Biomarkers, 2011; 16(4): 321–333

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2011.561367

informa
healthcare

RESEARCH ARTICLE

The effects of simvastatin treatment on plasma lipid-related biomarkers in men with dyslipidaemia

Fabian Chen, Vic Maridakis, Edward A. O'Neill, Brian K. Hubbard, Alison Strack, Chan Beals, Gary A. Herman and Peggy Wong

Merck Research Laboratories, Rahway, NJ, USA

Abstract

Background: Lipidomic biomarkers will facilitate the development of novel anti-atherosclerotic therapies.

Objective: To evaluate the responses of circulating biomarkers to simvastatin treatment.

Methods: A randomized, cross-over study in men with mixed dyslipidaemia was used to compare effects of simvastatin 40 mg/day with placebo.

Results: Plasma concentrations of nine fatty acids (FA; of 33 evaluated) were reduced significantly by simvastatin. No changes in the rates of FA synthesis or in hepatic lipase or lipoprotein lipase activities were apparent. Circulating proprotein convertase subtilisin/kexin type 9 (PCSK9) levels increased.

Conclusion: We identified lipidomic biomarkers of simvastatin treatment effect that are consistent with statin inhibition of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase (ClinicalTrials.gov: NCT00935259).

Keywords: *PCSK9, fatty acids, cardiovascular disease, atherosclerosis, hepatic lipase, lipoprotein lipase*

Introduction

Atherosclerosis and its clinical sequelae, coronary heart disease (CHD), stroke and peripheral vascular disease (PVD), are significant medical problems. Development of statins was a key breakthrough in pharmacological treatment of atherosclerosis (Cheung et al., 2004). But despite their medical success, the 20–30% reduction in CHD risk provided by statins still leaves a large, at-risk patient population (Superko and King, 2008).

For this reason, numerous additional approaches for identifying therapies directed at further reducing the risk of CHD by lipid modification are in development, including agents that lower low-density lipoprotein (LDL) more, or raise high-density lipoprotein (HDL) (Toutouzas et al., 2010). Evaluating the efficacy of such novel therapeutics requires analysis of biomarkers representing both target engagement and known risk factors.

Any novel therapy for CHD will likely be used as an add-on to statin therapy. Therefore, it is desirable to first document the status of various relevant biomarkers, which may be influenced by statin treatment. Statins reduce plasma cholesterol by blocking cholesterol synthesis through inhibition of the rate-limiting enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoA-R) (Endo, 1992), which results in increased expression of LDL-receptors and subsequent depletion of plasma LDL cholesterol (LDL-C) (Goldstein and Brown, 2009). However, statins may also have pleiotropic effects beyond LDL lowering that could contribute to therapeutic efficacy (Sparrow et al., 2001; Schönbeck and Libby, 2004). For example, the metabolism of fatty acids (FA) has been reported to change in the cell line THP-1 after treatment with simvastatin, atorvastatin or fluvastatin (Risé et al., 2003), in keratinocytes after treatment with fluindostatin or lovastatin (Williams et al., 1992) and in Monomac 6 and HepG2 cells after treatment with lovastatin (Hrboticky et al., 1994), as well as in human patients treated with simvastatin (Risé et al., 2001; Jula et al., 2005) or with pravastatin (Risé et al., 2001).

Address for Correspondence: Fabian Chen, Merck & Co., Experimental Medicine, RY34-A400, 126 East Lincoln Avenue, Rahway, NJ 07065-0900, USA. Tel: (732) 594-7425. Fax: (732) 594-5512. E-mail: fabian_chen@merck.com

(Received 17 December 2010; revised 03 February 2011; accepted 04 February 2011)

To confirm and expand these observations in preparation for the evaluation of novel therapies complementary to statins, we have undertaken an exploratory survey of a variety of accessible biomolecules that are likely to vary in response to treatment for dyslipidaemia. We have characterized a set of plasma lipids, two lipolytic enzymes, an LDL-receptor ligand, and indirectly, enzymes that regulate synthesis of some FA.

Plasma lipids are among the molecules most likely to be target engagement biomarkers of LDL or HDL therapy. Lipids are a heterogeneous population of synthetically complex biological molecules with a variety of roles, including structural, metabolic and signalling (Wenk, 2005). A variety of lipids circulate in human plasma, and high levels of several broad classes of easily quantitated lipids, such as triglycerides (TG) and cholesterol, are associated with diseased states like diabetes (Bitzur et al., 2009), coronary artery disease (Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report, 2002) and stroke (Labreuche et al., 2009). More diverse, and traditionally more difficult to quantify routinely, are the FA that circulate freely or as components of TG, cholesterol esters (CE) and phospholipids. Here, we report on changes in seven lipid classes and 33 FA, which occur after 2 weeks of treatment with 40 mg/day simvastatin.

Enzymes responsible for lipid synthesis may be affected by lipid-modifying therapy, whether or not they are directly targeted. A rate-limiting enzyme in long-chain FA synthesis, Δ5-desaturase, has been reported to become activated in the THP-1 cell line upon inhibition of HMG-CoA-R (Risé et al., 1997, 2003). We evaluated the activity of Δ5-desaturase, by examining relative changes of the product FA C20:4n-6 versus the substrate C20:3n-6 and the product C20:5n-3 versus the substrate C20:4n-3 in plasma (Nakamura and Nara, 2004), as well as the activity of various elongases (Wang et al., 2006), by comparing the changes of product 18:00 to substrate 16:00, product 20:3n6 to substrate 18:3n6 and product 22:4n6 to substrate 20:4n6.

The lipolytic enzymes lipoprotein lipase (LPL) (Mead et al., 2002) and hepatic lipase (HL) (Perret et al., 2002), either of which can influence the levels of circulating lipids, might be biomarkers of dyslipidaemia or targets for therapeutic agents designed to treat this disease. Either of these enzymes may also be affected by changes in plasma lipid composition, and both are accessible in plasma after heparin treatment, which releases them from blood vessel endothelium. We have evaluated the effect of simvastatin treatment on the activities of these enzymes in the post-heparin plasma fraction of treated patients.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a therapeutic target for dyslipidaemia (Horton et al., 2007). PCSK9 is a circulating protein that can influence plasma LDL-C concentrations by binding the LDL-receptor and causing its degradation (Lambert et al., 2009). Expression of the gene encoding PCSK9 is regulated by the transcription factor SREBP and thus can be affected by liver cholesterol levels (Horton et al., 2003). Here, we report changes in circulating PCSK9 levels associated with consumption of a high fat meal, as well as with simvastatin treatment.

Methods

Subjects and study design

This was a double-blind, placebo-controlled, randomized, two-period cross-over study (Figure 1) to evaluate the effects of 2 weeks of treatment with simvastatin 40 mg/day on blood biomarkers in men 30–70 years of age with mixed dyslipidaemia. Patients were whites (including Hispanic), with maximum waist circumference >40 inches, and LDL-C levels ≥130 mg/dL. Patients were to be

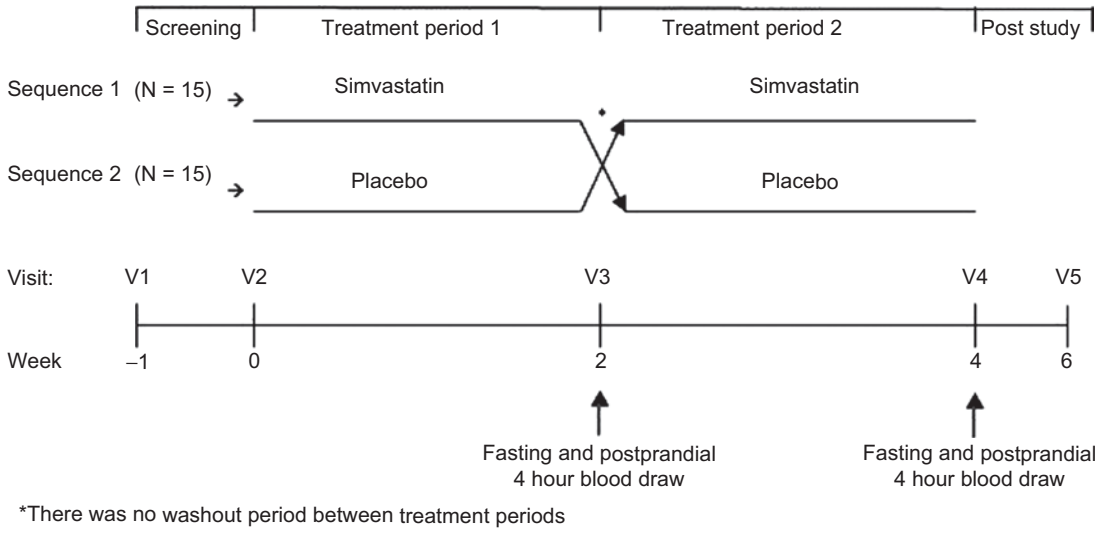


Figure 1. Study design. This was a double-blind, placebo-controlled, randomized, two-period cross-over study to evaluate the effects of 2 weeks of treatment with simvastatin 40 mg/day on blood biomarkers in men 30–70 years of age with mixed dyslipidaemia.

excluded if they had a history of severe or unstable cardiovascular disease, a history of major neurological disorder, an endocrine disorder, a metabolic disorder other than dyslipidaemia or any illness that, in the opinion of the study investigator, might confound the results of the study or pose an additional risk to the patient by their participation in the study. Patients were to be excluded if plasma creatinine was >2.0 mg/dL, if serum alanine aminotransaminase (ALT) or aspartate aminotransferase (AST) were >1.5 × the upper limit of normal range (ULN), or if creatine kinase (CK) was >2 × ULN. Patients were to be excluded if they consumed herbal, organic, homeopathic, dietary or nutritional remedies, or if they had taken lipid-lowering medications within 4 weeks of study initiation.

