Journal of Clinical Lipidology (2015) 9, 807–816

Journal of Clinical Lipidology

Biomarkers of cholesterol homeostasis in a clinical laboratory database sample comprising 667,718 patients

Thomas D. Dayspring, MD, FACP, FNLA*, Stephen A. Varvel, PhD, Leila Ghaedi, PhD, Dawn L. Thiselton, PhD, James Bruton, MS, Joseph P. McConnell, PhD, DABCC

Clinical Education Department, Foundation for Health Improvement and Technology (FHIT), Richmond, VA, USA (Dr Dayspring); and Clinical Affairs Department, Health Diagnostic Laboratory, Inc., Richmond, VA, USA (Drs Dayspring, Varvel, Ghaedi, Thiselton, Bruton, McConnell)

KEYWORDS:
Cholesterol homeostasis;
Sitosterol;
Campesterol;
Cholestanol;
Desmosterol;
APOE genotype

BACKGROUND: Circulating noncholesterol sterols/stanols (NCS) are used in clinical lipidology as surrogate measures of cholesterol synthesis and absorption, where they can be valuable tools in assessing cholesterol metabolism and personalizing therapies in patients with dyslipidemia.

OBJECTIVES: To describe the distributions of plasma NCS concentrations and inter-NCS correlations in a large cohort of American patients constituting a clinical laboratory database, and to investigate the relationship between circulating NCS, age, sex, and apolipoprotein E (*APOE*) genotype.

METHODS: A total of 667,718 patient blood samples submitted for testing to Health Diagnostic Laboratory, Inc. (Richmond, VA) were analyzed for cholesterol absorption markers (sitosterol, campesterol, and cholestanol) and one cholesterol synthesis marker (desmosterol). NCS percentiles were determined, along with intermarker correlations (Pearson's *R*). Analysis of variance was used to assess the effect of age and sex on NCS level, and to evaluate the relationship between cholesterol synthesis/absorption status and *APOE* genotype in a subset of 336,866 patients.

RESULTS: Mean NCS concentrations were: sitosterol, 2.45 μg/mL; campesterol, 3.3 μg/mL; cholestanol, 2.92 μg/mL; and desmosterol 0.99 μg/mL. The correlations between each NCS and its ratio to total cholesterol ranged from 0.72 (cholestanol) to 0.94 (desmosterol). NCS levels were significantly affected by age and sex ($P < .0001$), and prevalence of cholesterol hyperabsorption was higher in *APOE* ε4 allele carriers compared with the other *APOE* genotypes.

CONCLUSIONS: We have described sample distributions of NCS biomarkers and characterized their relationship to age, sex, and *APOE* genotype. These data may facilitate research into altered cholesterol homeostasis and human disease, and help physicians optimize lipid-lowering therapies.

Introduction

Sterols consist of a family of aromatic triterpine molecules found in plants (phytosterols) and animals (zoosterols). They perform a variety of functions, modulating cell membrane fluidity and influencing cell signaling through their structural role in lipid rafts. Although found

* Corresponding author. Foundation for Health Improvement and Technology (FHIT) and Health Diagnostic Laboratory, 737 N. 5th Street, Richmond, VA 23219, USA.
E-mail address: tdayspring@aol.com
Submitted April 29, 2015. Accepted for publication August 21, 2015.

http://dx.doi.org/10.1016/j.jacl.2015.08.003

in some plants, cholesterol is predominantly a zoosterol and serves as a precursor for steroid hormones and bile acids in humans. However, as excess cellular cholesterol may crystallize and lead to cell death, cholesterol homeostasis within the body is a tightly regulated balance between endogenous synthesis, exogenous (dietary) absorption, and excretion (bile acids or biliary cholesterol).[1,2]

The term noncholesterol sterols, or xenosterols, encompasses all sterols that are structurally similar to cholesterol, including phytosterols (eg, campesterol and sitosterol, which differ by methyl and ethyl groups at position C-24; Fig. 1), and several cyclical precursor molecules. More than 20 enzymes are used throughout the two cholesterol synthesis pathways, leading to either lathosterol or desmosterol, but because of a cholesterol "metabolon" there is much interaction between the two pathways.[3] Phytosterols cannot be synthesized by humans and are always derived from the diet.[4] Overall intestinal absorption of free cholesterol is regulated by an interplay between the enterocyte- and hepatobiliary-located sterol influx membrane transporter, Niemann-Pick C1-Like 1 (NPC1L1), and a heterodimer consisting of two ATP-binding cassette (ABC) efflux transporters, subfamily members 5 and 8 (ABCG5 and ABCG8, respectively).[2] The latter are found on the apical surface of enterocytes and the hepatobiliary interface, and are responsible not only for efflux of excess cholesterol into the gut lumen and bile to avoid cellular injury[1] but also for preventing the bodily accumulation of dietary phytosterols. High circulating levels of cholesterol, and potentially phytosterols, are thought to play a causal role in atherogenesis, which occurs when sterol-laden apolipoprotein B (apoB)–containing lipoproteins invade the arterial wall, are oxidized, and initiate a maladaptive inflammatory response.[5] Indeed, common genetic variants associated with serum phytosterol levels affect risk for coronary artery disease.[6,7] Stanols are sterols with a saturated double bond at the 5α position (Fig. 1) and are derived either from the diet or as a gut microbial byproduct of cholesterol metabolism; this family includes cholestanol and coprostanol.[8–10] Both phytosterols and stanols compete with cholesterol for absorption, and hence are used as functional foods or supplements in the treatment of hypercholesterolemia.[11]

