

The Positive Association between Plasma Myristic Acid and ApoCIII Concentrations in Cardiovascular Disease Patients Is Supported by the Effects of Myristic Acid in HepG2 Cells

Oliviero Olivieri,¹ Giulia Speziali,¹ Annalisa Castagna,¹ Patrizia Pattini,¹ Silvia Udali,¹ Francesca Pizzolo,¹ Laura Liesinger,^{2,3,4} Juergen Gindlhuber,^{2,3,4} Tamara Tomin,^{2,3,4} Matthias Schittmayer,^{2,3,4} Ruth Birner-Gruenberger,^{2,3,4} Daniela Cecconi,⁵ Domenico Girelli,¹ Simonetta Friso,¹ and Nicola Martinelli¹

¹Department of Medicine, University of Verona, Verona, Italy; ²Institute of Chemical Technologies and Analytics, Vienna University of Technology, Vienna, Austria; ³Diagnostic and Research Institute of Pathology, Diagnostic and Research Center of Molecular Medicine, Medical University of Graz, Graz, Austria; ⁴Omics Center Graz, BioTechMed-Graz, Graz, Austria; and ⁵Department of Biotechnology, University of Verona, Verona, Italy

ABSTRACT

Background: In the settings of primary and secondary prevention for coronary artery disease (CAD), a crucial role is played by some key molecules involved in triglyceride (TG) metabolism, such as ApoCIII. Fatty acid (FA) intake is well recognized as a main determinant of plasma lipids, including plasma TG concentration.

Objectives: The aim was to investigate the possible relations between the intakes of different FAs, estimated by their plasma concentrations, and circulating amounts of ApoCIII.

Methods: Plasma samples were obtained from 1370 subjects with or without angiographically demonstrated CAD (mean \pm SD age: 60.6 \pm 11.0 y; males: 75.8%; BMI: 25.9 \pm 4.6 kg/m²; CAD: 73.3%). Plasma lipid, ApoCIII, and FA concentrations were measured. Data were analyzed by regression models adjusted for FAs and other potential confounders, such as sex, age, BMI, diabetes, smoking, and lipid-lowering therapies. The in vitro effects of FAs were tested by incubating HepG2 hepatoma cells with increasing concentrations of selected FAs, and the mRNA and protein contents in the cells were quantified by real-time RT-PCR and LC-MS/MS analyses.

Results: Among all the analyzed FAs, myristic acid (14:0) showed the most robust correlations with both TGs ($R = 0.441$, $P = 2.6 \times 10^{-66}$) and ApoCIII ($R = 0.327$, $P = 1.1 \times 10^{-31}$). By multiple regression analysis, myristic acid was the best predictor of both plasma TG and ApoCIII variability. Plasma TG and ApoCIII concentrations increased progressively at increasing concentrations of myristic acid, independently of CAD diagnosis and gender. Consistent with these data, in the in vitro experiments, an ~ 2 -fold increase in the expression levels of the ApoCIII mRNA and protein was observed after incubation with 250 μ M myristic acid. A weaker effect ($\sim 30\%$ increase) was observed for palmitic acid, whereas incubation with oleic acid did not affect ApoCIII protein or gene expression.

Conclusions: Plasma myristic acid is associated with increased ApoCIII concentrations in cardiovascular patients. In vitro experiments indicated that myristic acid stimulates ApoCIII expression in HepG2 cells. *J Nutr* 2020;00:1–9.

Keywords: myristic acid, Apolipoprotein CIII, triglyceride-rich lipoproteins, lipid metabolism, coronary artery disease

Introduction

Despite therapeutic advances in treating cardiovascular disease, largely due to approaches that reduce plasma LDL cholesterol, during recent years, the decline in cardiovascular deaths has reached a plateau (1), so that a substantial hazard of cardiovascular mortality/morbidity (the so-called residual risk) still remains.

Such residual risk has been associated with current epidemic increases in obesity, metabolic syndrome, and type 2 diabetes, in turn leading to the so-called atherogenic dyslipidemia that

is mainly characterized by elevated plasma triglyceride (TG) and triglyceride-rich lipoprotein (TRL) concentrations and low concentrations of HDL cholesterol (2, 3). A renewed interest in TRLs has also been derived from a better knowledge of the peculiar role of some key molecules in TG-related atherogenesis, for which a body of evidence from clinical, epidemiological, and genetic studies has accumulated. Among these molecules, a prominent place is reserved for ApoCIII (4–7).

ApoCIII is a 79-amino-acid glycosylated protein expressed by hepatocytes and enterocytes that is present in all circulating lipoproteins (chylomicrons, VLDL, IDL, LDL, and even HDL),

but specifically characterizes the protein cargo of TRLs. Its presence on lipid particles implies several actions that can impair plasma lipoprotein metabolism, leading to hypertriglyceridemia. In fact, ApoCIII reduces the clearance of ApoB lipoproteins from circulation by interfering with their binding to hepatic ApoB/E receptors. At high concentrations, ApoCIII inhibits the actions of lipoprotein lipase, and at the liver cell level, it favors and increases VLDL formation [for reviews, see (4, 5)]. In addition, ApoCIII seems to possess direct atherogenic properties by provoking detrimental inflammatory responses in monocytes and endothelial cells (8, 9). All these actions clearly account for a primary predisposition toward the development of atherosclerotic disease (4, 5, 7). However, high concentrations of ApoCIII have also been demonstrated to be an important predictor of total and cardiovascular mortality in the setting of secondary prevention for coronary artery disease (CAD) (10, 11). Moreover, in earlier works, we demonstrated that elevated circulating concentrations of ApoCIII (but not other lipids or apolipoproteins) involve a progressive increase in Factor II coagulant activity in the plasma of cardiovascular patients (12). More recently, consistent with such an increase in procoagulant activity, we demonstrated that cardiovascular patients with high concentrations of ApoCIII are twice as likely to have future venous thromboembolic events within a follow-up period of 12 y (13).