All patients provided written informed consent to participate. The study (ClinicalTrials.gov: NCT00935259) was conducted in accordance with principles of Good Clinical Practice and was approved by the appropriate institutional review boards and regulatory agencies. All participants provided verbal and written informed consent before undergoing any study procedures.

Eligible patients were randomly assigned to one of two possible balanced treatment sequences, with 15 patients in each treatment sequence. Patients were to receive simvastatin or matching placebo. Study drug consisted of one tablet of simvastatin 40 mg (or matching placebo tablet) taken once daily for 2 weeks per treatment period.

Biochemical analyses

Two days of standardized pre-packaged meals were provided to each patient for consumption prior to the 10-h fast required before the biomarker blood collection at Visits 3 and 4. In addition, to assess how consumption of a meal would affect levels of plasma PCSK9, following each of the fasting blood draws, patients were provided with a high fat meal and were asked to consume it within 20 min. The high fat meal consisted of heavy whipping cream and vanilla ice cream in a mass ratio of one part heavy cream to four parts ice cream. The meal dose was to be 162 g of meal per square meter of body surface area. Body surface area was calculated according to the following formula: body surface area = 0.20247 × height $[m]^{0.725}$ × weight $[kg]^{0.425}$ (Weiss et al., 2008). For the duration of this test, the patients were to remain seated or recumbent until blood samples were drawn 4 h after completion of the meal.

Plasma was collected for lipid profiling and flash frozen. Lipid profiling (TrueMass Lipomic Panel) was conducted by Tethys Bioscience/Lipomics (Emeryville, CA). For the profiling analysis, lipids were extracted in the presence of authentic internal standards by the method of Folch et al. (1957) using chloroform:methanol (2:1 v/v). Individual lipid classes within each extract were separated by liquid chromatography (Agilent Technologies model 1100 Series). Each lipid class was transesterified in 1% sulphuric acid in methanol in a sealed vial under a nitrogen atmosphere at 100°C for 45 min. The resulting FA methyl esters were extracted from the mixture with hexane containing 0.05% butylated hydroxytoluene and prepared for gas chromatography by sealing the hexane extracts under nitrogen. FA methyl esters were separated and quantified by capillary gas chromatography (Agilent Technologies model 6890) equipped with a 30-m DB-88MS capillary column (Agilent Technologies Santa Clara, CA) and a flame-ionization detector. Total plasma concentrations of each lipid class (free cholesterol [FC], CE, TG, lysophosphatidylcholine [LY], phosphatidylcholine [PC] and phosphatidylethanolamine [PE]) were obtained by direct measurement. Total plasma concentrations of each FA analyte were obtained by summing its concentration in each complex lipid together with its free concentration.

Standard serum chemistry (includes CK, AST, ALT), serum lipids (total cholesterol [TC], high-density lipoprotein cholesterol [HDL-C], LDL-C and TG), serum cholesteryl ester transfer protein (CETP) and plasma apolipoprotein A1 (Apo-A1), Apolipoprotein B100 (Apo-B100), lipoprotein (a) (Lp(a)) and C-reactive protein (CRP) were measured by Global Central Labs at PPD (Highland Heights, KY).

PCSK9 in human plasma was measured with an antibody-based immunoassay by the Merck Clinical Development Laboratories (Rahway, NJ) using an electrochemiluminescence detection method from MesoScale Discovery (Gaithersburg, MD). A standard protocol provided by MesoScale was followed using a monoclonal antibody to human PCSK9, E07 (Merck Research Labs, Rahway, NJ) as the capture antibody and monoclonal antibody to human PCSK9-H23 (Merck Research Labs, Rahway, NJ) as the biotin-linked secondary antibody.

To measure LPL and HL, an intravenous bolus injection of 60 units/kg of heparin was administered 10 min before blood sampling to release these enzymes from the vessel wall. Assays for these enzymes, and for Apo-B48, were performed by Pacific Biometrics (Seattle, WA).

Data analysis

In order to assess the effects of simvastatin treatment when compared with placebo on each individual plasma lipid, the plasma lipid concentration was log transformed to the base 10 scale prior to running in a standard cross-over study design linear effects model with terms for period and treatment. In the exploratory portion of this study, plasma total FA that changed after administration of simvastatin when compared with placebo were similarly assessed as described above in a standard cross-over study design linear effects model. To control for multiple looks at the numerous analytes, a double false discovery rate (Mehrotra and Heyse, 2004) procedure was applied to the total FA analysis. In the first step, a P-value <0.025 was used to decide which of the FA components would warrant further investigation by lipid class. Then, the change in concentration of each of those FA within each lipid class was tested in the standard cross-over study design methodology as described above. A P-value <0.05 was the criterion used to discriminate lipid classes changing after administration of simvastatin.

The Δ5-desaturase and elongase enzyme activities were assessed by evaluating the change in concentration of specific products and precursors in the same model described above and the corresponding means and 95% confidence intervals for each precursor and product were plotted.

To assess the association of certain FA with HDL, LDL and triglycerides, the Spearman's rank correlation coefficient (r_s) of the concentrations of the set of FA with plasma lipoprotein or lipid levels, for all patients in the study, both after placebo treatment and after simvastatin treatment was calculated. Spearman's rank correlations above 0.38 were considered a significant association.

Results

Patient disposition and demographics

For this study, a total of 31 white, male patients with mixed dyslipidaemia were enrolled and randomized at a single site. Two patients discontinued due to scheduling conflicts and were lost to follow-up. The mean age of the 29 patients completing the study was 47 (SD = 8). Patients ranged in age from 30 to 64 years of age. The mean BMI of the 29 patients completing the study was 30.5 kg/m^2 (SD = 4.4) and ranged from 18.9 to 42.4 kg/m^2.

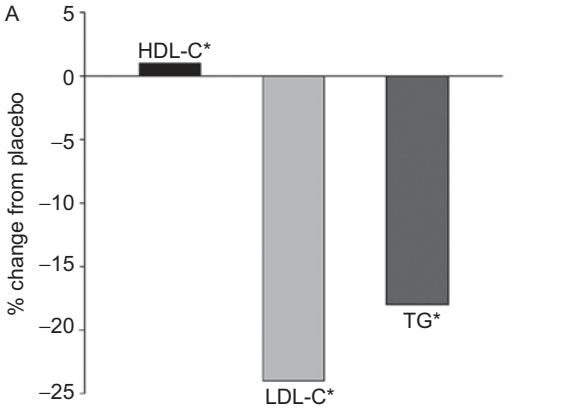
All of the following analyses include five patients who appeared resistant to treatment with simvastatin: two exhibited essentially no change in LDL-C associated with treatment, and three exhibited increased LDL-C associated with treatment. These patients were included in the analysis given these five patients completed the study as per protocol, and there was the intention to treat these patients after randomization. Based on patient visit, interviews and pill counts at visits, these patients were compliant with dosing.