Measuring plasma cholesterol directly does not reveal whether its origin is cellular synthesis or intestinal absorption. The sterol-binding domains of NPC1L1 and ABCG5/G8 have

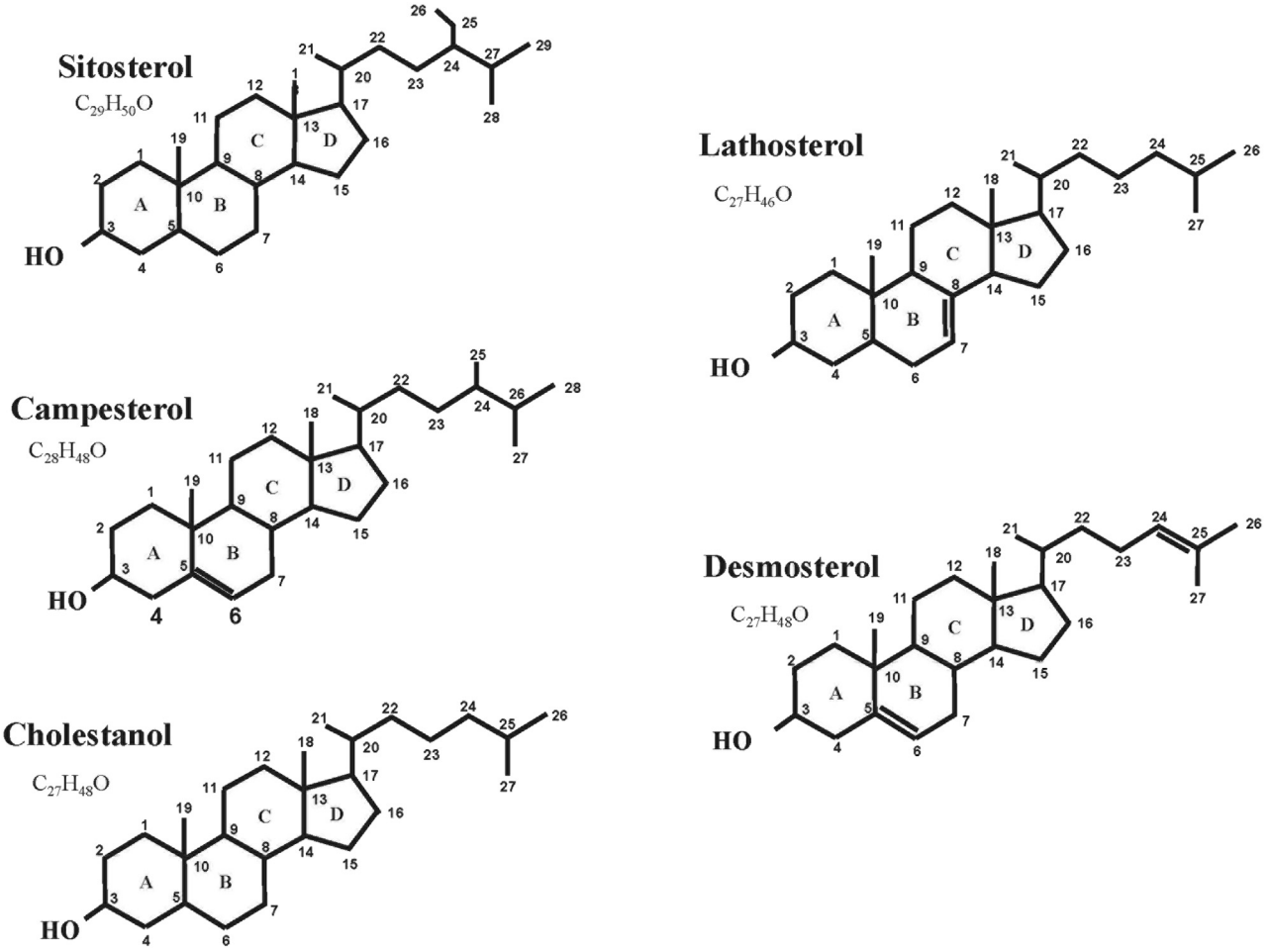

Figure 1 Chemical structures and formulae of the major cholesterol homeostasis biomarkers. With respect to cholesterol, sitosterol and campesterol differ by having ethyl or methyl groups, respectively, at carbon 24; cholestanol has no double bond at Δ5, lathosterol has no double bond at Δ5 but one at Δ7, and desmosterol has a diene at Δ5 and Δ24.

distinct affinities for cholesterol, and each of the phytosterols and stanols. Because they cannot be synthesized in the body and have significantly reduced absorption capability compared with cholesterol, the plasma measurement of noncholesterol sterols/stanols (NCS) can establish whether elevated cholesterol levels are due in part to hyperabsorption, hypersynthesis, or a combination of both or neither.[4,12–16] Serum concentrations of certain NCS have been validated in clinical trials as tools for the diagnosis of genetic NCS disorders, the evaluation of cholesterol homeostasis and cardiovascular risk, and clinical trial assessment of lipid-lowering therapies.[13,17–22] Measurement of NCS is required to diagnose most of the rare lipidoses such as phytosterolemia, cerebrotendinous xanthomatosis, Smith-Lemli-Opitz syndrome, desmosterolosis, and lathosterolosis.[23]