Taken together, these observations clearly underline the crucial, harmful, and multifaceted (both athero- and thrombotic) role played by ApoCIII in the pathogenesis of vascular diseases and in the residual risk. Therefore, knowledge of the determinants able to influence ApoCIII synthesis at the liver level may be of great potential relevance for targeted therapeutic approaches.

Several determinants have been previously identified (14), with a special emphasis devoted to the quality of dietary fatty acid (FA) intake. Long chain n-3 PUFAs, contained in fish and fish oil, have been described to reduce serum ApoCIII concentrations through a mechanism similar to that exerted by fibrates (15). Moreover, n-3 PUFA-based therapy (at elevated dosages: 2–4 g/d) has been shown to significantly decrease ApoCIII concentrations [for a systematic review and meta-analysis, see Sahebkar et al. (16)].

However, beyond n-3 PUFA data, much less information exists about the possible effects of other dietary FAs. For the accurate assessment of fat intake, several factors need to be considered, because this is a complex matter, as has been delineated by Hodson et al. (17).

We decided to investigate the plasma total FA pool as a potential biomarker of FA exposure status in our study population. This total FA pool is composed of a mixture of all plasma lipid fractions containing FA moieties, i.e., phospholipids, triacylglycerols, cholesteryl esters, and free nonesterified FAs. With our analyses of patient plasma by GC, we aimed to obtain general information about recent exposure to FAs, without preferentially considering one fraction among the others. This approach, based on the assessment of whole unfractionated plasma FAs, proved to be useful and has been recommended for obtaining an indication of FA intake (18, 19).

Therefore, the objective of the present study was to investigate the possible relations between the intakes of single FAs, as estimated by their plasma concentrations, and circulating amounts of ApoCIII in order to obtain useful dietary indications for prevention, especially for atherogenic dyslipidemia. This task was accomplished by means of combined analysis of epidemiological data in a large study cohort of cardiovascular patients based on the assay of plasma FAs as markers of exposure, as well as by evaluating the *in vitro* effects of selected FAs (chosen on the basis of epidemiological data) on the synthesis of ApoCIII in HepG2 hepatoma cells.

Methods

The primary endpoint of the study was to investigate the possible relations between the intakes of single FAs, as estimated by their plasma concentrations, and circulating amounts of ApoCIII in order to obtain useful dietary indications for prevention. As a secondary endpoint, we validated our correlative results of FAs as determinants of Apolipoprotein production in an *in vitro* model (HepG2 hepatoma cells). Other analyses conducted that were not prespecified were considered exploratory only.

Study subjects

Plasma samples were obtained from 1370 Caucasian subjects previously enrolled in the Verona Heart Study. Details on the enrollment criteria have been described elsewhere (20). The study complies with the Declaration of Helsinki and was approved by the ethics committee (protocol no. 1881/CE) of our institution (Azienda Ospedaliera Universitaria Integrata, Verona). Written informed consent was obtained from all participants. After patient consent, overnight fasting blood samples were collected in Vacutainer® tubes containing EDTA as an anticoagulant. The samples were centrifuged at $2500 \times g$ at 4°C for 15 min in order to remove the cellular components; plasma samples were stored at –80°C until use.

Among the selected samples, 1004 were from patients with angiographically demonstrated CAD, whereas the remaining 366 samples were derived from subjects, mainly with valvular heart disease, with completely normal coronary arteries (CAD-free). At the time of blood sampling, information regarding conventional cardiovascular disease risk factors, including the presence of diabetes and the consumption of lipid-lowering therapy, was also collected. A flowchart of the study design was made (Supplemental Figure 1).

Biochemical analyses

TG, HDL, LDL, and total cholesterol, as well as other routine biochemical parameters were determined as previously described (20). A fully automated turbidimetric immunoassay method was used in order to measure plasma ApoCIII (11). Reagents used for the assay (catalog number: KAI-006) were obtained from Kamiya, and the analysis was performed by means of a COBAS e501 autoanalyzer (Roche). Imprecision was assessed on 3 pools of control sera with low, medium, and high ApoCIII values, and the intra-assay CVs were 1.80%, 2.02%, and 1.98%, whereas the interassay CVs were 4.40%, 3.40%,

Supported by Austrian Science Fund (FWF) projects P26074, J-3983, KLI425, KLI645, doctoral school W1226 “DK Metabolic and Cardiovascular Disease,” and SFB F73 “Lipid hydrolysis” (to RB-G); by Austrian Herzfonds project “Metabolic and proteomic analysis of oxidative stress effects in heart diseases” (to RB-G), and by Medical University of Graz and Austrian Ministry of Science, Research and Economy project “Omics Center Graz” (to RB-G). NM and OO acknowledge the support of Fondazione Cariverona project B36J16002570003. Author disclosures: The authors report no conflicts of interest.

Present address for GS: Aptuit Verona, an Evotec Company, Verona, Italy. OO and GS contributed equally to this work.

Address correspondence to OO (e-mail: oliviero.olivieri@univr.it).

Supplemental Figure 1 and Supplemental Tables 1–4 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/jn/>.

Abbreviations used: ApoCIII, apolipoprotein CIII; CAD, coronary artery disease; FA, fatty acid; HNF4A, hepatocyte nuclear factor 4α; LC-MS/MS, Liquid Chromatography-tandem Mass Spectrometry; PPARα, peroxisome proliferator-activated receptor-α; PRIM, parallel reaction monitoring; TFE, trifluoroethanol; TG, triglyceride; TRL, triglyceride-rich lipoprotein.

and 2.29%, respectively. Moreover, ApoA1, ApoB, and ApoE plasma concentrations were measured by nephelometric immunoassays on a BNII Siemens nephelometer.

Plasma FA analysis

Plasma FAs were measured as previously described (21). Briefly, FAs were extracted from a 100- μ L plasma sample by means of a direct transesterification reaction. A GC method (Hewlett-Packard 5980 chromatograph equipped with an HP-INNOWax polyethylene glycol capillary column, 30.0 m, 250 μ m internal diameter, 0.25- μ m phase column, Agilent) was used. Peak identification was performed using commercially available reference FAs (Sigma). Quantification was performed by the addition of a known amount of an FA internal standard (nonadecylic acid 19:0) to all samples. Peak areas were measured and subsequently quantified by HP-3365 Chem Station software (Hewlett Packard). The concentration of each FA was expressed as g/100 g of total FAMES (% by wt). Each sample was analyzed in duplicate.