Plasma lipids

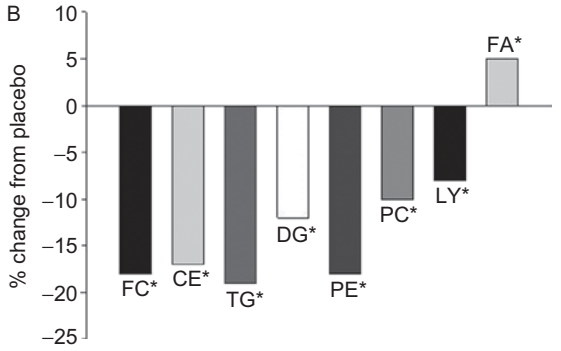
The mean plasma concentrations of HDL-C and LDL-C in placebo-treated patients were 44.3 and 150.5 mg/dL, respectively. The median plasma concentration of TG was 153 mg/dL. Daily treatment with 40 mg/day of simvastatin for 2 weeks was associated with the expected mean changes in bulk plasma lipid concentrations when compared with placebo (Figure 2A). Plasma HDL-C was not significantly affected by treatment. The mean percent difference in LDL-C after simvastatin treatment compared with placebo was −24% ($P < 0.001$) and the mean percent treatment difference in plasma triglycerides was −18% ($P = 0.016$). Plasma TC (mean plasma concentration in placebo-treated patients = 223.4 mg/dL) was also reduced significantly (mean difference of simvastatin from placebo −18%, $P < 0.001$). Analysis of FC, CE and TG with the True Mass Lipomic panel yielded quantitatively nearly identical results (Figure 2B), in terms of treatment-associated difference from placebo. Further, as reported by the True Mass Lipomic panel, treatment with simvastatin was associated with statistically significant reduction of PE and LY. Plasma concentrations of diglyceride (DG) and PC both trended towards reduction in association with simvastatin treatment, but the changes were not considered statistically significant because the *P*-values for their between treatment differences were >0.05. Total FA in plasma exhibited no change in association with statin treatment. The mean plasma concentrations of individual lipid classes (nanomoles/gram) in placebo-treated patients were 1333.7 (FC), 2855.5 (CE), 1196.7 (TG), 30.3 (DG), 178.45 (PE), 1824.5 (PC), 204.5 (LY) and 335.7 (FA).

Plasma proteins

The simvastatin-associated change in a variety of plasma proteins related to plasma lipids (Apo-A1, Apo-B100, Apo-B48, Lp(a) and CETP), along with a plasma protein marker of inflammation (CRP) compared with placebo, was evaluated (Table 1). There were no statistically significant changes in Apo-A1, CETP or CRP associated with statin treatment ($P > 0.050$). Compared with placebo, there was a 21% and a 34% reduction in Apo-B100



*Between treatment p-value HDL-C = 0.771, LDL-C < 0.001, TG = 0.016



*Between treatment p-value FC = 0.001, CE = 0.006, TG = 0.019, DG = 0.090, PE = 0.002, PC = 0.061, LY = 0.028, FA = 0.487

Figure 2. Lipid changes with simvastatin treatment. Changes in (A) concentrations of serum high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) and (B) concentrations of plasma free cholesterol (FC), cholesterol ester (CE), triglyceride (TG), diglyceride (DC), phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidylcholine (LY) and free fatty acids (FA) associated with simvastatin treatment.

Table 1. Plasma concentrations and treatment-associated changes in plasma concentration of selected lipid-related proteins.

			Simvastatin versus placebo		
				Between treatment	
Protein	Simvastatin 40 mg	Placebo	GMR† (±95% CI)	*P*-value†	% Change†
Apo-A1 (mg/dL)	141.24	140.56	1.00 (0.96, 1.05)	0.835	0%
Apo-B100 (mg/dL)	105.67	133.85	0.79 (0.71, 0.87)	<0.0001*	–21%
Apo-B48 (mg/dL)	0.58	0.88	0.66 (0.55, 0.79)	<0.0001*	–34%
Lp(a) (mg/dL)	44.13	39.14	1.13 (1.03, 1.23)	0.009*	13%
CETP (mg/dL)	1.48	1.61	0.92 (0.82, 1.03)	0.132	–8%
CRP (mg/dL)	2.40	2.07	1.16 (1.00, 1.34)	0.052	16%

†GMR = geometric mean ratio; back-transformed from the log base 10 scale.
*Between treatment difference considered statistically significant.

($P < 0.0001$) and Apo-B48 ($P < 0.0001$), respectively, and a 13% increase in Lp(a) ($P = 0.009$) associated with simvastatin treatment (Table 1).

Plasma FA

The True Mass Lipomic Panel consists of 33 FA, each of which is quantified both as free FA and as associated with six different complex plasma lipids. We calculated the total plasma concentration of each of the FA in all its forms in fasting patients who had been treated for 2 weeks with placebo, and for 2 weeks with 40 mg/day simvastatin (Table 2). Compared with placebo treatment, treatment with simvastatin was associated with a statistically significant reduction ($P < 0.025$) in the plasma concentration of nine FA. The plasma concentrations of another 16 FA were numerically reduced in the range of –9% to –26%, but these changes were not considered statistically significant. The plasma concentrations of the remaining eight FA analysed showed little evidence of change associated with simvastatin treatment. No plasma concentration of any FA measured increased with administration of simvastatin versus placebo.

Several statins have been reported to enhance the conversion of linoleic acid (C18:2n6) to long-chain polyunsaturated FA such as arachidonic acid (C20:4n6) in a variety of cultured cells (Williams et al., 1992; Hrboticky et al., 1994; Risé et al., 1997, 2003), and in human patients (Risé et al., 2001; Jula et al., 2005). In the present study, the fasting levels of total plasma arachidonic acid did not change in a statistically significant manner after statin treatment, compared with placebo (Table 2, $P = 0.573$).

Markers of FA metabolism

The rate-limiting step in conversion of linoleic acid to long-chain polyunsaturated FA like arachidonic acid is accomplished by Δ5-desaturase. The activity of this enzyme has been reported to increase with statin treatment of human subjects in a dose-dependent manner (Risé et al., 2001). To evaluate the activity of Δ5-desaturase, we compared the simvastatin treatment-associated changes in amount of precursors and products of this enzyme in plasma (Figure 3A). Although there appears to be a trend towards greater reduction in substrates of this enzyme (precursors), compared with their respective products, the 95% confidence intervals (CI) for each change of each precursor/product pair overlap. In addition, there was not a statistically significant difference in the change in plasma concentrations for both precursor and product pair after simvastatin compared with placebo treatment (20:3n6 and 20:4n6, $P = 0.077$ or 20:4n3 and 20:5n3, $P = 0.085$).

We also compared the amounts of various FA elongase precursors and products (Figure 3B). In two of the three sets examined, there appeared to be no difference in the mean changes in product and precursor after simvastatin treatment, compared with placebo. In one pair, there was a trend towards greater reduction in product (22:4n6) than precursor (20:4n6), but again the 95% CI for these changes overlap.

Composition of complex plasma lipids

A change in the total plasma concentration of a FA does not necessarily mean that it is equally depleted in every class of complex lipid in which it is found. It is possible that a particular FA in CE is preferentially associated with LDL particles, although the same FA associated with a particular phospholipid is found preferentially associated with HDL. In such a case, there would be a reduction of that FA in the CE pool, but not in the phospholipid pool. Therefore, we examined the distribution of several of the most abundant FA, for most of which the plasma concentration was significantly affected by simvastatin treatment compared with placebo treatment (Figure 4). The plasma concentration of 20:4n6 (arachidonic acid) was not significantly changed after simvastatin treatment, compared with placebo treatment. However, it was prespecified as a primary endpoint due to previous reports of its response to statin treatment (Risé et al., 2001; Jula et al., 2005). Therefore, we included it in the evaluation of its distribution among complex lipids (Figure 4A and 4B) and in the evaluation of treatment-associated changes in the amount of high abundance, overall significantly changed FA, by complex lipid association (Figure 4C and 4D). The FA 20:4n6 was the fourth most abundant among those we analysed, the most abundant being 18:2n6, 16:00, 18:1n9, in decreasing order of abundance (Table 2). The abundance of 18:00 was similar to 20:4n6. With regard to the distribution of each FA, there were several common features. There were relatively low levels of each associated with DG, LY, PE or FA. However, each of the

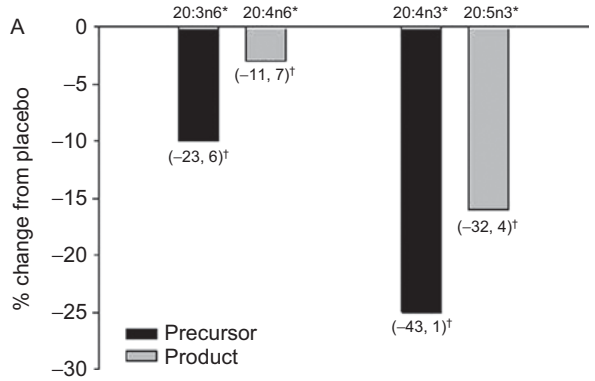
Table 2. Total plasma concentrations of fatty acids and simvastatin treatment-associated changes.

