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Full Length Article

Distribution of seven ApoC-III glycoforms in plasma, VLDL, IDL, LDL and HDL of healthy subjects

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ABSTRACT

Glycosylation of ApoC-III modulates its function in TG metabolism, with some variants being associated with a more atherogenic lipid profile. These associations have been studied in whole plasma but rarely in individual lipoprotein fractions. In this study, we aimed to measure the relative content of ApoC-III glycoforms in each lipoprotein fraction as a potential biomarker for TG metabolism and cardiovascular risk. Lipoprotein fractions were separated by differential ultracentrifugation of plasma samples from healthy subjects. Relative concentrations of seven ApoC-III variants were measured by MSIA. ApoC-III$_1$, ApoC-III$_{0b}$ and ApoC-III$_2$ were the most abundant glycoforms. There was high interindividual variability in the distribution of glycoforms across the study population but a uniform proportion in all lipoprotein fractions of each given subject. Two ApoC-III variants, ApoC-III$_{0b}$ and ApoC-III$_{1d}$, negatively correlated with plasma and VLDL triglycerides irrespectively of VLDL size and were associated with increased LDL size when transported in LDL particles. ApoC-III$_{0b}$ also showed a negative correlation with lipoprotein-insulin resistance score. We have been able to measure seven ApoC-III glycoforms in each lipoprotein fraction, setting the basis for future studies exploring their role on cardiovascular risk. Some glycoforms suggest a less proatherogenic role on TG and lipoprotein metabolism.

Significance: Apo CIII has an important role on plasma TG metabolism through different mechanisms and it is also involved in type 1 and type 2 Diabetes Mellitus. Different glycosylated forms of Apo CIII exist and they show different roles. For this reason, this protein has gained interest in the las years and the relationship between ApoC-III glycoforms and lipids, lipoproteins and metabolic disorders has been increasingly studied in the last years.

Apo CIII glycoforms have been previously analysed in plasma, and the function of the main four glycoforms has been assessed in a variety of cohorts; but in the present study, ApoC-III glycoforms are measured in each lipoprotein fraction, which may be of clinical interest.

1. Introduction

1.1. ApoC-III at the crossroads of TG metabolism, atherosclerosis, thrombosis, hepatic steatosis and pancreatic beta cell dysfunction

Apolipoprotein (Apo) C-III is an 8.8 kD glycoprotein that is primarily produced in the liver but is also expressed in the intestine. It is mostly known as a key triglyceride (TG) regulator, and this was clearly anticipated decades ago by the strong correlation observed between circulating ApoC-III and TG [1]. In animal models, overexpressing ApoC-III resulted in hypertriglyceridaemia [2], and targeted disruption of the gene decreased TG [3]. This role was confirmed in humans because mutations affecting the expression of the gene induced life-long reduced TG levels, which resulted in effective protection against cardiovascular diseases (CVDs) [4,5]. Therefore, several strategies to reduce residual CVD aim to lower ApoC-III levels [6].

The proposed mechanisms by which ApoC-III contributes to dyslipidaemia include inhibition of TG lipolysis by interacting with

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lipoprotein lipase [7,8] and stimulation of TG-rich lipoprotein secretion in the liver and intestine [9]. Notably, in vivo studies in humans have shown some controversies, suggesting that there may be other pathways linking ApoC-III with CVD [10].

In this regard, ApoC-III is also involved in other key steps promoting atherogenesis beyond lipid metabolism. It activates biological pathways increasing the expression of markers of endothelial dysfunction and monocyte adhesion [11], increases susceptibility to LDL hydrolysis and aggregation in the subendothelial space [12], and affects smooth muscle cell proliferation [13].

Interestingly, ApoC-III has been implicated in other physiological processes linked to CVD. Some evidence suggests that ApoC-III has a potential procoagulant capability associated with both TG-rich lipoproteins and HDL particles. This was supported by genetic studies showing an association with haemostatic processes [14].

ApoC-III is also proposed to be involved in hepatic steatosis, yet these results are controversial [15].

Finally, new roles of ApoC-III in type 1 and type 2 diabetes were reported. Observational studies describe a positive correlation between ApoC-III and the incidence of type 1 and type 2 diabetes, and this is suggested to apparently be independent of plasma TG levels. ApoC-III promotes an inflammatory state in the pancreas that drives apoptosis and insulin resistance in beta cells [16,17].