Statistical analysis

Statistical analyses were performed by SPSS software version 22.0 (IBM Corporation). Whereas normal continuous variables are expressed as the mean \pm SD, skewed variables (e.g., TG, ApoCIII, and ApoE) were logarithmically transformed, and the geometric means with 95% CIs are reported; in the latter case, all statistical analyses were performed on the log-transformed values. Normality was assessed by the Shapiro–Wilk test (data not shown). Correlations among continuous variables were analyzed by Pearson's correlation test. Significant correlations between the FA profile and plasma lipids/apolipoproteins were further evaluated by linear regression models estimating standardized β coefficients (in which both dependent and independent variables have the SD as their units). First, FAs showing a significant correlation by Pearson's analysis were included in a regression model with a forward stepwise selection of variables and an estimation of R^2 change, in order to disclose the most robust associations. Then, the FAs selected by this analysis were included in an ordinary regression model adjusted for sex, age, CAD diagnosis, BMI, diabetes, and lipid-lowering therapy. Further adjustments were performed for other potential confounding factors, such as ApoE and smoking status. The normal distribution of residuals in the linear regression models was controlled by checking residual plots (data not shown). Subjects with missing data were excluded from regression analyses [specifically, data were missing for BMI ($n = 88$), diabetes ($n = 45$), smoking ($n = 39$), and lipid-lowering therapy ($n = 128$)]. The statistical significance of interaction terms was tested by the Wald test for interaction in generalized linear models. ANOVA with polynomial contrasts for linear trend was then used to assess the distribution of lipid parameters across myristic acid (14:0) quintiles. Qualitative data were analyzed by the χ^2 test and χ^2 test for linear trend analysis when indicated. A P value < 0.05 was considered significant.

In vitro study: cell culture and FA/myristic acid incubation

The in vitro effects of incubation with myristic acid and other FAs, i.e., oleic acid (18:1 cis-9) and palmitic acid (16:0), were evaluated in a recognized model of human liver cells. HepG2 hepatoma cells were seeded in a 75-cm² flask (3 replicates/experimental group) with RPMI medium (RPMI 1640, Sigma Aldrich) supplemented with 10% FBS, 100 U penicillin/mL, 100 μ g streptomycin/mL, and 4 mM L-glutamine. Cells were grown in a humidified incubator at 37°C with 5% CO₂ until ~70% confluency. Six hours before the start of the experimental phase the medium was replaced by serum-free medium. In addition, each FA (Sigma Aldrich) was complexed with FA-free BSA (Sigma Aldrich) in a 6:1 molar ratio. In particular, the tested FAs were dissolved in either 0.1 M NaOH or EtOH and added to 10% FA-free BSA. The obtained solutions were then filtered and added to serum-free media. HepG2-treated cells were grown in the presence of 50, 125, or 250 μ M FAs, and control cells were supplemented with BSA alone. After 24 h of incubation, the cells were harvested with trypsin and washed 3 times with cold PBS. An aliquot of the cell pellet was stored at

–20°C for subsequent LC-tandem MS (LC-MS/MS) analysis, whereas TRI[®] reagent (T9424, Sigma Aldrich) was added to another aliquot of cells and stored at –80°C for RNA extraction. Cell viability was measured using CASY[®] technology (Roche) and the cytotoxicity of each FA was also assessed by Cell Counting Kit-8 (Sigma Aldrich). Independent experiments were conducted on each tested FA.

MS sample preparation

HepG2 cell pellets were resuspended in Tris-HCl-trifluoroethanol (TFE) buffer (25% TFE, 50% Tris-HCl 100 mM, pH = 8.5). After sonication on ice, the cell debris was removed by centrifugation at 3000 \times g for 10 min at 4°C. The protein concentration of the homogenates was estimated using a protein assay (Bio-Rad). Ten micrograms of total protein was reduced with 10 mM Tris(2-carboxyethyl) phosphine and alkylated with 40 mM chloroacetamide for 10 min at 95°C. Each sample was then diluted with 50 mM ammonium bicarbonate to 10% TFE, and overnight tryptic digestion (1:50 trypsin:protein, wt:wt) at 37°C was performed. After digestion, the peptides were diluted with 0.3% formic acid and 5% acetonitrile and stored at –20°C until LC-MS/MS analysis.

LC-MS/MS analysis

Five hundred nanograms per sample were analyzed by nano-HPLC (Dionex Ultimate 3000) with a C18, 5 μ m, 100 Å, 20 \times 0.1 mm enrichment column and an Acclaim PepMap RSLC nano column (C18, 2 μ m, 100 Å, 500 \times 0.075 mm) (all Thermo Fisher Scientific). Samples were concentrated on the enrichment column for 5 min at a flow rate of 15 μ L/min with 0.1% formic acid as the isocratic solvent. Separation was carried out on the nanocolumn at a flow rate of 400 nL/min at 60°C using the following gradient, where solvent A is 0.1% formic acid in water and solvent B is acetonitrile containing 0.1% formic acid: 0–5 min: 2%–5% B; 5–90 min: 5%–35% B; 90–100 min: 35%–95% B; 100–110 min: 95% B; 111–125 min: 2% B. The maxis II ETD mass spectrometer (Bruker) was operated with the captive source in positive mode with the following settings: mass range: 200–2000 m/z , 7 Hz, capillary 1300 V, dry gas flow 3 L/min at 150°C, nanoBooster 0.2 bar, scan mode parallel reaction monitoring (PRM) (CID).