			Simvastatin versus placebo		
Lipid subclass	Simvastatin 40 mg (nmol)	Placebo (nmol)	GMR[†] (±95% CI)	Between treatment *P*-value[†]	% Change[†]
Between treatment *P*-value ≤ 0.025 (step one of double FDR)					
16:00	2311.98	2716.44	0.85 (0.75, 0.96)	0.013[*]	−%51
18:00	737.76	855.24	0.86 (0.77, 0.97)	0.015[*]	−%41
18:1n9	1751.68	2046.41	0.86 (0.76, 0.97)	0.017[*]	−%41
18:2n6	2602.55	3278.98	0.79 (0.70, 0.90)	0.001[*]	−%12
18:3n3	52.91	69.66	0.76 (0.61, 0.94)	0.013[*]	−%42
20:00	4.93	6.29	0.78 (0.68, 0.91)	0.002[*]	−%22
20:2n6	25.66	30.82	0.83 (0.73, 0.95)	0.008[*]	−%71
22:2n6	0.46	0.67	0.69 (0.53, 0.90)	0.009[*]	−%13
dm18:1n9	9.74	11.08	0.88 (0.79, 0.98)	0.020[*]	−%21
Between treatment change ≤ –9%, *P*-value > 0.025					
14:00	117.18	138.99	0.84 (0.68, 1.04)	0.113	−%61
16:1n7	217.02	242.82	0.89 (0.74, 1.08)	0.242	−%11
18:1n7	151.45	167.48	0.90 (0.81, 1.00)	0.06	−%01
18:3n6	54.11	60.13	0.90 (0.74, 1.09)	0.275	−%01
18:4n3	2.24	2.75	0.81 (0.63, 1.05)	0.112	−%91
20:1n9	10.64	11.89	0.90 (0.79, 1.02)	0.096	−%01
20:3n6	163.17	181.15	0.90 (0.77, 1.06)	0.194	−%01
20:4n3	5.92	7.84	0.75 (0.57, 0.99)	0.044	−%52
20:5n3	46.22	54.9	0.84 (0.68, 1.04)	0.11	−%61
22:00	3.36	4.37	0.77 (0.54, 1.09)	0.136	−%32
22:4n6	28.44	31.18	0.91 (0.80, 1.04)	0.165	−%9
22:5n3	51.05	59.6	0.86 (0.74, 0.99)	0.035	−%41
22:6n3	146.01	164.57	0.89 (0.80, 0.99)	0.029	−%11
24:00:00	4.55	5.33	0.85 (0.60, 1.20)	0.351	−%51
24:1n9	2.26	3.07	0.74 (0.42, 1.28)	0.269	−%62
dm18:1n7	1.81	2.01	0.90 (0.80, 1.02)	0.084	−%01
Between treatment change ≥ -8%, *P*-value > 0.025					
14:1n5	10.02	10.1	0.99 (0.80,1.22)	0.933	−%1
15:00	20.29	20.95	0.97 (0.85, 1.10)	0.609	−%3
20:3n9	11.51	11.28	1.02 (0.85, 1.22)	0.817	2%
20:4n6	860.86	883.93	0.97 (0.89, 1.07)	0.573	−%3
22:1n9	2.24	2.36	0.95 (0.80, 1.12)	0.528	−%5
22:5n6	19.08	20.56	0.93 (0.82, 1.04)	0.202	−%7
dm16:0	36.25	39.12	0.93 (0.84, 1.03)	0.141	−%7
dm18:0	25.9	28.3	0.92 (0.81, 1.03)	0.143	−%8

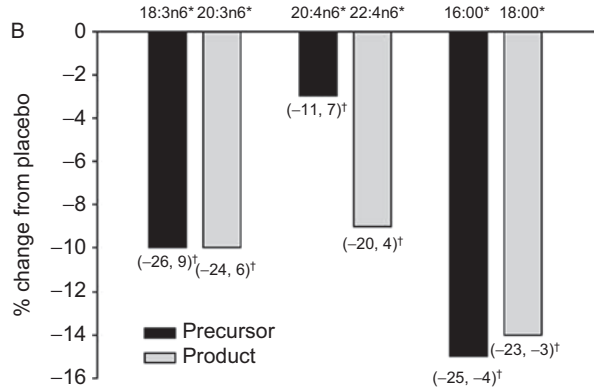
[†]GMR = geometric mean ratio; back-transformed from the log base 10 scale.
[*]Between treatment difference considered statistically significant ($P < 0.025$).

FA was unique in its distribution. For example, 16:00 was equally distributed between TG and PC, whereas 18:00 was primarily associated with PC; 18:1n9 was primarily associated with TG and 18:2n6 was primarily associated with CE. Like 18:00, 20:4n6 was primarily associated with PC, but unlike 18:00, a significant proportion of 20:4n6 was associated with CE.

As noted above, total plasma CE was reduced by 17% after statin treatment, compared with placebo, and with the exception of 20:4n6, the abundant FA associated with CE were reduced in the range of 14–21% (Figure 4C). The amount of 20:4n6 associated with CE was reduced by 6% but this change was not considered statistically significant ($P = 0.310$). Similarly, most of the abundant FA associated with TG and PC were reduced by a proportion similar to the overall reduction in PC, although the amount of 20:4n6 associated with PC did not change. In general, with the exception of 20:4n6, the percent change of each of these FA was similar to the percent change in the complex lipid it was associated with. In no case did the free form of any FA change significantly (Figure 4D). The abundant FA associated with LY and PE were reduced to the same extent as total LY and PE (compare Figures 4D and 2B). The less abundant FA that exhibited statistically significant simvastatin-associated overall change in plasma concentration exhibited similar patterns of change when evaluated by lipid class association (data not shown). The only lipid class in which the amount of 20:4n6 changed in a statistically significant manner was that associated with PE, in which it was reduced by 14%, consistent with the overall reduction in PE of 18%.



*p-value: 20:3n6 = 0.194, 20:4n6 = 0.573, 20:4n3 = 0.044, 20:5n3 = 0.11
†95% CI



*p-value: 18:3n6 = 0.275, 20:3n6 = 0.194, 20:4n6 = 0.573, 22:4n6 = 0.165, 16:00 = 0.013, 18:00 = 0.015
†95% CI

Figure 3. Desaturase and elongase activity in response to simvastatin treatment. (A) Δ5-Desaturase product–precursor analysis, mean % change from baseline of total plasma concentration of indicated fatty acids and (B) elongase product–precursor analysis, mean % change from baseline of total plasma concentration of indicated fatty acids.

Relationships between FA levels or phospholipids and lipoprotein levels

In this study, reductions in the plasma concentration of specific FA after administration of simvastatin treatment (Table 2) reflected simvastatin-associated reductions in LDL-C, triglycerides and certain phospholipids (Figure 2A and 2B). Consistent with the hypothesis that circulating levels of those FA that are reduced in association with simvastatin treatment are reduced primarily as the result of LDL or IDL particle uptake by the LDL-receptor, the concentration of each of these FA correlates reasonably well with the plasma concentrations of both LDL-C (16:00 $r_s = 0.61$, $P < 0.0001$; 18:00 $r_s = 0.53$, $P < 0.001$; 18:1n9 $r_s = 0.58$, $P < 0.001$; 18:2n6 $r_s = 0.67$, $P < 0.001$) and TG (16:00 $r_s = 0.73$, <0.001; 18:00 $r_s = 0.69$, <0.001; 18:1n9 $r_s = 0.71$, <0.001; 18:2n6 $r_s = 0.50$, <0.001), and not at all with HDL-C (16:00 $r_s = 0.13$, $P = 0.32$; 18:00 $r_s = 0.11$, $P = 0.41$; 18:1n9 $r_s = 0.10$, $P = 0.46$ 18:2n6 $r_s = 0.19$, $P = 0.17$) (Figure 5). In several cases (LDL vs. 18:00, vs. 18:1n9, vs. 18:2n6), the significant correlation is driven by the treated arm. A FA that exhibits no overall change in plasma concentration in association with simvastatin treatment (20:4n6) exhibits a moderate correlation with the plasma concentration of LDL-C ($r_s = 0.46$, $P = 0.0004$), and no correlation with triglycerides ($r_s = 0.21$, $P = 0.12$) or HDL-C ($r_s = 0.17$, $P = 0.21$).

HL and LPL

Consistent with the observations we have made in this study, simvastatin has been shown to reduce TG (Branchi et al., 1999). This may be mediated by increased clearance as demonstrated by stable isotope TG turnover studies (Isley et al., 2006). Statin-associated increased TG clearance has been proposed to result from an increase in plasma LPL activity (Isley et al., 2006). Therefore, we measured the activities of this and another lipolytic enzyme, HL, in the post-heparin fraction of plasma from patients treated for 2 weeks with 40 mg/day simvastatin and compared it with the same fraction from those individuals after 2 weeks on placebo. We observed no significant change in the activity of either of these enzymes after treatment with simvastatin when compared with placebo ($P = 0.918$). After 2 weeks of placebo treatment, mean LPL activity was 19.72 U/L, although after simvastatin treatment it was 19.96 U/L. Mean HL activity after 2 weeks of placebo treatment was 245.42 U/L, and after simvastatin treatment it was 221.41 U/L and there was no statistically significant difference in the HL activity between treatments ($P = 0.146$).