1.2. ApoC-III function is lipoprotein- and glycoform-dependent

ApoC-III exists in different glycoforms that originate from post-translational modifications. Up to 16 glycoforms have been described [18–20]. The most relevant forms are ApoC-III_{0a} (native form) and three variants glycosylated at Thr74: ApoC-III_{0b}, with one galactose and one N-galactosamine (GalNAc); ApoC-III_1 and ApoC-III_2, with one and two sialic acid residues added to the ApoC-III_{0b} form, respectively [21]. The influence of ApoC-III on TG metabolism varies significantly depending on the relative presence of each glycoform. Previous studies have shown that ApoC-III_2 is associated with a less pro-atherogenic profile than ApoC-III_0 and ApoC-III_1 in relation to TG levels and lipoprotein parameters in several populations [20–23]. However, all these observations were made from total plasma ApoC-III glycoforms and did not take into account the relative distribution in each lipoprotein particle.

The presence or absence of ApoC-III in specific lipoproteins has significant clinical implications. LDL containing ApoC-III contributes to atherosclerotic CVD [24], and even HDL containing ApoC-III increases the risk of CVD [25], which otherwise would be atheroprotective. Moreover, the protective role against type 2 diabetes mellitus attributed to HDL is only observed in the fraction not containing ApoC-III [26].

Altogether, these results suggest that being able to analyse ApoC-III glycoforms in individual lipoproteins seems to be an indispensable step in unravelling the role of this protein in linking atherosclerosis, thrombosis, hepatic steatosis and pancreatic beta cell dysfunction.

We hypothesize that ApoC-III glycoforms can be reliably measured in VLDL, IDL LDL and HDL of normolipidaemic subjects with normal ApoC-III concentrations. This would establish a methodological basis to deepen our knowledge of ApoC-III as a multifunctional protein.

2. Material and methods

2.1. Volunteers

Twenty-four apparently healthy young men older than 18 years of age were recruited. All of them practised physical activity and exhibited a normal lipid profile. Two of them were excluded because they were taking lipid-lowering medication. The Ethics Committee of the Hospital Universitari Sant Joan de Reus approved the study, and all patients gave their written consent to participate. Data were coded for anonymity in accordance with current Spanish law on biomedical research.

2.2. Biochemical analyses

Fasting venous blood samples were collected in EDTA tubes and centrifuged immediately for 15 min at 4 °C at 1500 x g to obtain plasma. Samples were divided into aliquots and stored at −80 °C until analyses.

Standard laboratory methods were used to quantify total cholesterol, TG and HDLc. LDLc values were calculated using the Friedewald formula. Apolipoproteins were quantified using an immunoturbidimetric assay with specific antibodies against ApoA1 (Horiba, Japan), ApoB100 (Horiba, Japan) and ApoC-III (Spinreact, Spain). These analyses were adapted to the Cobas-Mira-Plus autoanalyzer (Roche Diagnosis, Spain).

2.3. Lipoprotein separation

Lipoprotein fractions were separated via sequential ultracentrifugation after progressively increasing the solvent density using an Optima XPN-100 ultracentrifuge (Beckman Coulter, California, USA) and a Kontron 45.6 fixed-angle rotor at 37,000 rpm and 4 °C for different time intervals: 16 h for chylomicrons and VLDL, 20 h for IDL, 20 h for LDL and 40 h for HDL. To separate these fractions, density solutions were used to set the density of the sample at 1.006, 1.019, 1.063 and 1.210 g/ml.

2.4. ApoC-III glycoforms using mass spectrometric immunoassay (MSIA)

Analysis of ApoC-III was performed using immunoprecipitation of ApoC-III complexes coupled to a mass spectrometric analysis of the intact ApoC-III protein, as previously described [24], in the Centre for Omic Sciences (Reus, Spain). Briefly lipoprotein samples were dialyzed with PBS using a Slice-A-Lyzer MINI Dialysis Unit, 2KDa MWCO (ThermoScientific, Spain). Samples were incubated with 5 μg of Biotinylated Goat Anti-Human Apolipoprotein CIII antibody (Academy Bio-Medical Co., Germany) and immobilized on AssayMAP Streptavidin cartridges (Agilent Technologies, Spain) using the automated AssayMap Bravo platform (Agilent Technologies, Spain).