These cholesterol homeostasis biomarkers have been brought to the bedside over recent years, as liquid chromatography and/or mass spectrometry lipidomic assays have replaced the more complex, invasive methods of evaluating cholesterol synthesis and absorption.[22,24–27] Knowledge of an individual's cholesterol absorption and synthesis status can help clinicians better understand how these processes are related to atherosclerosis and how NCS supplements and other drugs modulate cholesterol homeostasis.[28,29] Such information is increasingly used to help guide lipid-lowering strategies and therapeutic lifestyle choices in individual patients.[30] Sitosterol and campesterol are the most commonly tested NCS, along with cholesterol synthesis precursors desmosterol and lathosterol (Fig. 1).[13,14,31] Levels of sitosterol, campesterol, and cholestanol have been evaluated in many studies relating increased cholesterol absorption to cardiovascular risk.[19,20,22] NCS markers reflective of cholesterol synthesis or absorption are reported either as absolute concentrations or as ratios to total cholesterol (TC), the latter to normalize differences caused by variable levels of lipoproteins, in which NCS are trafficked.[18,21] Although the two measures are correlated, and there is scientific evidence to support the predictive value of each, there is also debate as to which should be used in clinical practice.[13,16,18,21,30,32,33]

Various factors are known to influence cholesterol homeostasis, including nutritional, physiological, pharmacological, and genetic factors; for example, specific polymorphisms of the *NPC1L1*,[7] *ABCG5/ABCG8*,[6] or apolipoprotein E (apoE) genes (*APOE*).[18,34] *APOE* has three allele "genotypes"—ε3, ε4, and ε2—encoding 3 protein isoforms (respectively, E2, E3, and E4). The three apoE protein isoforms are defined by the presence or absence of cysteine (Cys) or arginine (Arg) at amino acid positions 112 and 158, in turn determined by the haplotype of two underlying single nucleotide polymorphisms (SNPs) in exon 4 of the *APOE* gene. SNP rs429358 determines the amino acid at 112 and SNP rs7412 at 158. The E2 isoform consists of Cys112/Cys158, the E3 isoform comprises Cys112/Arg158, and E4 is Arg112/Arg158. The three apoE isoforms differentially affect plasma lipid and lipoprotein concentrations as they have different affinities for various membrane receptors and lipases.[35]

Given the increasing interest in using NCS as biomarkers of cholesterol homeostasis in the clinical setting, it is important to establish age- and sex-based norms against which physicians can evaluate their patients. The aims of this study were three fold: first, to describe the concentration distributions of four cholesterol homeostasis biomarkers in a large and unselected cohort of clinical laboratory serum samples collected over the course of 2.5 years; second, to investigate the relationship between these cholesterol homeostasis biomarkers and traditional lipid levels; and third, to establish the relationship between NCS concentrations and *APOE* genotype, along with other demographic variables such as age and sex.

Material and methods

Subjects

This retrospective cross-sectional analysis included data from blood samples submitted for testing to Health Diagnostic Laboratory, Inc (HDL, Inc., Richmond, VA) as part of routine clinical cardiovascular risk assessment between January 2012 and May 2014. Absolute concentrations of cholesterol absorption biomarkers (sitosterol, campesterol, and cholestanol) and the cholesterol synthesis biomarker (desmosterol), their ratios to TC, traditional lipid assessments, and *APOE* genotype (in a subset of patients) were extracted without any linked patient identifiers except age and sex. No family history, medical history, current medications, or vital signs were available. A waiver of informed consent and Health Insurance Portability and Accountability Act authorization requirements for this study (using only deidentified and aggregated laboratory data) was obtained from the Copernicus Group IRB (Research Triangle, NC).

Laboratory methods

Blood samples were drawn after an overnight fast and shipped with cold packs to HDL, Inc for biomarker testing. Samples were prepared at each clinical site according to standardized instructions appropriate for specimen type (BD Vacutainer PPT "Pearl Top" with EDTA and gel were used for sterol tests, BD Vacutainer SST "Tiger Top" tubes for traditional lipid tests, and BD Vacutainer K2 Whole Blood "Lavender Top" tubes for *APOE* genotyping), received by HDL, Inc within 24 hours, and tested immediately. For the NCS assays, plasma samples were saponified and extracted, then rapid chromatographic separation was achieved with a water:methanol:acetonitrile gradient using a reversed phase high-performance liquid chromatography column. Detection was performed in positive ion mode on an AB Sciex Triple Quad 5500 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) System. *APOE* genotyping was performed by TaqMan assay (Applied Biosystems Inc, Carlsbad, CA). Low-density lipoprotein

cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured using direct enzymatic assays (Beckman-Coulter Biomedical Ltd, Co Clare, Ireland). Triglyceride assay was performed using standard automated enzymatic methods (Roche Diagnostics, Indianapolis, IN).

Statistical methods

Population distributions and percentile cutpoints were determined for each NCS, along with intermarker correlations (Pearson's *R*). The effects of age and sex on NCS level were assessed by multifactorial analysis of variance (ANOVA). Biomarkers were inspected for normality and were transformed using the natural logarithm as necessary to improve the normality assumption for ANOVA. In the subset of patients with *APOE* genotype results, values for each marker were compared across genotype by ANOVA, with post hoc testing compared with the *APOE* ε3/ε3 group controlled using Dunnett-adjusted *P* values $< .05$ for statistical significance. NCS levels for each patient sample were categorized according to the 25th and 75th percentiles of the large cohort as reflecting hypoabsorption/hyperabsorption or synthesis. Pearson's chi-square test was used to compare incidence of each category according to *APOE* genotype. All analyses were performed using SAS software (version 9.4; SAS Institute).