For fragmentation of ApoCIII peptides, previously measured peptide m/z values were added to a PRM mass list using a width of 2–3 m/z and calculated collision energies (R.GWVTDGFSSSLK.D 2+, K.DALSSVQESQVAQQAR.G 2+/3+, K.DYWSTVK.D 2+, K.TAKDALSSVQESQVAQQAR.G 3+, and R.GWVTDGFSSSLKDYWSTVK.D 2+). Furthermore, a mass of 300.00 with a width of 0 and collision energy of 5 eV was added to the target list to obtain full MS spectra.

Processing of LC-MS/MS data

The LC-MS/MS data were analyzed by Data Analysis software (Bruker) using the Sum Peak algorithm. MSConvertGUI (22) was used to generate .mgf files, which were searched by Proteome Discoverer 1.4 (Thermo Fisher Scientific) and Mascot 2.4.1 (MatrixScience) against the public SwissProt database with taxonomy *Homo sapiens* and common contaminants (downloaded on 8 November, 2017; 20,304 sequences). Carbamidomethylation on Cys was entered as a fixed modification, and oxidation on Met was entered as a variable modification. Detailed search criteria were used as follows: trypsin, maximum missed cleavage sites: 2; search mode: MS/MS ion search with decoy database search included; precursor mass tolerance: ± 10 ppm; product mass tolerance: ± 0.05 Da. The resulting .dat files were used to build a spectral library in Skyline (version 4.1.0.11796) (23) and quantify ApoCIII peptides on the MS/MS level in the raw data (Bruker .d files) of the same samples as those used for generating the .mgf files for the spectral library. Detailed Skyline settings were as follows: retention time prediction: ± 2.5 min for peptide R.GWVTDGFSSSLK.D (measured retention time: 61.08 min) and K.DALSSVQESQVAQQAR.G (measured retention time: 43.22 min); pick peptides matching library; filter for 6 ion transitions from $m/z >$ precursor (y,b,p); ion match tolerance: 0.5 m/z ; method match tolerance: 0.005 m/z ; and use only scans within 5 min of predicted retention times. Transition areas of accepted peptides

TABLE 1 Clinical and biochemical features of Verona Heart Study participants with or without CAD¹

	Total study sample (n = 1370)	CAD-free (n = 366)	CAD (n = 1004)	P ²
Age, y	60.6 ± 11.0	58.6 ± 12.5	61.4 ± 10.3	<0.001
Male sex, %	75.8	65.0	79.8	<0.001
Diabetes, %	15.2	7.1	18.1	<0.001
Smoking, %	61.6	42.7	68.5	<0.001
BMI, kg/m ²	25.9 ± 4.6	25.0 ± 4.4	26.3 ± 4.7	<0.001
Lipid-lowering therapy, %	22.6	4.8	29.1	<0.001
Total cholesterol, mmol/L	5.49 ± 1.15	5.48 ± 1.08	5.50 ± 1.18	0.701
HDL cholesterol, mmol/L	1.25 ± 0.35	1.43 ± 0.42	1.18 ± 0.30	<0.001
LDL cholesterol, mmol/L	3.64 ± 0.98	3.51 ± 0.92	3.69 ± 1.00	0.004
TGs, mmol/L	1.61 (1.57, 1.64)	1.37 (1.31, 1.43)	1.70 (1.65, 1.75)	<0.001
ApoA-I, g/L	1.31 ± 0.27	1.39 ± 0.30	1.27 ± 0.24	<0.001
ApoB, g/L	1.12 ± 0.30	1.05 ± 0.25	1.16 ± 0.31	<0.001
ApoCIII, mg/dL	10.8 (10.6, 11.0)	10.1 (9.8, 10.5)	11.1 (10.8, 11.3)	<0.001
ApoE, g/L	0.041 (0.040, 0.042)	0.038 (0.037, 0.040)	0.041 (0.040, 0.043)	0.004

¹Variables with normal distribution are expressed as means ± SDs. Skewed (log-transformed) variables are expressed as geometric means (95% CIs). Lipid parameters and apolipoproteins values refer to plasma concentrations. Apo A-I, apolipoprotein A-I; Apo B, apolipoprotein B; ApoCIII, apolipoprotein CIII; Apo E, apolipoprotein E; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride.

²Comparing CAD with CAD-free subjects by *t* test or χ^2 test, when appropriate.

(threshold ≥ 3 out of 6 matched transitions) were exported to a Microsoft Excel sheet, and product ions were summed per sample. In addition, samples with a wide variation in retention time were excluded from statistical analysis with Perseus (version 1.6.1.1), performing log (2) transformation, ANOVA, and post hoc testing ($P < 0.01$).

APOC3 gene expression analysis

Gene expression analysis was performed by real-time RT-PCR with TaqMan chemistry on a 7500 Real-Time PCR System (Applied Biosystems). Total RNA was extracted according to the TRI[®] reagent protocol, and the amount of RNA was quantified by a Qubit 3.0 fluorometer with a specific Qubit RNA BR assay kit (Thermo Fisher Scientific). The reverse transcription reaction was performed with a SuperScript[™] Vilo[™] cDNA synthesis kit (Invitrogen) starting from 400 ng total RNA for each sample. Relative gene expression analysis of Apolipoprotein C3 (*APOC3*) was assessed by the TaqMan[®] Gene expression assay (Hs00163644_m1, Applied Biosystems) using human *GAPDH* as the endogenous control. The changes in *APOC3* gene expression after FA treatment were expressed as fold change according to the $2^{-\Delta\Delta C_t}$ method comparing the FA-treated cells with the untreated controls (considering the mean value of the 3 replicates with 0 μ M FA). To express the variability of the replicates even in the untreated cells, the SD of the 0- μ M point was calculated by comparing each single replicate with the mean value of the 3 replicates.

Results

Human study

Clinical and laboratory characteristics of the study sample were considered as a whole and subdivided into CAD and CAD-free groups (Table 1). As expected, the CAD group was characterized by an unfavorable lipid profile, including high plasma concentrations of TGs and ApoCIII. Plasma FAs were measured (Table 2). The flowchart of the study participants is also reported (Supplemental Figure 1). Several nominally significant correlations were found among the plasma lipid profile and FAs, as well as among the different FAs (Supplemental Tables 1, 2). Separate regression analysis was performed using plasma lipids and apolipoproteins as independent variables and FAs as dependent variables (Supplemental Table 3). To further dissect the most relevant associations between plasma lipids and FAs, we considered only the associations showing a substantial impact on plasma lipid variability, defined by R^2 change $> 5\%$.