PCSK9

PCSK9 is an endogenous negative regulator of the LDL-receptor (Horton et al., 2007; Lambert et al., 2009). Recent studies have reported that statin treatment is associated with an ~35% increase in circulating PCSK9 levels in patients (Careskey et al., 2008). In the study reported here, we measured the plasma concentration of this protein in both fed and fasted states, and compared these levels after 2 weeks of placebo and 2 weeks simvastatin treatment. After 2 weeks of placebo treatment, the mean plasma concentration of PCSK9 was 4.92 nM in the fasted state, and 5.78 nM in the fed state. Simvastatin treatment was associated with a 29% increase in PCSK9 levels compared with placebo in the fed state ($P = 0.001$) and a 16% increase in the fasted state ($P = 0.027$) (Figure 6). The fed versus fasted state within the placebo subjects was significantly different (–0.86 nM; $P = 0.005$), whereas the fed versus fasted state within the simvastatin treatment did not differ significantly (–0.35 nM, $P = 0.119$). When the simvastatin fed versus fasted state difference was compared with the placebo treatment, the mean difference of 0.50 nM was not significant ($P = 0.106$).

Discussion

In this study, we characterized the responses to simvastatin treatment of a unique set of circulating plasma biomarkers relevant to the understanding and treatment

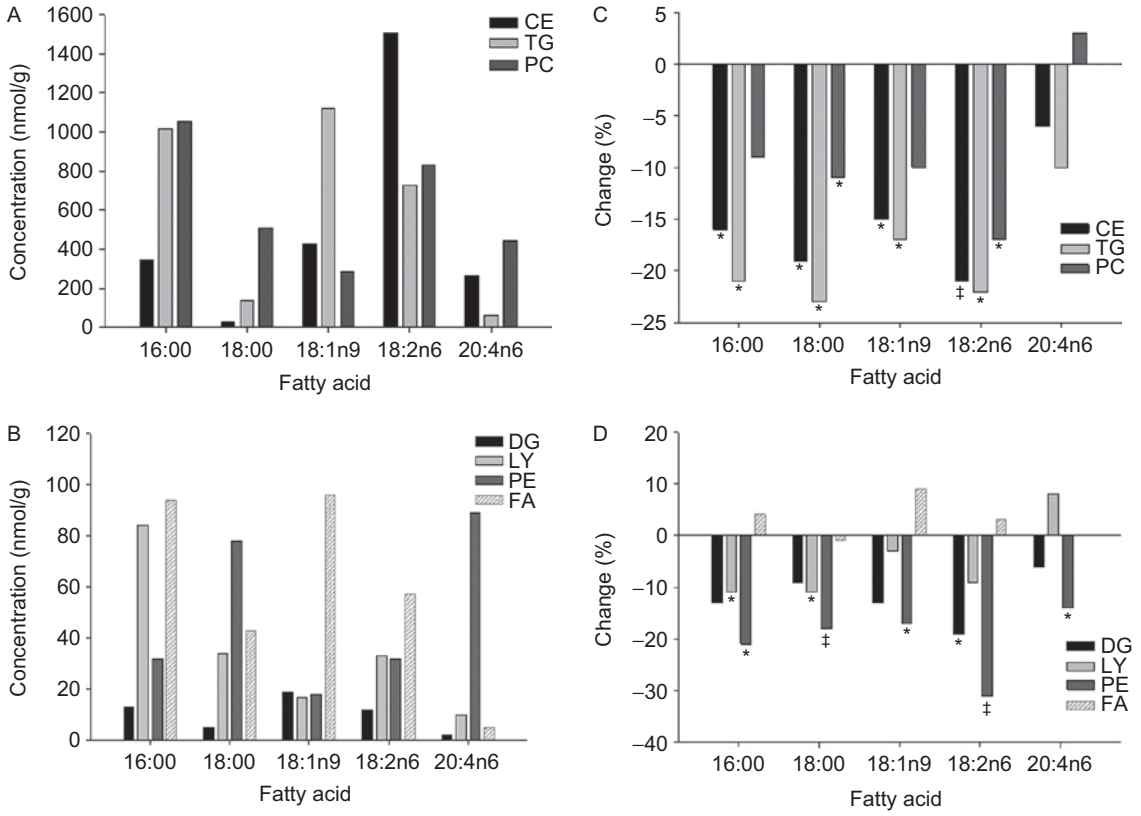


Figure 4. Distribution of selected fatty acids in complex lipids (A, B) and simvastatin treatment-associated change from placebo (C, D). In (C, D) * between treatment *P*-values < 0.05 and ‡*P*-values < 0.001. The complex lipids are cholesterol ester (CE), triglyceride (TG), phosphatidylcholine (PC), diglyceride (DG), lysophosphatidylcholine (LY), phosphatidylethanolamine (PE) and free fatty acids (FA).

of dyslipidaemia. Using a cross-over study, we evaluated the effect of simvastatin treatment on the absolute amount of seven plasma lipid classes and 33 different FA, on the distribution of the FA on complex lipids, on the activity of two lipases, and on the plasma concentration the LDL-receptor antagonist PCSK9.

No previous study has examined these biomarkers together and at this level of detail. Further, unlike previous studies that have examined the effects of simvastatin on some of the same biomarkers, the cross-over design of this study minimizes the influence of confounding covariates because each patient serves as his or her own control.

Treatment with 40 mg/day of simvastatin was associated with a 24% reduction in LDL-C (Figure 2A). Although greater % reductions in LDL-C levels have been reported for simvastatin (e.g. –38% for 20 mg/day in the 4S Study (Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S), 1994), in most published simvastatin studies the patient population is extremely hyperlipidaemic, often with mean LDL-C levels in the 170 mg/dL range. In our study, the patient population selected had a mean LDL-C level of 150 mg/dL. This difference may create an inherent limit to the % reduction in serum lipids one can expect. In addition, five of the 29 (17%) patients included in the analysis appeared resistant to simvastatin treatment. Subject compliance with statin and placebo was monitored by interviews and pill counts at patient visits. Based on these assessments, study drug compliance was satisfactory in this study. In this study, the observed 24% reduction suggests that the expected up-regulation of LDL-receptor occurred in response to reduced cholesterol synthesis in the liver caused by inhibition of HMG-CoA-R by simvastatin (Goldstein and Brown, 2009). TG was also reduced by 18%, as is typically observed in association with statin treatment (Branchi et al., 1999).

We used a commercial lipidomics platform to evaluate changes in the absolute amount and the distribution of a large number of plasma FA, FC and several classes of complex plasma lipids. To help validate this platform, we first compared changes in lipids measured by the lipidomics platform with changes in the same lipid classes assessed by standard clinical laboratory techniques. The lipidomics platform yielded changes in TC, CE and TG that closely approximated changes in these analytes observed with the standard techniques: 18% decreases in both TC (HDL-C + LDL-C) and TG were observed by standard techniques although 18% and 19% decreases were observed with the lipidomics platform for total cholesterol (CE + FC) and TG, respectively.

The lipidomics platform yields information on complex lipids not directly measured by clinical laboratories (Table 2). Consistent with expectations, CE was reduced by 17% in association with simvastatin treatment.