Results

NCS population distributions and percentile cutpoints

Fasting lipid and NCS data were available for 667,718 patients (mean age 56 ± 15 years, 47% male). The distribution of plasma concentrations for sitosterol, campesterol, cholestanol, and desmosterol are shown in Figure 2, with the dashed lines representing the 25th, 50th, and 75th percentile of each marker. Distributions were not normal (kurtosis for all markers was >17) and are presented in Figure 2 only through the 99th percentile, as each marker had a long tail extending at least 30 standard deviations above the mean. The mean values and percentile cutpoints for each marker, and their respective ratios with TC, are presented in Table 1. The absolute mean concentrations in μg/mL were as follows: sitosterol, 2.45 (interquartile range [IQR]: 1.56–3.01); campesterol, 3.3 (IQR: 2.10–4.12); cholestanol, 2.92 (IQR: 2.23–3.42); and desmosterol, 0.99 (IQR: 0.58–1.18). The 99th percentile cutpoint values for the absorption markers (μg/mL) were as follows: sitosterol ≥6.94, campesterol ≥9.49, and cholestanol ≥6.05. We identified 18 patients with sitosterol values >30 μg/mL; these individuals most likely have significant loss of function of ABCG5 or ABCG8, with an incidence in this sample of 0.0027% (ie, 3 per 100,000). Furthermore, the 99th percentile cutpoint for desmosterol (used to support a diagnosis of desmosterolosis) was ≥2.83 μg/mL in this cohort.

Relationships between different cholesterol homeostasis markers

As shown in Table 2, sitosterol and campesterol were highly correlated ($R = 0.90$, $P < .0001$), sitosterol and cholestanol slightly less so ($R = 0.63$, $P < .0001$), and sitosterol and desmosterol hardly at all ($R = 0.01$, $P < .05$). Interestingly, cholestanol was more strongly correlated with TC ($R = 0.56$), LDL-C ($R = 0.45$), and HDL-C ($R = 0.39$) than were either of the sterol absorption markers or desmosterol. Reflecting the imperfect correlation between concentrations of the cholesterol absorption markers, only 13% of the cohort was above the 75th percentile of all three, whereas 38% of the cohort was above the 75th percentile of any one. The pairwise

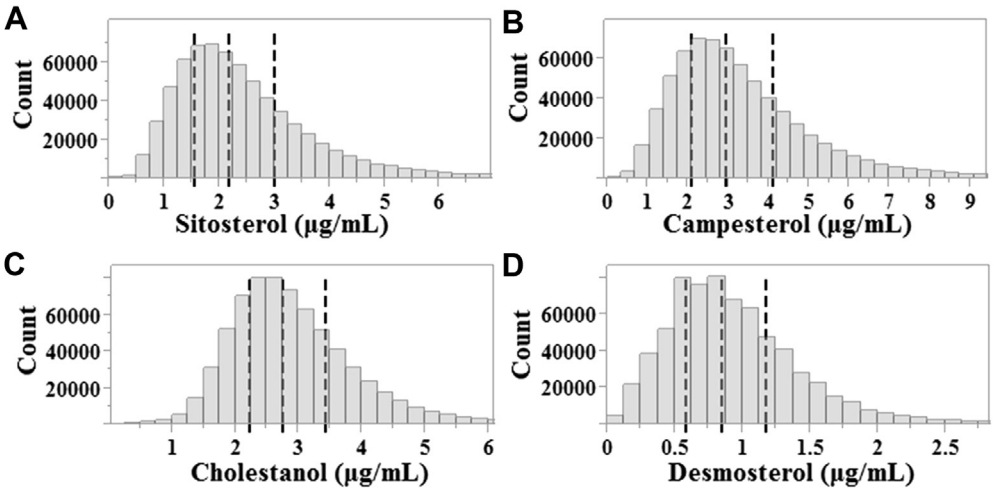

Figure 2 Distributions of assessed noncholesterol sterols/stanols through the 99th percentile. (A) Sitosterol, (B) campesterol, (C) cholestanol, (D) desmosterol. Vertical dotted lines indicate the 25th, 50th, and 75th percentile cutpoints (N = 667,718).

Table 1 Percentile cutpoints for noncholesterol sterols/stanols and their ratios to total cholesterol (N = 667,718)

Biomarker	Mean (SD)	Minimum	1st	25th	50th	75th	99th	Maximum
Sitosterol	2.45 (1.39)	0.10	0.66	1.56	2.18	3.01	6.94	165.60
Sitosterol/TC	129.16 (73.96)	0.00	37.00	83.00	114.60	158.00	367.00	13,119.00
Campesterol	3.33 (1.83)	0.18	0.81	2.10	2.96	4.12	9.49	112.80
Campesterol/TC	181.23 (98.68)	0.00	49.00	115.00	160.50	222.90	520.00	6256.30
Cholestanol	2.92 (1.00)	0.28	1.26	2.23	2.76	3.42	6.05	40.37
Cholestanol/TC	161.90 (46.06)	0.00	83.00	130.30	155.00	186.00	304.00	3355.00
Desmosterol	0.99 (1.24)	0.06	0.14	0.58	0.85	1.18	2.83	127.40
Desmosterol/TC	54.37 (71.91)	0.00	9.50	36.00	48.00	62.00	139.00	8056.00
TC	181.41 (43.38)	37.00	99.00	151.00	178.00	207.00	301.00	1386.00
LDL-C	108.00 (36.32)	9.00	41.00	82.00	105.00	130.00	207.00	628.00
HDL-C	56.39 (16.94)	3.00	29.00	44.00	53.00	65.00	110.00	216.00

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SD, standard deviation; TC, total cholesterol.

correlations between each cholesterol homeostasis marker and its ratio to TC were as follows: 0.89 ($P < .0001$) for sitosterol vs sitosterol/TC ratio, 0.89 ($P < .0001$) for campesterol vs campesterol/TC ratio, 0.72 ($P < .0001$) for cholestanol vs cholestanol/TC ratio, and 0.94 ($P < .0001$) for desmosterol vs desmosterol/TC ratio (Table 2).

