al. 2010). The opposing actions of Apo C-III and Apo E on subspecies of VLDL and LDL represent important factors modulating Apo B lipoprotein metabolism: it was indeed suggested that, in presence of Apo E, lipoproteins are cleared more rapidly from the circulation (Sacks 2015). We did not analyze the single species of lipoproteins, however, in light of the findings obtained (positive and negative correlation for Apo C-III1 and Apo C-III0, respectively; see **Table 7**) Apo C-III1/Apo C-III0 ratio may reflect the relative abundance of Apo E on these particles. Accordingly, for the same concentration of total Apo C-III, individuals with highest Apo C-III1/Apo CIII0 ratio should have more Apo E in their TG- rich lipoproteins.

Furthermore the design of the study and the patients selection were planned to clarify how fatty acids, and more specifically PUFA, affect the glycoforms distribution. While total Apo C-III decreased with increasing PUFA ($R=-0.530$; $P<0.001$, **Table 9**), the pattern of distribution of the single isoforms did not change in presence of large variations of the dietary intake of total PUFA (i.e. including both ω3 and ω6 families). When the group of patients was separated by low or high PUFA intake, an association trend was present for Apo C-III0 and Apo C-III1 but this was limited

to the situation of "low risk" (low total apolipoprotein). The results were particularly clear for ω3 PUFA that showed a null capability to interfere with the glycoforms (**Table 9**).

A similar situation was also observed for total MUFA that correlated with total Apo C-III but not with its isoforms. Thus, these findings confirm the interaction between dietary FA intake and apolipoprotein production and metabolism but do not suggest relevant dietary influences on the Apo C-III glycosilation. The relative proportions of Apo C-III glycoforms are probably unaffected by changing the quality of FAs in the diet or by administering ω3 PUFA for therapeutic purposes.

The data obtained provided information on the complex relation among Apo C-III glycoforms, lipids and lipoproteins defining the dynamics of the sialylation process through the entire range of concentrations of Apo C-III. Regardless of the cause (pharmacologically by statins and/or genetically induced), the findings obtained in presence of low levels of Apo C-III should be of general value in terms of pathophysiology. In the opposite case, the value of the present data is instead specifically of interest for CAD patients in the setting of secondary prevention, one of the most important challenge for future cardiovascular research.

The work suffers from some limitations that need to be acknowledged. First, the sample size of patients is relatively limited so that possible statistical associations may result overlooked. The selection of patients with very low or very high concentrations of Apo C-III should however amplify the differences arising from such opposite conditions.

In second instance, the individuals investigated were patients affected by CAD; therefore all conclusions have to be restricted to this specific condition. Similarly, it is necessary to take into consideration that all patients were treated with statins. Such treatment may have influenced some results; for example, the cholesterol-lowering effect of these drugs may have weakened the correlations with plasma lipids. Nevertheless, accurate information on the Apo C-III metabolism is probably valuable in the context of the secondary prevention of CAD patients presenting a "residual risk" in spite of the best available therapy.

### 5.4. LPL activity study and Apolipoprotein C-III glycoforms correlation analysis

According to inhibitory effect of the Apo C-III on the lipoprotein lipase (Brown and Baginsky 1972, Wang, McConathy et al. 1985, Yamamoto, Morita et al. 2003), worth of interest cold be observing how the three different Apo C-III glycoforms could affect this function. In 51 CAD patients, we measured LPL activity in order to observe possible correlations and/or modulation of the inhibitory action of the three different glycoforms.

Our result on LPL activity suggest that the total Apo C-III concentration of the apolipoprotein is more important in modulating (with an inhibitory influence) LPL activity than the relative proportions of glycoforms. LPL activity decreased progressively by increasing total Apo C-III plasma levels and similar trends were observed for each of the three glycoforms (**Figure14-15**), thereby suggesting that if Apo C-III glycoforms could have different functional role such difference would be not related to the influence on LPL.

### 5.5. Proteomics analysis by SWATH

Different proteomic pathways are involved in CAD condition, this is the reason why a SWATH analysis (untargeted proteomics approach) for the characterization of proteomic profile of CAD patients could be useful to get new insights into the molecules playing major roles in this pathology. The monovariate analysis underlined the presence of 21 up and down regulated proteins associated with high levels of Apo C-III. In particular among these protein there were 7 up regulated and 4 down regulated with a FC >1.5 (**Table 13**). By the Cytoscape analysis (**Figure 17**) of these 21 proteins we could reveal the presence of four functional clusters linked to the complement activation, the low-density lipoprotein metabolism, the acute phase response and the intermediated filament based process.