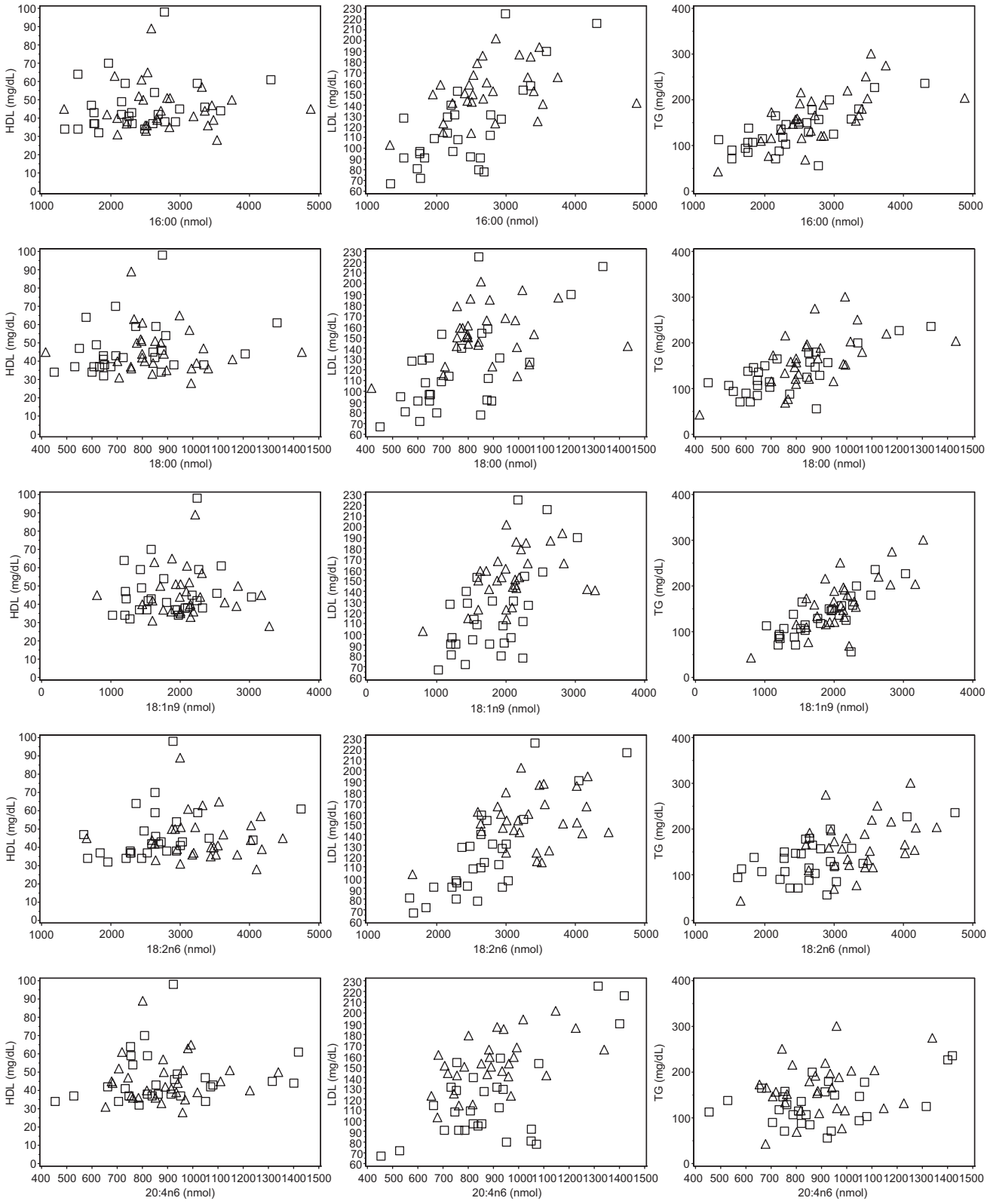


Figure 5. Scatter plots demonstrating the relationships between high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglyceride (TG) with most abundant fatty acids. Samples were assayed after placebo treatment (Δ) and after simvastatin treatment (□).

Changes in plasma phospholipids associated with simvastatin treatment, to our knowledge, have not previously been reported. In this study, we observed reduction in the plasma concentrations of PE, PC and LY associated with simvastatin treatment. We speculated that the lipid classes that are significantly reduced in association with simvastatin treatment (17–19%) are preferentially associated with either LDL or IDL/VLDL or all and that the lipid classes that are reduced, but not to as great an extent (8–12%), are more evenly distributed among all plasma

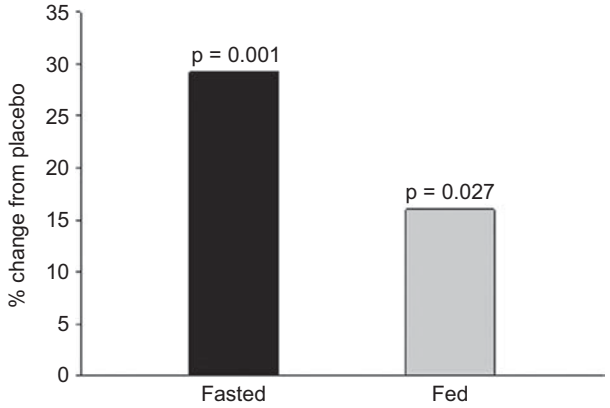


Figure 6. Simvastatin treatment increases proprotein convertase subtilisin/kexin type 9 (PCSK9) in the fasted and fed state.

lipoproteins. These conclusions are consistent with previous observations by Bergheanu et al. (2008) that changes in certain sphingomyelins and PC correlated with changes in LDL-C/HDL-C associated with either rosuvastatin or atorvastatin treatment.

Consistent with the reductions in LDL-C and TG, and with the expected mechanism of this decrease, Apo-B100 and Apo-B48 were both significantly reduced in association with simvastatin treatment compared with placebo (Table 1). Consistent with the absence of change in HDL-C, total plasma Apo-A1 did not change (Table 1). Of three other plasma proteins evaluated, only Lp(a) levels changed in a statistically significant manner in association with simvastatin treatment, increasing 13% compared with placebo. This observation is consistent with previous reports of Lp(a) increase associated with simvastatin treatment (Branchi et al., 1995; Bredie et al., 1996). Though in another study, simvastatin did not have an effect on Lp(a) levels (Haffner et al., 1995). These inconsistent observations appear to have resulted in a consensus that in general statins do not affect Lp(A) (Maron et al., 2000). There was no statistically significant change in either CETP or CRP levels. There is no reason to expect CETP levels to change in association with simvastatin treatment, and we are aware of no other studies in which this biomarker has been evaluated after treatment with simvastatin or any other statin. And while there have been many reports of statin-associated reduction in plasma CRP, we noted that the CRP levels in patient population reported here was within the normal range (mean < 3 mg/L), and hence may be largely resistant to reduction by simvastatin treatment. Note, for example, that in the Jupiter trial patients receiving rosuvastatin achieved a 37% reduction in the median CRP level compared with placebo (Ridker et al., 2009), but the median and range of CRP levels in the patient population studied was 4.3 (2.8–7.1) mg/L (Ridker et al., 2007). We also noted that the correlation between LDL-C lowering and CRP reduction in the Jupiter trail was small ($r = 0.1$) (Ridker et al., 2009). Thus it may not be surprising that we have seen no statin-dependent change in CRP levels in the study reported here. It is also possible that this study was underpowered to observe simvastatin-associated reduction in CRP levels due to high within individual variation in CRP levels (Macy et al., 1997).

In addition to reductions in C and TG observed after treatment with simvastatin, we noted statistically significant reductions in a variety of FA (Table 2), many likely to be primary constituents of the CE and TG in the IDL/VLDL and LDL particles removed from plasma by the increased number of LDL-receptors. Further, most of these FA were reduced by 14–24%, similar to the observed reductions in CE and triglyceride. As noted above for the complex lipids, we have speculated that those FA reduced by somewhat more than 15–20% may be preferentially associated with LDL, and possibly IDL/VLDL, that those reduced in the range of 8–15% are evenly distributed among plasma lipoproteins, and those that change little or not at all are preferentially associated with HDL, and/or other lipophilic components of plasma. However, with this study we cannot rule out the possibility of differential metabolism of various FA, some of which may be oxidized upon entering the liver with LDL, others simply recycled onto VLDL or nascent HDL. Another possibility is that the rate of synthesis of some FA is increased in association with simvastatin treatment.

We observed no increase in the plasma concentration of any FA. However, the mean plasma concentrations of two products of Δ5-desaturase were less reduced after statin treatment than were the mean plasma concentrations of their precursors (Figure 3A), though the 95% CI of the changes in pairs of precursors and products overlap, suggesting no statistically significant change in precursor versus product in either case. Thus, we concluded that we have observed no evidence of change in the activity of Δ5-desaturase associated with simvastatin treatment. Similarly, we observed no statistically significant differences in the changes in the any of three precursor/product pairs of FA elongase associated with simvastatin treatment.

Our observation that plasma total arachidonic acid (20:4n6) levels did not increase in association with simvastatin treatment, compared with placebo treatment, is consistent with previous reports (Harris et al., 2004; Jula et al., 2005) in which no changes in the plasma concentration of arachidonic acid after simvastatin treatment, compared with baseline, were seen. But our observation is inconsistent with the report of a study by Risé et al. in which statins increased plasma levels of arachidonic acid (Risé et al., 2001). A possible explanation for this discrepancy is that the population of patients we studied and conditions of treatment we used were quite different from the study reported by Risé et al. In the study reported here, the patients exhibited mean LDL-C levels of ~150 mg/dL, whereas in the Risé study the patients were hyperlipidaemic, with mean LDL-C levels of ~230 mg/dL. Further, in the Risé study, out of nine patients treated with statins, six were treated with pravastatin (10–40 mg/day) and three were treated with simvastatin (10–20 mg/day). Finally, in the Risé study, patients were not only treated with statins,

but also advised to follow a tailor-made prudent diet. It is not clear how these many differences from our protocol would affect arachidonic acid levels.