In this way, the most robust associations were found for TGs and ApoCIII (Table 3). Myristic acid explained $\sim 20\%$ of the TG variability and 11% of the ApoCIII variability (Table 3). The association of myristic acid with TGs and ApoCIII remained significant even when including all the analyzed FAs in the regression models for TGs (standardized β coefficient = 0.333, $P < 0.001$) or for ApoCIII (standardized β coefficient = 0.224, $P < 0.001$). This association was confirmed after adjustment

TABLE 2 Plasma FAs in the Verona Heart Study participants¹

FA g/100 g total FAMES	Total study sample (n = 1370)
SFAs	35.2 ± 2.3
12:0	0.118 (0.114, 0.122)
14:0	1.03 (1.01, 1.06)
16:0	24.0 ± 1.8
18:0	8.07 (8.02, 8.13)
20:0	0.34 ± 0.07
22:0	0.74 ± 0.17
24:0	0.73 ± 0.21
UFAs	64.8 ± 2.2
MUFAs	27.0 ± 4.0
16:1n-7	2.02 (1.98, 2.06)
18:1n-9	24.5 ± 3.8
20:1n-9	0.214 (0.211, 0.217)
22:1n-9	0.13 ± 0.11
PUFAs	37.7 ± 4.4
Total n-3s	3.76 (3.71, 3.82)
18:3n-3	0.386 (0.38, 0.39)
18:4n-3	0.26 (0.25, 0.27)
20:5n-3	0.68 (0.66, 0.70)
22:6n-3	2.41 ± 0.77
Total n-6s	33.8 ± 4.3
18:2n-6	23.9 ± 3.9
20:2n-6	0.229 (0.226, 0.232)
20:4n-6	9.68 ± 2.14

¹Variables with normal distribution are expressed as means ± SDs. Skewed variables (log-transformed) are expressed as geometric means (95% CIs). FA, fatty acid; FAMES, fatty acid methyl esters; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFAs, saturated fatty acids; UFA, unsaturated fatty acid.

TABLE 3 The most significant associations of FAs with either TGs or ApoCIII¹

Dependent variable	Std β -coeff.	P^2	R^2	R^2 change	Adjusted std β -coeff.	P^3
TGs						
14:0	0.324	<0.001	0.195	0.195	0.338	<0.001
22:0	−0.112	<0.001	0.326	0.131	−0.130	<0.001
ApoCIII						
14:0	0.288	<0.001	0.107	0.107	0.310	<0.001
20:0	−0.150	<0.001	0.173	0.065	−0.140	<0.001

¹Only predictors of variability with R^2 change >5% are shown. Complete results of the linear regression model with forward stepwise selection of variables are reported in Supplemental Table 3. FA, fatty acid; std β -coeff., standardized β coefficient; TG, triglyceride.

²By linear regression analysis with forward stepwise selection of variables, including all the FAs showing significant association in univariate analysis.

³By linear regression analysis adjusted for sex, age, BMI, diabetes, coronary artery disease diagnosis, and lipid-lowering therapy.

for sex, age, BMI, diabetes, CAD diagnosis, and lipid-lowering therapy (Table 3). The association remained significant after including ApoE, another key apo of TRLs, in the regression models for TGs (standardized β coefficient = 0.387, P < 0.001), ApoCIII (standardized β coefficient = 0.251, P < 0.001), and smoking status (standardized β coefficient for TG = 0.370, P < 0.001, and standardized β coefficient for ApoCIII = 0.259, P < 0.001). No significant differences in myristic acid concentrations were found between the CAD and CAD-free subgroups (mean: 1.03%; 95% CI: 1.00%, 1.06% compared with mean: 1.05%; 95% CI: 1.02%, 1.09%; P = 0.317). Plasma TG and ApoCIII concentrations were analyzed in relation to the different myristic acid concentrations, categorized according to the quintile distribution in the study sample considered as a whole (Figures 1, 2). TG and ApoCIII concentrations increased progressively by increasing myristic acid concentrations from the lowest to the highest quintile (Figures 1A, 2A, respectively). This result was confirmed in subgroups of subjects with or without CAD (Figures 1B, C, 2B, C, for TGs and ApoCIII, respectively), as well as in males or females (Figures 1D–G, 2D–G, for TGs and ApoCIII, respectively). There were no

statistically significant interactions between myristic acid and CAD in determining TG or ApoCIII concentrations (P = 0.891 and P = 0.436, respectively, by the Wald test for interaction in generalized linear models), or between myristic acid and gender (P = 0.304 and P = 0.624, respectively, by the Wald test for interaction in generalized linear models). No significant results were found also considering a model of myristic acid \times CAD diagnosis \times gender interaction (P > 0.05 by the Wald test for interaction in generalized linear models).

In vitro analyses of *APOC3* mRNA and ApoCIII protein in HepG2 cells

Because of the aforementioned results, myristic acid was selected for further in vitro analysis supposing a direct promotion effect on *APOC3* gene transcription and ApoCIII protein synthesis at the liver cell level. This working hypothesis was investigated by using an in vitro cell model of human hepatoma cells (HepG2) that was considered suitable for this purpose, analogous to what was previously demonstrated for ApoB-100 secretion (24).