Effect of age and sex on circulating NCS levels

Levels of all four NCS markers were significantly affected by both age and sex, as shown in Figure 3 ($P < .0001$). Multifactorial ANOVA found significant age × sex interactions for sitosterol (F = 161, $P < .0001$), campesterol (F = 218, $P < .0001$), cholestanol (F = 1623, $P < .0001$), and desmosterol (F = 807, $P < .0001$). In women, levels of all three absorption markers increased significantly with advancing age—most notably during the fifth decade—while desmosterol levels tended to increase slightly from the third to fifth decade and decline thereafter. These trends may be at least partly due to the shift toward cholesterol absorption vs synthesis that begins in women in the third and/or fourth decade and peaks at or just after the fifth decade, when most women experience menopause. In contrast, despite age-related increases in cholesterol absorption markers during the third decade (most pronounced for sitosterol, which continued to increase through the fourth decade), levels of all four markers declined significantly with advancing age in men.

For a subset of the cohort described previously (n = 336,866), *APOE* genotype data were available. Table 3 summarizes the mean concentrations of NCS markers according to *APOE* genotype and the observed frequency of each genotype. The mean age of this subgroup was 54 ± 16 years, of whom 56% were women. The *APOE* genotype distribution was 1% 2/2, 11% 2/3, 2% 2/4, 61% 3/3, 23% 3/4, and 2% 4/4. In general, carriers of the *APOE* ε2 allele (ie, *APOE* genotypes 2/2, 2/3, and 2/4) tended to have lower levels of absorption markers compared with the *APOE* ε3 homozygotes, whereas carriers of the *APOE* ε4 allele (ie, *APOE* genotypes 3/4 and 4/4) tended to have higher levels. The proportions of cholesterol hyper- (above the 75th percentile) and hypo-absorbers (below the 25th percentile) or -synthesizers are shown for each *APOE* genotype in Figure 4. The percentage of patients who were hyperabsorbers (defined by increased sitosterol, campesterol, and cholestanol) was higher for the *APOE* 3/4 and 4/4 genotype groups (and the percentage of those with hypoabsorption was correspondingly lower) than that for the *APOE* 3/3 genotype, whereas the reverse was true

Table 2 Relationships between levels of cholesterol absorption/synthesis markers and traditional cholesterol measures

Biomarker	Sitosterol	Campesterol	Cholestanol	Desmosterol	TC	LDL-C	HDL-C
Sitosterol	1.00						
Campesterol	0.90	1.00					
Cholestanol	0.63	0.65	1.00				
Desmosterol	0.004*	0.003*	0.05	1.00			
TC	0.24	0.27	0.56	0.21	1.00		
LDL-C	0.17	0.20	0.45	0.19	0.86	1.00	
HDL-C	0.27	0.23	0.39	0.02	0.37	0.09	1.00

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol.
Pearson's correlations (*R*) determined by pairwise correlation. All correlations were statistically significant, $P < .0001$, except for $^*P < .05$.