Protein samples were eluted on a 96-well formatted MALDI target, dried under N2 flow and resuspended using a sinapinic acid matrix in a sinapinic acid matrix (15 mg/ml of sinapinic acid with 33% acetonitrile and 0.4% trifluoroacetic acid). Linear mass spectra were acquired from each sample spot (1 ul) using an UltrafleXtrem III MALDI-TOF/TOF instrument (Bruker, Germany) operating in positive ion mode and a range of 4 to 20 kDa. An average of 3000 laser shot mass spectra were saved for each sample spot. Mass spectra were internally calibrated using protein calibration standard-I (Bruker, Germany) and further processed using Flex Analysis 3.0 software (Bruker Daltonics). All peaks representing ApoC-III and their glycoforms were integrated. To assess the consistency of the ionization efficiency and reproducibility between and within runs, a quality control plasma sample was run in triplicate with each analysis.

2.5. Liposcale® test: nuclear magnetic resonance (NMR) lipoprotein profile

Total plasma lipids and distributions of lipoprotein subclasses were analysed via NMR spectroscopy by Biosfer Teslab (Reus, Spain) The Liposcale® test is a novel advanced lipoprotein test based on 2D diffusion-ordered ^{1}H NMR spectroscopy and using diffusion coefficients [27]. This technique provides the particle size and number of the main types of lipoprotein (VLDL, LDL, and HDL), divided each one in large, medium and small size according to increases in molecular weight. Moreover, ^{1}H NMR approaches based on regression methods also allow for the determination of the cholesterol and triglycerides concentrations of lipoprotein [28]. The ^{1}H NMR was carried out on EDTA plasma stored and thawed just prior to the analysis.

2.6. Hierarchical clustering

Hierarchical clustering was performed using metaboanalyst.ca online software. Euclidean distance measure and Ward's method were used to create a heatmap hierarchical clustering. One case (subject 18) was excluded from this analysis because data from ApoC-III glycoforms in the IDL fraction were missing.

2.7. LP-IR score

The Lipoprotein Insulin Resistance (LP-IR) score was calculated as described by Shalaurova et al. [29]. Briefly, six parameters of lipoproteins (VLDL size, large VLDL particles, LDL size, small LDL particles, HDL size and large HDL particles), measured by NMR, were divided into several categories and a value was given to each category. The six values were added to obtain a score between 0 and 100 indicating insulin resistance.

2.8. Statistical analyses

Normality distribution of variables was assessed with the Shapiro-Wilk test. Partial correlations among ApoC-III proteoforms and lipid and lipoprotein profile were adjusted for age, BMI, and total ApoC-III or TG levels.

Statistical significance was set at $p < 0.05$, and all statistical analyses were evaluated using SPSS Statistical Software version 23 (SPSS Inc., Chicago, USA).

3. Results

3.1. Study population

We studied 22 young healthy males. Anthropometric and conventional lipid profile data showed that all parameters were within the normal range (Table 1).

3.2. Detection of ApoC-III glycoforms in plasma, VLDL, IDL, LDL and HDL

Seven ApoC-III glycoforms were detected and quantified: the native ApoC-III$_{0a}$ form and six variants glycosylated at Thr74. These include ApoC-III$_{0b}$, with one GalNac glycan core; ApoC-III$_1$, with one sialic acid molecule added to the 0b form ((Gal)1(GalNAc)1(NeuAc)1); ApoC-III$_2$, with two sialic acid molecules added to the 0b form, ((Gal)1(GalNAc)1(NeuAc)1); ApoC-III$_{0f}$, with two molecules of galactose, two *N*-acetylgalactosamine and three molecules of fucose ((Gal)2(GalNAc)2(Fuc)3); and the truncated forms ApoC-III$_{1d}$ and ApoC-III$_{2d}$, which are variants of ApoC-III$_1$ and ApoC-III$_2$ without the terminal Ala residue, respectively (Supplemental Fig. S1).

All seven glycoforms could be detected and quantified in all 22 subjects except for the ApoC-III$_{0f}$ glycoform, which was not detected in some lipoprotein fractions (mostly VLDL, IDL and HDL) in 4 individuals. ApoC-III$_1$ was the most abundant glycoform in plasma (52.6% of all glycoforms in plasma), followed by ApoC-III$_2$ (13.4%) and ApoC-III$_{0b}$ (13.1%); then ApoC-III$_{2d}$ (6.7%), ApoC-III$_{1d}$ (5.9%) and ApoC-III$_{0a}$ (5.7%); and finally, ApoC-III$_{0f}$ (2.6%), which had the lowest relative abundance. Similar values were obtained in all lipoprotein fractions (Fig. 1).