Among these four highlighted groups, the low-density lipoprotein cluster is the most prominent and the proteins included in it were all upregulated. A predominance of small, low-density lipoprotein (LDL) in plasma has been already accepted as an emerging cardiovascular risk factor (Rizzo and

Berneis 2006). Moreover several evidence supports a functional role for complement activation in the pathogenesis of cardiovascular disease through pleiotropic effects on endothelial and hematopoietic cell function and hemostasis. Prospective and case control studies have reported strong relationships between numerous complement components and cardiovascular outcomes. Moreover, in vitro studies and animal models support a functional effect of complement activation on cardiovascular diseases (Carter 2012). In this cluster both up and down regulated proteins were present. On the other hand proteins included in the acute phase response cluster were down-regulated. The acute phase reaction is a systemic response, which usually follows a physiological condition that takes place in the beginning of an inflammatory process. Cardiovascular diseases are characterized by the elevation of several positive acute phase reactants but are also associated with the reduction of negative acute phase reactants, as evidenced by Ahmed at al. (Ahmed, Jadhav et al. 2012).

The network related to lipoproteins, already highlighted by Cytoscape, shows that *APOC3* and *APOC2* have a co-expression connection while *APOC3* - *APOE* and *APOC2* - *APOE* are characterized by an interaction linked to literature works. The strict coexistence on TRL of Apo C-III and Apo C-II is well known, so that the finding is largely expected. Apolipoprotein E (Apo E) was initially described as a lipid transport protein and major ligand for LDL receptors with a role in cholesterol metabolism and cardiovascular disease. Apo E is often reported to modify the effects of environmental risk factors such as diet, smoking, or physical activity on cardiovascular outcomes (Mahley 2016).

Another protein linked to the regulation of lipoprotein metabolism is Retinol binding protein 4 (RBP4). RBP4 concentrations were weakly correlated with both total cholesterol and triglycerides. Several studies have confirmed the associations of circulating RBP4 with obesity, insulin resistance, type 2 diabetes and cardiovascular risk factors (Ingelsson, Sundstrom et al. 2009).

Proteins related to the complement activation did not show any connections in STRING, but, among these, there is Vitronectin (VTN), an adhesive glycoprotein, that is involved in various

functions including complement activation, blood coagulation, binding to proteoglycans, and modification of the matrix. The levels of vitronectin in plasma increased in patients with coronary artery diseases, showing a positive correlation with the severity of the disease. Indeed, patients with high levels of Apo C-III have an up-regulation of this protein (Derer, Barnathan et al. 2009).

Our data reflect these results. It is also known that vitronectin accumulates in atherosclerotic plaques by both diffusion from plasma and in situ synthesis (Dufourcq, Louis et al. 1998) and that extent of coronary artery disease correlates with its plasma levels (Ekmekci, Ekmekci et al. 2005).

Other proteins as Complement C2, Complement C1q (C1QC) and immunoglobulin IGLL5 were found upregulated. During recent years complement mediated inflammation has been shown to be an important player in a variety of heart diseases. Evidence points to an association between the complement system and heart diseases. Thus, complement seems to be important in coronary heart disease as well as in heart failure, where several studies underscore the prognostic importance of complement activation (Lappegard, Garred et al. 2014).

Serum amyloid A-1 (SAA1) protein and alpha-1-antitrypsin are acute phase proteins. In particular several studies show as high expression of SAA1 may contribute to atherosclerosis (Fyfe, Rothenberg et al. 1997, Schillinger, Exner et al. 2005) and an elevated SAA1 concentrations associated with an increased risk of cardiovascular disease events (Johnson, Kip et al. 2004). Regarding Alpha- 1-antitrypsin, one of the major serine proteinase inhibitor in human plasma, it inhibits overexpressed proteinases during inflammation (Kalsheker 1994). It is important to underline as some pathological conditions, where oxidative stress could play a major role, are characterized by an impairment of the tight regulation between proteases and their inhibitors (Banfi, Brioschi et al. 2008). A failure of alpha 1-antitrypsin levels may be associated with a worse clinical course (Gilutz, Siegel et al. 1983).

By the multivariate analysis, we obtained 42 significant variables which characterized Apo C-III CAD state (**Table 14**). This analysis confirmed the monovariate results as it is shown in **Figure 20.**

In particular the complement system, which plays a central role in innate immunity and also regulates adaptive immunity, is significantly prominent both for patients with low and for patients with high Apo C-III.

Low density lipoprotein are clearly significant for patients with high levels of Apo C-III as already confirmed by the fold change analysis: in fact this class of protein is up-regulated for patients with high level of Apo C-III.

The multivariate analysis identified as significant one more lipoprotein, the apolipoprotein H (Apo H). Apo H is a single chain glycoprotein involved in clotting mechanisms and lipid pathways. Plasma concentrations of Apo H are strongly associated with the metabolic syndrome and cardiovascular disease in type 2 diabetic patients and could be considered as a clinical marker of cardiovascular risk. The increased Apo H concentration is commonly associated to its increased liver synthesis (Crook 2010). Lipopolysaccharide-binding protein (LPB) has been shown to bind to lipopolysaccharides, lipoproteins, and lipopeptides and is a soluble acute-phase protein. LPB is the first protein to encounter lipopolysaccharide and to deliver it to its cellular targets. Its presence might be a reliable biomarker that indicates activation of innate immune responses. Elevated levels of circulating LBP represent a strong and independent predictor of the presence of CAD in men (Lepper, Schumann et al. 2007).