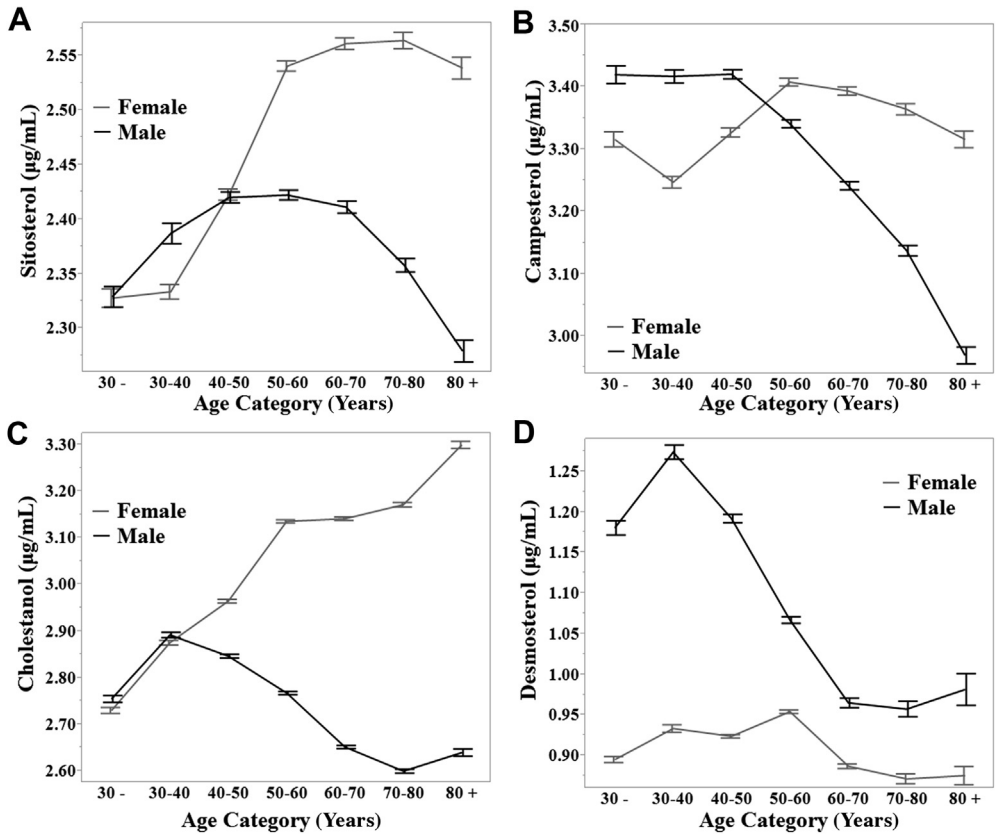

Figure 3 Effect of age and sex (in 10-year increments from age 30 to 80 years) on noncholesterol sterol/stanol concentrations, N = 667,718. (A) Sitosterol, (B) campesterol, (C) cholestanol, (D) desmosterol.

for the *APOE* ε2 allele carriers. These differences between the *APOE* genotype groups were similar, but much less pronounced, for desmosterol.

Discussion

Clinical testing of NCS markers of cholesterol homeostasis has been increasing in recent years, allowing this descriptive presentation of distributions and percentiles from a very large clinical laboratory cohort in the setting of routine clinical care (Table 1). As this random sample of our clinical laboratory cohort consists entirely of patients of providers who deal with cardiometabolic issues and order beyond typical laboratory testing, caution should be used when generalizing these results to the "apparently healthy" US population. However, in this study, the mean circulating NCS values were broadly consistent with those of other studies,[17,18,25,36,37] whereas the sheer sample size—to our knowledge, the largest to date reporting NCS distributions—allowed us to generate percentile cutpoints, which may serve as a potential population reference to facilitate research into altered cholesterol homeostasis and human disease. Although routine use of such testing is not advocated in the general population, these data may help physicians select the most optimal lipid-lowering therapies for their patients by enabling prompt recognition of individuals with hyper- or hypo-absorption or -synthesis. It may also

Table 3 Patient characteristics and noncholesterol sterol/stanol levels by *APOE* genotype

APOE genotype	2/2	2/3	2/4	3/3	3/4	4/4
N (% of total)	2013 (0.6)	37,258 (11.1)	7555 (2.2)	205,452 (61.0)	76,815 (22.8)	7773 (2.3)
Sex (% F)	56	56	56	56	56	57
Age, y	54.1 (16.0)	53.8 (15.8)*	53.3 (15.5)	53.3 (15.8)	52.9 (15.5)*	52.0 (15.9)*
Sitosterol	2.28 (1.21)*	2.28 (1.22)*	2.39 (1.26)*	2.43 (1.45)	2.52 (1.34)*	2.67 (1.47)*
Campesterol	3.25 (1.77)	3.12 (1.66)*	3.29 (1.78)	3.30 (1.80)	3.43 (1.84)*	3.61 (1.99)*
Cholestanol	2.83 (1.09)*	2.79 (0.97)*	2.92 (0.97)	2.94 (0.99)	3.06 (1.05)*	3.20 (1.10)*
Desmosterol	1.05 (1.65)	1.01 (1.22)*	1.01 (0.98)*	1.06 (1.14)	1.07 (1.14)*	1.06 (0.99)

F, female.

*Significant differences compared with *APOE* 3/3 genotype adjusted for age and sex, $P < .0001$ (N = 336,866).