Table 1
Characteristics of the study population.

Age, years	25.5 ± 5.1
Body Mass Index, kg/m^2	23.3 ± 2.4
Cholesterol, mg/dl	170.5 ± 22.2
LDLc, mg/dl	92.1 ± 23.2
HDLc, mg/dl	58.5 (52.8–70.8)
Triglycerides, mg/dl	79.6 ± 19.2
ApoA1, mg/dl	142.0 ± 17.8
ApoB100, mg/dl	79.0 ± 17.2
ApoC-III, mg/dl	7.6 ± 2.8

Values of normally distributed variables are presented as the means ± SD, and variables that are not normally distributed are presented as medians (IQR).

3.3. Distribution of ApoC-III glycoforms

Fig. 1 shows the mean values of the relative distribution of each glycoform in plasma, VLDL, IDL, LDL and HDL. Averaging the glycoform content of all individuals in each lipoprotein shows that the same relative distribution of ApoC-III that can be observed in plasma, is maintained in the four isolated lipoproteins.

Supplemental Fig. S2 shows the relative distribution in plasma and lipoproteins for each individual glycoform in each of the 22 study participants. There was high interindividual variability in the relative distribution of ApoC-III glycoforms, but again, for each subject, the proportion of each glycoform was maintained in all lipoprotein fractions.

We studied whether the variability between individuals was dependent on total ApoC-III concentrations. Stratification of the population by total ApoC-III plasma concentration showed no significant differences in the proportion of ApoC-III glycoforms (in plasma and in lipoprotein fractions) between total ApoC-III tertiles (data not shown).

Since there was such interindividual variability in the distribution of ApoC-III glycoforms, we looked for different populations defined by the relative abundances of glycoforms in plasma and lipoprotein fractions. Glycoforms were grouped according to sialic acid content: no sialic acid (ApoC-III$_{0a}$, ApoC-III$_{0b}$ and ApoC-III$_{0f}$), 1 sialic acid residue (ApoC-III$_1$ and ApoC-III$_{1d}$), and 2 sialic acid residues (ApoC-III$_2$ and ApoC-III$_{2d}$). These groups were used to perform hierarchical clustering of the study population, which revealed 3 sub-groups among our participants (Fig. 2).

Sub-group 1 was composed of subjects showing a high content of non-sialylated glycoforms, sub-group 2 showed a higher proportion of di-sialylated glycoforms, and sub-group 3 showed a high proportion of mono-sialylated glycoforms; the means of each glycoform are shown in Supplemental Table S1.

We explored associations between ApoC-III glycoforms and lipid and

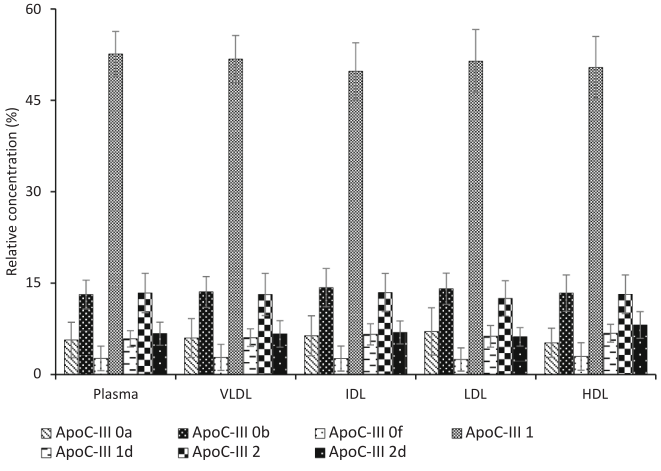

Fig. 1. Distribution of the different glycoforms of ApoC-III in plasma and lipoproteins in healthy men. Means of the relative concentrations of the ApoC-III glycoforms in each lipoprotein fraction and the SD are shown. The relative concentration of each glycoform was constant in all lipoprotein fractions and plasma. ApoC-III$_1$ is the most abundant glycoform, followed by ApoC-III$_{0b}$ and ApoC-III$_2$; then ApoC-III$_{0a}$, ApoC-III$_{1d}$ and ApoC-III$_{2d}$; and finally ApoC-III$_{0f}$, the least abundant of all the glycoforms analysed.