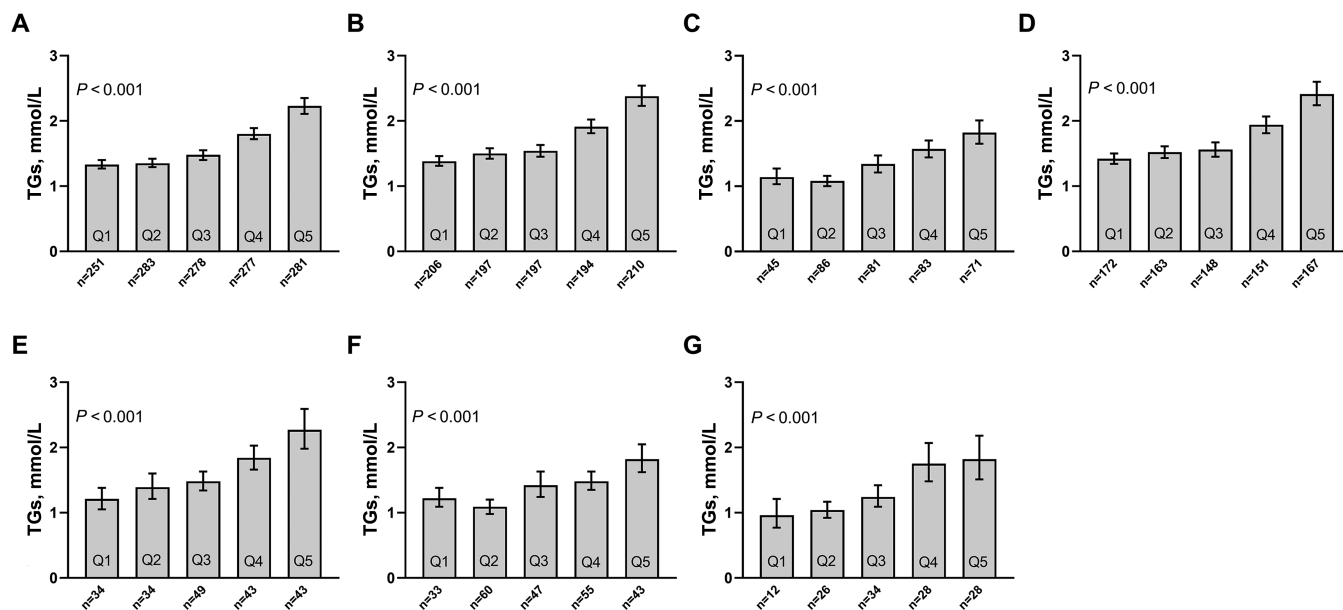


FIGURE 1 Plasma TG concentration distribution by quintiles of plasma myristic acid (14:0) concentrations in all participants (A), CAD patients (B), CAD-free participants (C), males with CAD (D), females with CAD (E), CAD-free males (F), and CAD-free females (G). Plasma myristic acid concentrations in the quintiles were as follows: Q1: <0.73; Q2: 0.73–0.90; Q3: 0.91–1.12; Q4: 1.13–1.47; and Q5: >1.47 g/100 g plasma total FAMES. P values were calculated by ANOVA with polynomial contrasts for linear trend. There were no statistically significant interactions among 14:0, CAD diagnosis, and gender in determining TG concentrations (P > 0.05 by the Wald test for interaction in generalized linear models). CAD, coronary artery disease; TG, triglyceride.

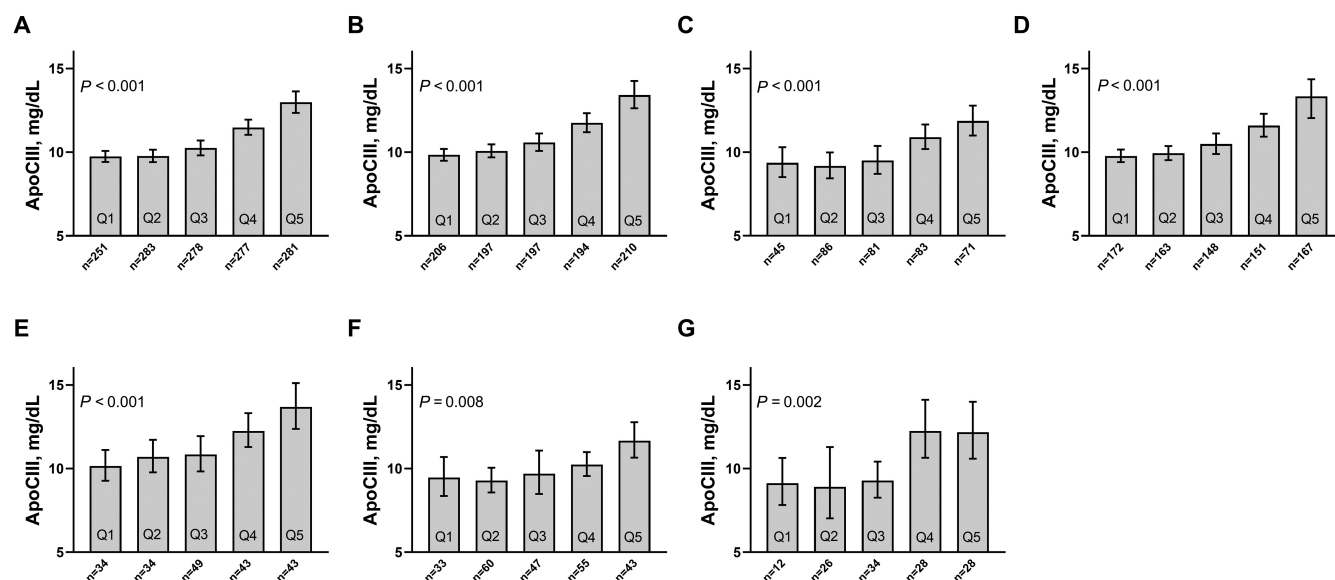


FIGURE 2 Plasma ApoCIII concentration distribution by quintiles of plasma myristic acid (14:0) concentrations in all participants (A), CAD patients (B), CAD-free participants (C), males with CAD (D), females with CAD (E), CAD-free males (F), and CAD-free females (G). Plasma myristic acid concentrations in the quintiles were as follows: Q1: <0.73; Q2: 0.73–0.90; Q3: 0.91–1.12; Q4: 1.13–1.47; and Q5: >1.47 g/100 g plasma total FAMES. *P* values were calculated by ANOVA with polynomial contrasts for linear trend. There were no statistically significant interactions among 14:0, CAD diagnosis, and gender in determining ApoCIII concentrations (*P* > 0.05 by the Wald test for interaction in generalized linear models). CAD, coronary artery disease.