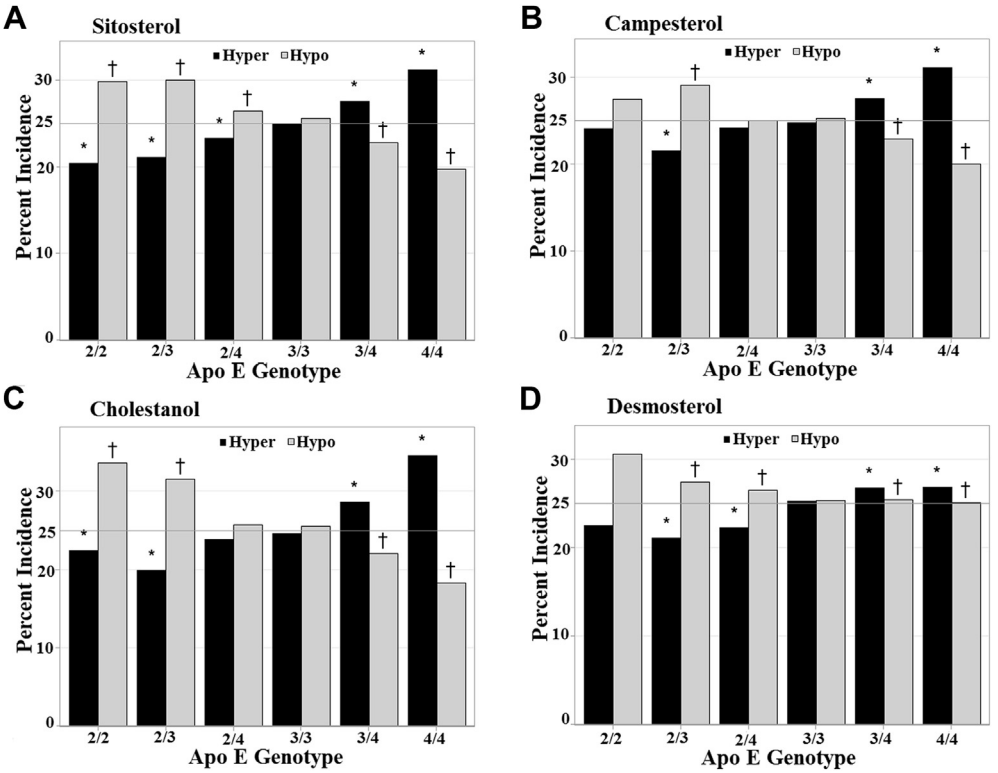

Figure 4 Prevalence of hypoabsorption (below 25th percentile) or hyperabsorption (above 75th percentile) or synthesis according to *APOE* genotype, as indicated by noncholesterol sterol/stanol levels (A) sitosterol, (B) campesterol, (C) cholestanol, (D) desmosterol. Asterisks and daggers indicate significant differences compared to *APOE* 3/3 genotype, $P < .001$.

help in detecting statin-induced compensatory increases in sterol absorption that could inform decisions about multitherapy options directed at reducing absorption (eg, ezetimibe, phytosterol esters, stanol esters, probiotics, or other functional food supplements).[38–40]

We measured both absolute NCS concentrations and their ratios to TC in this study, to explore differences and relationships between the alternate metrics. The absolute measures were strongly but variably correlated to their corresponding ratios to TC, although the imperfect correlations among absolute levels of the cholesterol absorption markers highlights the increased sensitivity afforded by assessing more than one.[41] In this era of widespread statin use, although the ratio is the most commonly used NCS measure, it may not be the best indicator of cholesterol absorption and/or synthesis because TC can change—due to variations in LDL-C—even when there is no change in the absolute concentration of the NCS. Indeed, van Himbergen et al (2009) advise using the ratios to demonstrate the biomarkers are independent of cholesterol when analyzing absorption and/or synthesis differences between 2 groups, but using absolute levels of the biomarkers when investigating relationships between the absorption and/or synthesis markers and cholesterol, to avoid masking the variable TC and LDL-C that is of primary interest.[32] Others have suggested using an absorption-to-synthesis marker ratio, such as phytosterol:lathosterol or phytosterol:desmosterol, as a tracer of cholesterol absorption.[42] More recently, given that NCS marker levels can be affected by common factors such as diet, metabolic disease (diabetes), and drugs (statins), it has been recommended that measuring multiple NCS biomarkers, with inclusion of at least one absolute concentration and serial testing to establish individual setpoints, is the best approach for the evaluation of cholesterol homeostasis.[16,30]

Patients with absolute NCS markers above 99th percentile readings almost certainly have a genetic issue at play (eg, variable loss of function of ABCG5 or ABCG8), and our database identified more than 6000 individuals in this clinical laboratory cohort with such markedly elevated concentrations. The long-term implications are not known, unless the absorption markers enter into the currently accepted phytosterolemic range (eg, sitosterol >100–300 μg/mL).[43,44] Of particular interest are those individuals with 99th percentile concentrations of desmosterol. No known clinical entity has been described in such patients, but abnormal accumulation of desmosterol underlies many of the homeostatic, including inflammatory responses, of foam cells and hepatic Kupffer cells and is also related to hepatitis C replication, fatty liver disease, and diabetes.[45,46] It is also worth noting that the cholesterol synthesis inhibitor triparanol, formerly used to reduce total and LDL-C, but which blocks the conversion of desmosterol to cholesterol, has been shown to cause significant desmosterolemia, associated with cataracts, alopecia, and accelerated atherosclerosis.[47] In general, patients with

insulin resistance, metabolic syndrome, or excess visceral fat tend to have significantly elevated markers of cholesterol synthesis driven by hyperinsulinemia.[21,48] On the other hand, reduced serum desmosterol correlates with cerebrospinal fluid desmosterol and has been validated as a potential biomarker of Alzheimer's disease.[45,49]

Our findings further suggest that age, sex, and *APOE* genotype are important considerations for the clinical interpretation of NCS values. Women showed increased cholesterol absorption with advancing age (especially after menopause) whereas in men, a slight increase in cholesterol absorption toward middle age was followed be a decline thereafter in both absorption and synthesis. The decline in cholesterol synthesis with advancing age has been reported in at least one prior study, although circulating cholesterol levels tend to increase with age.[50] However, prospective analysis of the Framingham Offspring Study cohort over a 10-year period found this to be true for some markers of cholesterol synthesis, but not all.[51] Previous investigations into sex-specific differences in NCS levels have generated conflicting findings.[18] In the Framingham Offspring cohort study, after controlling for standard CVD risk factors, cholesterol synthesis markers were associated with reduced incidence of myocardial infarction and coronary death in women, but higher risk in men.[51] It is interesting in this regard that elevated desmosterol levels have been shown to predict worsening hyperglycemia and 5-year conversion to type 2 diabetes in a male population cohort.[46] Our results suggest that the postmenopausal shift toward cholesterol hyperabsorption may constitute an important and modifiable cardiovascular risk factor for women in their later years.

Data regarding the effect of *APOE* genotype on the efficiency of cholesterol absorption is mixed, but has never been investigated in a database of this enormity.[18,52–56] In this clinical cohort, a subset (n = 136,701) of which we have previously shown has an *APOE* genotype distribution reasonably representative of the US population,[57] we observed the prevalence of hyperabsorption biomarkers to be higher, and that for hypoabsorption to be lower, in those with the *APOE* 3/4 and 4/4 genotypes. Perhaps the decreased LDL receptor–mediated clearance of apoB particles conferred by the *APOE* ε4 allele triggers a reflex hyperabsorption. If true, ezetimibe might be a useful therapy in achieving apoB (LDL-C) goals of therapy.