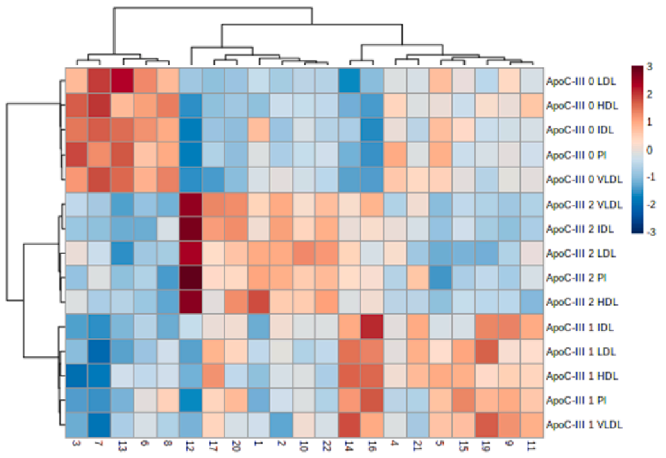

Fig. 2. Hierarchical clustering of ApoC-III glycoforms. Study subjects can be divided into three subgroups according to their distribution of ApoC-III glycoforms. Sub-group 1 consisted of subjects with high non-sialylated ApoC-III glycoforms, sub-group 2 consisted of subjects with high di-sialylated ApoC-III glycoforms, and sub-group 3 contained subjects with high mono-sialylated ApoC-III glycoforms. One subject (number 18) was excluded from this analysis because of missing data in the IDL fraction.

lipoprotein parameters and found that total ApoC-III$_{0b}$ and ApoC-III$_{1d}$ measured in plasma, negatively correlated with both plasma and VLDL-TG levels (Supplemental Table S2). When determining the glycoforms in each lipoprotein only the ApoC-III$_{0b}$ glycoform determined in the VLDL fraction showed a significant negative correlation with VLDL-TG levels (Supplemental Table S2); together with the amount of all the VLDL subfractions (large, medium, small) (Supplemental Table S3). When determined in the LDL fraction, glycoforms ApoC-III$_{0b}$ and ApoC-III$_{1d}$ showed a positive correlation with the size of LDL particles (Supplemental Table S4). Finally, plasma ApoC-III$_{0b}$ negatively correlated with a lipoprotein-based insulin resistance score (LP-IR) (Supplemental Fig. S3) [29].

4. Discussion

There is convincing evidence that life-long reduced TG levels due to APOC3 mutations result in effective protection against cardiovascular disease [4,5], and ApoC-III is also linked to the onset of type 1 and type 2 diabetes mellitus. Therefore, ApoC-III is a very promising biomarker to study the cardiovascular risk associated with diabetes. Understanding the mechanism of action of ApoC-III is complex because scientific evidence indicates that the effects of ApoC-III depend on the lipoprotein to which it is bound [24,25] and the relative abundance of the ApoC-III glycoforms [20,21,23]. To date, previous studies have measured only the non-glycated, mono-sialo and di-sialo forms in VLDL, LDL and HDL [23], and most of them only considered total ApoC-III glycoforms and not their distribution in individual lipoproteins.

Because of its potential as a biomarker, we attempted to measure the most frequent ApoC-III glycoforms in plasma and each of the individual lipoproteins (VLDL, IDL, LDL and HDL) and characterized their inter-individual variability. To assess the robustness of the method, we analysed healthy young subjects with normal-to-low ApoC-III concentrations, thus limiting the metabolic variability that could be expected from diseased groups.

Our results demonstrate that up to seven ApoC-III glycoforms can be reliably measured in plasma and each separate lipoprotein (VLDL, IDL, LDL and HDL) in young healthy subjects. Based on our data, we made two main observations. There was significant interindividual variability in the relative distribution of the seven glycoforms, but in each given subject, the distribution was maintained throughout the different lipoproteins. Consistent with several previous observations [21,22], the correlations between TG and lipoproteins differed depending on the ApoC-III glycoform (data not shown).