Other studies have directly observed statin-enhanced conversion of linoleic to long-chain polyunsaturated FA, including arachidonic acid, using labelled tracer experiments in a variety of cultured cell types (Williams et al., 1992; Hrboticky et al., 1994; Risé et al., 1997, 2003). These studies may not be relevant to the present study. Observations made on isolated cells, cultured in defined medium, with no serum are unlikely to translate to a complex, autonomous, self-regulating system.

Changes in proportions of specific FA, understood to have product and precursor relationship with regard to specific FA-metabolizing enzymes, have been interpreted as reflecting metabolic enzyme activity (Risé et al., 2001; Jula et al., 2005; Kaddurah-Daouk et al., 2010). We too have observed apparent changes in the ratios of a set of products and precursors, associated with simvastatin treatment. However, these are not statistically significant. Further, appropriately interpreting change or apparent absence of change, in the ratio of precursors and products of FA synthetic enzymes in a complex system like the one under study, is difficult. There is variability in the distribution of FA in various complex lipids, which may lead to variability in their association with different lipoprotein particles (Figure 4A and 4B; Hodson et al., 2008). Since lipoprotein particles are differentially affected by statin treatment, this could lead to differential depletion of precursors and products of specific enzymes, changing their ratios but not in ways that reflect the activity of the enzymes. There is also the possibility that precursors and products of certain enzymes would be catabolized differently once taken up by the liver along with LDL particles, some to be oxidized, some recycled. This would also compromise the interpretation of changes, or absence of changes in precursor/product ratios. Finally, there may be an up-regulation of the synthesis of some FA, to compensate for their depletion in association with increased LDL uptake, leading to an apparent absence of change in their plasma concentration. In any event, as there is a clear decrease in the plasma concentration of some FA and not others, there is also a relative increase in the mole % of a set of FA. How this proportional change in plasma FA concentration would affect the physiology or pathology of the system is unknown.

The correlations we observed between the amounts of many of the FA that are significantly reduced in association with simvastatin treatment, and LDL-C and triglycerides (Figure 5), are consistent with our hypothesis that the simvastatin-associated changes in all these analytes are mechanistically related. Similarly the weak or absent correlation between the FA whose plasma concentrations do not change in association with simvastatin treatment (e.g. 20:4n6) and LDL-C or triglycerides strengthens the hypothesis that these FA are not preferentially associated with LDL or IDL/VLDL, and may be only minimally associated with these particles.

We observed no changes in post-heparin LPL activity associated with simvastatin treatment. This observation is consistent with a variety of previous reports (The effect of aggressive versus standard lipid lowering by atorvastatin on diabetic dyslipidemia: the DALI study: a double-blind, randomized, placebo-controlled trial in patients with type 2 diabetes and diabetic dyslipidemia, 2001; Kobayashi et al., 2001; Nordøy et al., 2001; Castro Cabezas et al., 2004). Though, others have observed an increase in LPL in patients with diabetes, hyperlipidaemia and CHD after treatment with simvastatin (Castro Cabezas et al., 1993; Piorunska-Stolzmann et al., 2003; Schneider et al., 2004).

Atorvastatin has been reported to decrease post-heparin plasma HL activity in patients with type 2 diabetes (Berk-Planken et al., 2003) and the combination of lovastatin and colestipol reduces HL activity in patients with dyslipidaemia (Zambon et al., 2001). We observed a trend towards reduction in HL activity associated with simvastatin treatment, but it was not statistically significant, possibly due to a combination of the absence of diabetes in the study population, and the genetic influence on the response of HL activity to pharmacological treatment (Zambon et al., 2001). Given these results, and the inconsistent effects of statins on LPL, compared with the consistent effect of statins on triglycerides (Branchi et al., 1999), we have seen no reason to postulate mechanisms for plasma triglyceride reduction associated with simvastatin treatment beyond that suggested by Branchi et al. (1999): the up-regulation of LDL-receptor and subsequent depletion of IDL, and possibly VLDL particles, both rich in triglycerides.

A 10-h fast yields a small but statistically significant increase in mean plasma PCSK9 concentration, which is not apparent after simvastatin treatment. This observation is consistent with the recent report by Browning and Horton (2010) in which they observed reduction in circulating PCSK9 levels occurring during the period of 12–48 h after fasting was initiated. Our analysis took place ~10 h after fasting was initiated, at a time when Browning and Horton observe a transient rise in circulating PCSK9 levels. Plasma PCSK9 levels increase significantly in association with statin treatment. This is not surprising as expression of the gene encoding PCSK9 is regulated in a manner similar to that of the gene encoding the LDL-receptor (Horton et al., 2003), and would therefore tend to be up-regulated when cellular cholesterol levels decrease subsequent to HMG-CoA inhibition. This observation is consistent with previous reports of atorvastatin-associated increases in circulating PCSK9 (Dubuc et al., 2004; Careskey et al., 2008; Lakoski et al., 2009). These data suggest that anti-PCSK9 therapy would enhance the cholesterol-lowering effects of statins.

In summary, an optimal study design was used to assess the effect of simvastatin treatment on a set of circulating biomarkers relevant to dyslipidaemia, including an examination of circulating plasma lipids in unprecedented detail. The observations reported are all consistent with well-documented effects of statins, which result

from HMG-CoA-R inhibition, that is, depletion of cellular cholesterol, up-regulation of gene expression regulated by SREBP-1, resulting in increased expression of LDL-receptors (among other proteins, including PCSK9) and subsequent depletion of plasma IDL and LDL, together with a subset of plasma FA.

Declaration of interest

All authors are employees of Merck & Co., Inc. All research was supported and funded by Merck & Co., Inc.

References

Bergheanu SC, Reijmers T, Zwinderman AH, Bobeldijk I, Ramaker R, Liem AH, van der Greef J, Hankemeier T, Jukema JW. (2008). Lipidomic approach to evaluate rosuvastatin and atorvastatin at various dosages: investigating differential effects among statins. *Curr Med Res Opin* 24:2477–2487.

Berk-Planken II, Hoogerbrugge N, Stolk RP, Bootsma AH, Jansen H; DALI Study Group. (2003). Atorvastatin dose-dependently decreases hepatic lipase activity in type 2 diabetes: effect of sex and the LIPC promoter variant. *Diabetes Care* 26:427–432.

Bitzur R, Cohen H, Kamari Y, Shaish A, Harats D. (2009). Triglycerides and HDL cholesterol: stars or second leads in diabetes? *Diabetes Care* 32 (Suppl 2):S373–S377.

Branchi A, Fiorenza AM, Rovellini A, Torri A, Muzio F, Macor S, Sommariva D. (1999). Lowering effects of four different statins on serum triglyceride level. *Eur J Clin Pharmacol* 55:499–502.

Branchi A, Rovellini A, Fiorenza AM, Sommariva D. (1995). Effects of bezafibrate and of 2 HMG-CoA reductase inhibitors on lipoprotein (a) level in hypercholesterolemic patients. *Int J Clin Pharmacol Ther* 33:345–350.

Bredie SJ, Westerveld HT, Knipscheer HC, de Bruin TW, Kastelein JJ, Stalenhoef AF. (1996). Effects of gemfibrozil or simvastatin on apolipoprotein-B-containing lipoproteins, apolipoprotein-CIII and lipoprotein(a) in familial combined hyperlipidaemia. *Neth J Med* 49:59–67.

Browning JD, Horton JD. (2010). Fasting reduces plasma proprotein convertase, subtilisin/kexin type 9 and cholesterol biosynthesis in humans. *J Lipid Res* 51:3359–3363.

Careskey HE, Davis RA, Alborn WE, Troutt JS, Cao G, Konrad RJ. (2008). Atorvastatin increases human serum levels of proprotein convertase subtilisin/kexin type 9. *J Lipid Res* 49:394–398.

Castro Cabezas M, de Bruin TW, Van Linde-Sibenius Trip M, Kock LA, Jansen H, Erkelens DW. (1993). Lipoprotein(a) plasma concentrations associated with lipolytic activities in eight kindreds with familial combined hyperlipidemia and normolipidemic subjects. *Metab Clin Exp* 42:756–761.

Castro Cabezas M, Verseyden C, Meijssen S, Jansen H, Erkelens DW. (2004). Effects of atorvastatin on the clearance of triglyceride-rich lipoproteins in familial combined hyperlipidemia. *J Clin Endocrinol Metab* 89:5972–5980.

Cheung BM, Lauder IJ, Lau CP, Kumana CR. (2004). Meta-analysis of large randomized controlled trials to evaluate the impact of statins on cardiovascular outcomes. *Br J Clin Pharmacol* 57:640–651.