After incubation with myristic acid, HepG2 cells were harvested and whole-cell RNA was extracted. *APOC3* mRNA expression was evaluated by real-time RT-PCR, and the results were normalized against *GAPDH* expression. mRNA expression was greater after incubation of the hepatic cells in medium enriched with myristic acid (ANOVA *P* = 0.047) (Figure 3A).

In order to further analyze the effects of myristic acid on *APOC3* expression, intracellular protein contents after myristic acid conditioning were also investigated. Owing to the low abundance of the protein in HepG2 cell lysates, identification and relative measurement were performed by means of a high-resolution MS PRM approach, where specific and previously known *m/z* values of ApoCIII peptides were used for targeted LC-MS/MS analysis. Lysate ApoCIII protein concentrations tended to be greater in myristic acid-treated cells (ANOVA *P* = 0.054) (Figure 3B). This result was consistent with the findings obtained from *APOC3* mRNA expression. Considering that, in patients, palmitic acid showed a significant correlation with ApoCIII (*P* < 0.001; *R*² = 0.244) (see Supplemental Table 4) and is the most common SFA, accounting for 20%–30% of the total FAs in the human body as well as in this study (see Table 2), a second set of experiments was repeated using palmitic acid instead of myristic acid. According to previous information on a possible neutral role of MUFAs (25, 26), parallel experiments were performed using oleic acid, the most common MUFA. Dynamic changes in *APOC3* mRNA after incubation of HepG2 cells with different amounts of myristic acid, palmitic acid, and oleic acid were observed (Figure 3A), as well as changes in ApoCIII protein concentrations (Figure 3B). *APOC3* mRNA expression was induced mainly by myristic acid and, to a lesser extent, by palmitic acid. On the protein side (Figure 3B), although a limited stimulatory activity was observed for increasing concentrations of palmitic acid, myristic acid was a more effective inducer of ApoCIII increase at the

concentrations of 125 and 250 μ M. In summary, an ~2-fold increase in the expression levels of ApoCIII mRNA and protein was observed after incubation with 250 μ M myristic acid. A weaker effect (~30% increase) was observed after incubation with palmitic acid, whereas incubation with oleic acid did not affect either ApoCIII protein or *APOC3* gene expression.

Discussion

The present work provides evidence in support of a link between myristic acid and circulating concentrations of ApoCIII. Despite previous data concerning the unfavorable effects on circulating lipids (i.e., an increase in total and LDL cholesterol) derived from diets enriched in SFAs (26, 27), to the best of our knowledge, specific information related to the effects on ApoCIII has not been reported so far.

To reach this conclusion, 2 different types of experimental evidence were obtained. The first was derived from the evaluation of plasma FA and ApoCIII concentrations in a large cohort of cardiovascular patients. Plasma total FAs reflect both endogenous metabolic processes and previous dietary intake over a relatively short period (~1 wk) and provide information on multiple FAs in an objective way that is superior in terms of variability to traditional dietary assessment methods (28). Our approach was based on the assessment of the plasma total FA pool, which is considered a good biomarker of recent FA exposure (17, 19).

Myristic acid was significantly associated with both TGs and ApoCIII; this association was independent of several other variables, including other FAs as well as gender and CAD diagnosis. Taken together, these results suggest the hypothesis that a single FA—accounting for only 1% of the total extracted FAs from plasma—is in some way able to systematically

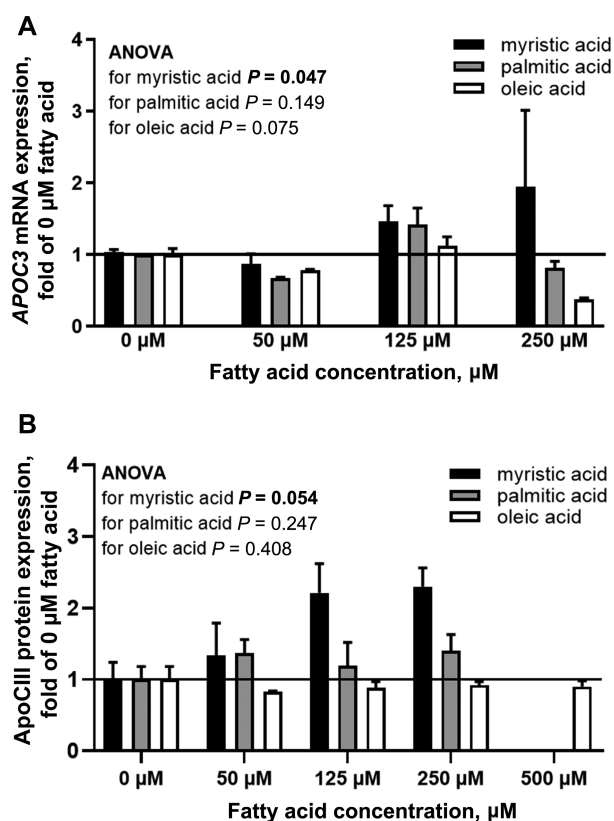


FIGURE 3 Effect of different concentrations of myristic acid, palmitic acid, and oleic acid in cultured HepG2 cell media on *APOC3* mRNA expression measured by real-time RT-PCR (A), and on intracellular ApoCIII protein content measured by high-resolution MS (B). Data are presented as means \pm SDs, $n = 3$ independent cell cultures. ApoCIII, apolipoprotein CIII.

influence the metabolism of TRLs by means of modulation of ApoCIII concentrations.

Myristic acid is an SFA that is mainly present in dairy fats and coconut oil and usually accounts for small amounts of the total FAs in human tissues. Dietary SFAs are usually thought to have negative consequences on human health because of their hypercholesterolemic effects (26, 27). According to such negative assumptions, SFAs are often considered as a single group. The current results, however, suggest specific effects on TRL metabolism for myristic acid, shared only in part by the more abundant SFA palmitic acid. However, although suggestive, such a conclusion was based on data derived from an association study, i.e., an approach intrinsically characterized by relatively limited evidence strength. A statistical association, although strong, does not necessarily mean biological causality. In addition, the results did not allow us to draw any conclusions on the possible metabolic mechanisms involved.