The fact that the ApoC-III glycoform distribution was maintained across the different lipoproteins naturally raises the question of the need to measure ApoC-III glycoform distribution in each lipoprotein when plasma may be an equally valid indicator. This observation could be explained in part because ApoC-III is a highly exchangeable protein mainly among VLDL and HDL lipoprotein particles [30]. Although this is one possible scenario, another possibility is that this distribution is a characteristic of normal metabolism, which may be disrupted in the presence of metabolic alterations, with such disruption itself being a useful biomarker. Obviously, this hypothesis deserves further study in diabetic, obese or metabolic syndrome patients. As mentioned above, to set up the methodology for ApoC-III quantification in individual lipoproteins, we chose a population of healthy young males because we assumed that the ability to detect the seven glycoforms in low-concentration lipoproteins and with low ApoC-III concentrations would be an indicator of the robustness of the method. This ensures reliable results when applied to subjects with metabolic alterations and most likely higher ApoC-III concentrations.

We found that ApoC-III$_1$ was the glycoform with the highest proportion in all individuals, followed by ApoC-III$_{0b}$ and ApoC-III$_2$; and no differences in the distribution of glycoforms were observed between tertiles of total ApoC-III concentration; thus, the proportion of each glycoform was not dependent on total ApoC-III. However, in a study by Olivieri et al. [31] with CAD patients, a higher proportion of ApoC-III$_2$ was observed, and changes in the proportion of certain glycoforms were observed across total ApoC-III tertiles. This may suggest that the increase in total plasma ApoC-III that is associated with CVD conditions may be associated with changes in the proportions of certain glycoforms.

The main objective of this work was to study only the distribution and to assess the detection and quantification method for ApoC-III glycoforms but, despite the reduced sample size, the observed correlations between lipid and lipoprotein parameters and two glycoforms are promising. The negative correlation between lipid and lipoprotein parameters and the glycoforms ApoC-III$_{0b}$ and ApoC-III$_{1d}$ suggest a different role of these glycoforms in lipid metabolism. Several previous works have observed associations between different glycoforms and TG levels or lipoprotein particle size in a variety of cohorts (subjects with T2DM, coronary artery disease or obesity) [20,22,31]. They mostly focus on the four main ApoC-III glycoforms and found a predominant role of ApoCIII$_2$ and ApoC-III$_1$, while in the present study, in a small group of healthy and young subjects, we could not find any correlation regarding these glycoforms. To study the distribution of the seven ApoC-III glycoforms in subjects with hypertriglyceridemia, T2DM or other metabolic conditions, and its associations with cardiometabolic parameters will be of interest in future approaches.

The molecular mechanisms behind the associations described between ApoC-III glycoforms and lipoprotein parameters are yet unknown, and it is unclear whether glycosylation of ApoC-III is responsible of these lipid and lipoprotein profiles or it is just a consequence of the metabolic state of the subject with no direct modification of the role of ApoC-III. Notably, studies in mice show that glycosylation of ApoC-III directly affects TRL clearance, thus suggesting a causal role in mice [32]. One possible mechanism for the observed associations could be that glycosylation of ApoC-III would influence its affinity for TRL. Khetarpal et al., described a missense variant in ApoC-III with reduced affinity for TRL, with carriers of this mutation having lower TG and higher HDL-cholesterol [33]. However, this is beyond the scope of the present article and our results cannot confirm whether there is a different affinity between some glycoforms and lipoprotein particles.

The strengths of this study are, mainly, those related to the technology used for ApoC-III glycoforms determination. Mass Spectrometry Immuno-Assay allows for a reliable and robust detection of a large number of ApoC-III glycoforms in samples with low total ApoC-III concentrations, such as lipoprotein fractions of normolipidemic

subjects.

This study has some limitations; first of all, the number of study subjects is limited. Despite this has been enough for a qualitative observation of the distribution of ApoC-III glycoforms; a larger sample size would have given more reliable and complete information about the relationship between certain glycoforms and lipid and lipoprotein parameters as well as the clustering of individuals.

5. Conclusions

In conclusion, the present study shows that Apo CIII proteoforms can be measured in all lipoprotein fractions of healthy subjects and present different interindividual distributions, setting the basis for future studies exploring their role on cardiovascular risk. Some of them are associated with a less pro-atherogenic lipid and lipoprotein profile. Thus, more in-depth study of these associations and their mechanisms in other groups associated with high cardiovascular risk such as hypertriglyceridemic or T2DM patients will be of great value.

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Author's contribution

MG and JR designed the project, interpreted the data, and wrote the manuscript. MR and PR analysed the data and wrote the manuscript. MR, VI, JM, CB contributed to data collection, sample extraction and data analyses. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2021.104398.

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