For what concern the acute phase response proteins, which are significant for patients with low Apo C-III, the multivariate analysis was able to identify the alpha-1 antichymotrypsin in addition to SAA1 and Alpha-1-antitrypsin,. The serine protease inhibitor alpha-1 antichymotrypsin (ACT) has been implicated in the pathology of a number of devastating human diseases including chronic obstructive pulmonary disease (COPD), Parkinson's disease (PD), Alzheimer's disease (AD), Stroke, Cystic Fibrosis, Cerebral Hemorrhage and Multiple System Atrophy. ACT is an acute phase protein and its gene expression is stimulated by the presence of cytokines. ACT acts as an inhibitor of several serine proteases is a typical acute phase protein, with the amount of circulating protein dramatically increasing in response to inflammation (Baker, Belbin et al. 2007).

It is very interesting to note that the multivariate analysis uncovered two new significant proteins related to the oxygen transport: Hemoglobin subunit alpha (HBA) and Hemoglobin subunit beta (HBB). Although HBA and HBB resulted not modulated from the monovariate analysis, patients with high level of Oxygen proteins are often linked to anemia, which is a risk factor for adverse cardiovascular disease outcomes. Anemia has been shown to be an independent cardiovascular risk factor and a negative predictor of survival in patients with congestive heart failure. Furthermore, a lower hemoglobin level is also a risk factor for worse outcome in patients with coronary artery disease after myocardial infarction and percutaneous coronary intervention. Recently, there has been considerable interest in the relation between hemoglobin levels and cardiac outcomes.

The analysis of the plasma profiles of these stable CAD patients revealed the strong implication of lipoproteins (Apo C-II and Apo E), retinol-binding protein 4 and vitronectin. Surprisingly the alpha 1-antitrypsin was down-regulated in patients with high Apo C-III: this modulation could explain the worsening of the clinical course of this group of patients. Although our findings need to be verified in larger groups, this proof of concept allowed to study the physiologically expression of plasma proteins through the entire range of concentrations of Apo C-III and to link them to secondary cardiovascular prevention in CAD patients.

### 5.6. Lipidomics approach

The lipidomic study of the CAD and CAD free patients stratified according to TG, Apo C-III and Apo E levels, could give new insights into the knowledge of the burden of parameters associated with CAD and how the lipid profile could be associated with the CAD pathological condition.

The population was subdivided in six different groups with different distribution of the three selected parameters (**Table 17**) and was subjected to both Gas-chromatography(GC) and Liquid Chromatography-MS (LC-MS).

The first analysis, by GC didn't allows a subdivision and classification of the patients, being all the parameters similar. On the contrary the LC-MS analysis allowed a classification of the patients, on

the basis of all the lipids quantified, according to the Apo E levels. In particular the PCA analysis underlined a distribution of all the patients in two groups: high Apo E versus low Apo E according to PC1 (**Figure 22**) and in three groups according to PC2 (**Figure 24**). The following PLS-DA analysis (**Table 18**) confirmed the significance of the distribution of all the patient in two and three groups (Q2>0.5). Than the OPLS-DA analysis identified the metabolites that characterized the distribution of the patients in the two and three groups (**Figure 23-25**).

Of note the group characterized by high levels of Apo E presented among all the metabolites Cholesteryl ester oxidized (CE-Ox) species which were absent in patients characterized by low levels of Apo E (**Table 19-20**). This finding is of particular relevance and deserve further investigation. Earlier studies estimated that 2% of the total CE is oxidized in human plaque (Suarna, Dean et al. 1995) and also by recent study employing mass spectrometry on atherosclerosis lesion it could be possible to observe the presence of CE-Ox in atherosclerosis lesion (Hutchins, Moore et al. 2011). Even if preliminary, these results on the lipidomic profiles of CAD patients seem to point out an association of Apo E with an unfavorable lipid distribution. Further analysis are ongoing in order to disclose other correlations and potential effect of the different lipid species observed in the different groups.

## 6. CONCLUSIONS

In conclusion our study suggest that:

- ✓ The shotgun topdown MS approach is a more reliable method than IEF to quantify the three different Apo C-III glycoforms
- ✓ The more abundant Apo C-III glycoforms is the monosialylated and it is associated with the highest TG levels, showing a positive correlations with all the lipoproteins and lipid profile associated with CAD.
- ✓ The validation analysis confirmed that high levels of Apo C-III are associated with a pro-atherogenic and pro-inflammatory proteomic profile
- ✓ LPL activity analysis highlighted how the total Apo C-III plasma concentration is more important in modulating (with an inhibitory influence) LPL activity than the relative proportion of glycoforms.
- ✓ The SWATH analysis revealed a set of proteins associated with a "high and low Apo C-III state"
- ✓ Lipidomic approach illustrated a different point of view on the possible markers associated to the plasma levels of Apo C-III and Apo-E in CAD.

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