Thus, a second type of evidence was obtained by *in vitro* experiments on a cell model of human hepatoma cells (HepG2), which was considered suitable for this purpose, as previously demonstrated in studies investigating ApoB-100 secretion (24). *In vitro* experiments were designed based on the results previously obtained *ex vivo*, i.e., to verify the hypothesis that myristic acid may act at the liver cell level by promoting an increase in ApoCIII production. Three different FAs were tested: 1) myristic acid (as the main possible culprit); 2) palmitic acid (as the most representative and common SFA); and 3) oleic acid

(as a neutral reference and the most representative and common MUFA).

We observed a significant rise in the mRNA expression of *APOC3* in the presence of increasing concentrations of myristic acid supplied in the culture media. A parallel increase in ApoCIII intracellular protein content was determined by high-resolution MS. In contrast, under the same experimental conditions, incubation with increasing concentrations of palmitic acid produced a more limited increase in *APOC3* mRNA expression, whereas oleic acid appeared substantially neutral.

Taken together, these results indicate that MUFAs do not affect ApoCIII expression in contrast to SFAs, but individual SFAs show different efficacies. A specific, higher impact of myristic acid on the *in vitro* hepatic synthesis of ApoCIII was found, which is consistent with the evident statistical correlations between this FA and the plasma concentrations of ApoCIII and TGs observed in patients. The results of the other tested FAs were consistent with previous studies. Oleic acid was reported to have a low effect on the expression of *APOC3* in HepG2 cells treated with insulin and/or oleate (29); in addition, in a human study, a negligible effect of placebo (oleic acid treatment) on the increase in ApoCIII concentration was demonstrated (30).

To our knowledge, there are no previous works investigating the relations between myristic acid and ApoCIII. Some reports on low HDL-cholesterol concentrations and elevated TG concentrations, i.e., features common in atherogenic dyslipidemia, indirectly support the present results. Myristic acid has been associated with low plasma HDL-cholesterol concentrations in a Mediterranean population, where a weaker but significant direct correlation between myristic acid and TGs was also observed (31). Similarly, de Oliveira Otto et al. (32) reported a significant increase in plasma TGs in relation to plasma myristic acid quintiles in a multiethnic cohort of 2837 patients. Finally, a strong association between plasma myristic acid concentration and plasma TG concentrations was observed in a Western Alaskan Native population (33).

Cellular myristic acid may be derived from the shortening of palmitic acid by peroxisomal β -oxidation or the elongation of lauric acid (12:0), but the amount of endogenously biosynthesized myristic acid appears to be far smaller than the amounts assumed from dietary sources (34). Whatever the origin of myristic acid, its cellular concentration appears to be highly regulated in consideration of the fact that this molecule is crucial in the N-terminal myristoylation of many proteins (35). Therefore, the first reasonable hypothesis to explain the present results is that increased dietary myristic acid may affect the myristoylation of proteins involved in *APOC3* gene transcription. Because *APOC3* transcription is under negative control from peroxisome proliferator-activated receptor- α (PPAR α), it is, for example, possible to speculate an influence of myristic acid on the activity of this nuclear receptor, qualitatively similar but opposite to the effects exerted by n-3 FAs. Popeijus et al. (36) reported a negative influence of high concentrations of myristic acid on the activity of this receptor in a HepG2 cell model, although myristic acid was not the FA with the most evident effect. Moreover, negative control of PPAR α is likely exerted through the displacement of hepatocyte nuclear factor 4 α (HNF4A) from the C3P location of the *APOC3* promoter region, as suggested by Hertz et al. (37), and HNF4A binding activity can be increased by SFAs (38), causing an increase in ApoCIII expression; it is therefore reasonable to suppose, in our specific experimental setting, that

myristic acid can cause an increase in HFN4A binding activity on the APOC3 promoter.

It is evident that further investigation is needed to better clarify the involved mechanisms. Our exploratory study has some significant limitations, including the potential biases related to multiple statistical testing and the limited set of FAs tested in the *in vitro* analyses, as well as the lack of a food questionnaire for dietary data collection. As in any similar study, statistical results should be viewed with caution because of the probability of type I errors. Our findings should be replicated in other population cohorts and validated by further experiments.

In conclusion, the present work reports a robust association between myristic acid and plasma concentrations of TGs and ApoCIII, the latter being one of the main players in TRL metabolism. *In vitro* evidence in myristic acid-treated HepG2 cells convincingly suggests that this correlation is derived from the facilitating activity of myristic acid cellular content on APOC3 mRNA expression, in turn leading to an increase in plasma TG concentrations.

As a practical consequence, if confirmed and validated by further studies, our results may imply novel dietary approaches in the treatment of hypertriglyceridemia and/or ApoCIII-linked cardiovascular disease risk, for instance by limiting the consumption of myristic acid-rich foods, such as dairy products, palm kernel oil, and coconut oil (39). The results of the present work introduce the elements of specificity and quality (rather than quantity) into the general view focused on the presumed harms of a single macronutrient category (SFA) on a single cardiovascular disease risk marker (LDL cholesterol). The SFA myristic acid, one of the relatively less abundant SFAs, seems to possess unexpected features in modulating TRL metabolism. For this reason, our data suggest that limiting myristic acid in the diet may be beneficial for patients affected by atherogenic dyslipidemia.

Acknowledgments

This study was performed in part in the LURM (Laboratorio Universitario di Ricerca Medica) Research Center, University of Verona. The authors' responsibilities were as follows—OO and NM: designed the research; GS, RB-G, and DC: performed the data analysis and interpretation and wrote the initial draft of the manuscript; SF, FP, and DG: critically revised the manuscript; GS, PP, AC, SU, LL, JG, TT, and MS: conducted the research; OO: has primary responsibility for the final content; and all authors: contributed to critically reviewing the manuscript and read and approved the final manuscript.

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