Dubuc G, Chamberland A, Wassef H, Davignon J, Seidah NG, Bernier L, Prat A. (2004). Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 24:1454–1459.

Endo A. (1992). The discovery and development of HMG-CoA reductase inhibitors. *J Lipid Res* 33:1569–1582.

Folch J, Lees M, Sloane Stanley GH. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226:497–509.

Goldstein JL, Brown MS. (2009). The LDL receptor. *Arterioscler Thromb Vasc Biol* 29:431–438.

Haffner S, Orchard T, Stein E, Schmidt D, LaBelle P. (1995). Effect of simvastatin on Lp(a) concentrations. *Clin Cardiol* 18:261–267.

Harris JI, Hibbeln JR, Mackey RH, Muldoon MF. (2004). Statin treatment alters serum n-3 and n-6 fatty acids in hypercholesterolemic patients. *Prostaglandins Leukot Essent Fatty Acids* 71:263–269.

Hodson L, Skeaff CM, Fielding BA. (2008). Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Prog Lipid Res* 47:348–380.

Horton JD, Cohen JC, Hobbs HH. (2007). Molecular biology of PCSK9: its role in LDL metabolism. *Trends Biochem Sci* 32:71–77.

Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, Goldstein JL. (2003). Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc Natl Acad Sci USA* 100:12027–12032.

Hrboticky N, Tang L, Zimmer B, Lux I, Weber PC. (1994). Lovastatin increases arachidonic acid levels and stimulates thromboxane synthesis in human liver and monocytic cell lines. *J Clin Invest* 93:195–203.

Isley WL, Miles JM, Patterson BW, Harris WS. (2006). The effect of high-dose simvastatin on triglyceride-rich lipoprotein metabolism in patients with type 2 diabetes mellitus. *J Lipid Res* 47:193–200.

Jula A, Marniemi J, Rönnemaa T, Virtanen A, Huupponen R. (2005). Effects of diet and simvastatin on fatty acid composition in hypercholesterolemic men: a randomized controlled trial. *Arterioscler Thromb Vasc Biol* 25:1952–1959.

Kaddurah-Daouk R, Baillie RA, Zhu H, Zeng ZB, Wiest MM, Nguyen UT, Watkins SM, Krauss RM. (2010). Lipidomic analysis of variation in response to simvastatin in the Cholesterol and Pharmacogenetics Study. *Metabolomics* 6:191–201.

Kobayashi J, Maruyama T, Masuda M, Shinomiya M. (2001). Effect of atorvastatin treatment on lipoprotein lipase mass in the pre-heparin plasma in Japanese hyperlipidemic subjects. *Clin Chim Acta* 314:261–264.

Labreuche J, Touboul PJ, Amarenco P. (2009). Plasma triglyceride levels and risk of stroke and carotid atherosclerosis: a systematic review of the epidemiological studies. *Atherosclerosis* 203:331–345.

Lakoski SG, Lagace TA, Cohen JC, Horton JD, Hobbs HH. (2009). Genetic and metabolic determinants of plasma PCSK9 levels. *J Clin Endocrinol Metab* 94:2537–2543.

Lambert G, Charlton F, Rye KA, Piper DE. (2009). Molecular basis of PCSK9 function. *Atherosclerosis* 203:1–7.

Macy EM, Hayes TE, Tracy RP. (1997). Variability in the measurement of C-reactive protein in healthy subjects: implications for reference intervals and epidemiological applications. *Clin Chem* 43:52–58.

Maron DJ, Fazio S, Linton MF. (2000). Current perspectives on statins. *Circulation* 101:207–213.

Mead JR, Irvine SA, Ramji DP. (2002). Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med* 80:753–769.

Mehrotra DV, Heyse JF. (2004). Use of the false discovery rate for evaluating clinical safety data. *Stat Methods Med Res* 13:227–238.

Nakamura MT, Nara TY. (2004). Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. *Annu Rev Nutr* 24:345–376.

Nordøy A, Hansen JB, Brox J, Svensson B. (2001). Effects of atorvastatin and omega-3 fatty acids on LDL subfractions and postprandial hyperlipemia in patients with combined hyperlipemia. *Nutr Metab Cardiovasc Dis* 11:7–16.

Perret B, Mabile L, Martinez L, Tercé F, Barbaras R, Collet X. (2002). Hepatic lipase: structure/function relationship, synthesis, and regulation. *J Lipid Res* 43:1163–1169.

Piorunska-Stolzmann M, Piorunska-Mikolajczak A, Mikolajczyk Z. (2003). Effect of simvastatin on trioleylglycerol hydrolysis and transacylation with cholesterol in serum of outpatients with coronary heart disease. *Drugs Exp Clin Res* 29:37–43.

Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). (1994). *Lancet* 344:1383–1389.

Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, Macfadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, Glynn RJ; JUPITER Trial Study Group. (2009). Reduction in C-reactive protein and LDL cholesterol and cardiovascular event rates after initiation of rosuvastatin: a prospective study of the JUPITER trial. *Lancet* 373:1175–1182.

Ridker PM, Fonseca FA, Genest J, Gotto AM, Kastelein JJ, Khurmi NS, Koenig W, Libby P, Lorenzatti AJ, Nordestgaard BG, Shepherd J, Willerson JT, Glynn RJ; JUPITER Trial Study Group. (2007). Baseline characteristics of participants in the JUPITER trial, a randomized placebo-controlled primary prevention trial of statin therapy among individuals with low low-density lipoprotein cholesterol and elevated high-sensitivity C-reactive protein. *Am J Cardiol* 100:1659–1664.

Risé P, Colombo C, Galli C. (1997). Effects of simvastatin on the metabolism of polyunsaturated fatty acids and on glycerolipid, cholesterol, and *de novo* lipid synthesis in THP-1 cells. *J Lipid Res* 38:1299–1307.

Risé P, Ghezzi S, Galli C. (2003). Relative potencies of statins in reducing cholesterol synthesis and enhancing linoleic acid metabolism. *Eur J Pharmacol* 467:73–75.

Risé P, Pazzucconi F, Sirtori CR, Galli C. (2001). Statins enhance arachidonic acid synthesis in hypercholesterolemic patients. *Nutr Metab Cardiovasc Dis* 11:88–94.

Schneider JG, von Eynatten M, Parhofer KG, Volkmer JE, Schiekofer S, Hamann A, Nawroth PP, Dugi KA. (2004). Atorvastatin improves diabetic dyslipidemia and increases lipoprotein lipase activity *in vivo*. *Atherosclerosis* 175:325–331.

Schönbeck U, Libby P. (2004). Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? *Circulation* 109:II18–II26.

Sparrow CP, Burton CA, Hernandez M, Mundt S, Hassing H, Patel S, Rosa R, Hermanowski-Vosatka A, Wang PR, Zhang D, Peterson L, Detmers PA, Chao YS, Wright SD. (2001). Simvastatin has anti-inflammatory and antiatherosclerotic activities independent of plasma cholesterol lowering. *Arterioscler Thromb Vasc Biol* 21:115–121.

Superko HR, King S 3rd. (2008). Lipid management to reduce cardiovascular risk: a new strategy is required. *Circulation* 117:560–568; discussion 568.

The effect of aggressive versus standard lipid lowering by atorvastatin on diabetic dyslipidemia: the DALI study: a double-blind, randomized, placebo-controlled trial in patients with type 2 diabetes and diabetic dyslipidemia. (2001). *Diabetes Care* 24:1335–1341.

Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. (2002). *Circulation* 106:3143–3421.

Toutouzas K, Drakopoulou M, Skoumas I, Stefanadis C. (2010). Advancing therapy for hypercholesterolemia. *Expert Opin Pharmacother* 11:1659–1672.

Wang Y, Botolin D, Xu J, Christian B, Mitchell E, Jayaprakasam B, Nair MG, Nair M, Peters JM, Peters JM, Busik JV, Busik J, Olson LK, Jump DB. (2006). Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. *J Lipid Res* 47:2028–2041.

Weiss EP, Fields DA, Mittendorfer B, Haverkort MA, Klein S. (2008). Reproducibility of postprandial lipemia tests and validity of an abbreviated 4-hour test. *Metab Clin Exp* 57:1479–1485.

Wenk MR. (2005). The emerging field of lipidomics. *Nat Rev Drug Discov* 4:594–610.

Williams ML, Menon GK, Hanley KP. (1992). HMG-CoA reductase inhibitors perturb fatty acid metabolism and induce peroxisomes in keratinocytes. *J Lipid Res* 33:193–208.

Zambon A, Deeb SS, Brown BG, Hokanson JE, Brunzell JD. (2001). Common hepatic lipase gene promoter variant determines clinical response to intensive lipid-lowering treatment. *Circulation* 103:792–798.