Numerous studies have evaluated the effect of *APOE* alleles on statin responsiveness, with mixed results. Some have shown decreased statin-induced reductions of LDL-C in those with *APOE* ε4 alleles.[58] One could hypothesize that any statin hyporesponsiveness that may exist in ε4 carriers could be related to the increased incidence of cholesterol hyperabsorption as reported here. Variation in study findings may be at least partly attributable to differences in ethnic composition and medication use of the different populations under study. Nevertheless, monitoring NCS levels for evidence of cholesterol hyperabsorption might therefore be prudent in *APOE* ε4 allele carriers, who are already at heightened risk of coronary artery disease.[59]

The major strengths of our study include the measurement and comparison—using state-of-the-art sterol assay methodology—of both absolute NCS levels and their ratio to cholesterol, along with the sheer size of the cohort analyzed, which has allowed us to establish NCS level percentiles and explore the effect of *APOE* genotype, sex, and gender in a cohort broadly representative of the US population. We acknowledge limitations to this study: there was no direct (invasive) clinical determination of cholesterol absorption or synthesis, only one synthesis sterol biomarker (desmosterol) was analyzed, there was a lack of information on drug use, and patients were not stratified according to clinical diagnosis (eg, diabetes and/or metabolic disease) or menopausal status (women).

In conclusion, the information presented here, from a very large clinical laboratory data set of cholesterol synthesis and absorption biomarkers, may be useful in helping to establish US population percentiles, recognizing the incidence of several genetic lipidoses. Such cutpoints might also aid in selecting therapeutic lifestyles that recommend or do not recommend absorption-related functional foods and in the optimization of statin therapy or cholesterol absorption blocker to achieve desired lipid and/or lipoprotein goals.

Financial disclosures

This work was supported by Health Diagnostic Laboratory, Inc.

T.D.: Employee, Health Diagnostic Laboratory, Inc; Consultant Merck, Consultant, Speaker, AstraZeneca; S.V.: Employee, Health Diagnostic Laboratory, Inc; L.G.: Employee, Health Diagnostic Laboratory, Inc; D.T.: Employee, Health Diagnostic Laboratory, Inc; J.B.: Employee, Health Diagnostic Laboratory, Inc.; M.S.: Employee, Health Diagnostic Laboratory, Inc; and Joseph McConnell: CEO, Health Diagnostic Laboratory, Inc.

References

1. Abela GS, Aziz K. Cholesterol crystals cause mechanical damage to biological membranes: a proposed mechanism of plaque rupture and erosion leading to arterial thrombosis. *Clin Cardiol*. 2005;28:413–420.
2. van der Wulp MY, Verkade HJ, Groen AK. Regulation of cholesterol homeostasis. *Mol Cell Endocrinol*. 2013;368:1–16.
3. Luu W, Hart-Smith G, Sharpe LJ, Brown AJ. The terminal enzymes of cholesterol synthesis, DHCR24 and DHCR7, interact physically and functionally. *J Lipid Res*. 2015;56:888–897.
4. Calpe-Berdiel L, Escola-Gil JC, Blanco-Vaca F. New insights into the molecular actions of plant sterols and stanols in cholesterol metabolism. *Atherosclerosis*. 2009;203:18–31.
5. Shibata N, Glass CK. Macrophages, oxysterols and atherosclerosis. *Circ J*. 2010;74:2045–2051.
6. Teupser D, Baber R, Ceglarek U, et al. Genetic regulation of serum phytosterol levels and risk of coronary artery disease. *Circ Cardiovasc Genet*. 2010;3:331–339.

7. Stitziel NO, Won HH, Morrison AC, et al. Inactivating mutations in NPC1L1 and protection from coronary heart disease. *N Engl J Med*. 2014;371:2072–2082.
8. Gurr MI, Harwood JL, Frayn KN. Lipids in Cellular Structures. Oxford, UK: Blackwell Science; 2002.
9. Nelson DL, Cox MM. Lehninger: Principles of Biochemistry. New York, NY: WH Freeman and Co; 2005.
10. Behrman EJ, Gopalan V. Cholesterol and plants. *J Chem Educ*. 2005; 82:1791–1793.
11. Jones PJ. Dietary agents that target gastrointestinal and hepatic handling of bile acids and cholesterol. *J Clin Lipidol*. 2008;2: S4–s10.
12. Levy E, Spahis S, Sinnett D, et al. Intestinal cholesterol transport proteins: an update and beyond. *Curr Opin Lipidol*. 2007;18: 310–318.
13. Gylling H. Clinical utility of serum markers of cholesterol absorption and synthesis. *Curr Opin Lipidol*. 2014;25:207–212.
14. Miettinen TA, Tilvis RS, Kesaniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am J Epidemiol*. 1990;131:20–31.
15. von Bergmann K, Sudhop T, Lutjohann D. Cholesterol and plant sterol absorption: recent insights. *Am J Cardiol*. 2005;96:10d–14d.
16. Miettinen TA, Gylling H, Nissinen MJ. The role of serum noncholesterol sterols as surrogate markers of absolute cholesterol synthesis and absorption. *Nutr Metab Cardiovasc Dis*. 2011;21: 765–769.
17. Matthan NR, Pencina M, LaRocque JM, et al. Alterations in cholesterol absorption/synthesis markers characterize Framingham offspring study participants with CHD. *J Lipid Res*. 2009;50:1927–1935.
18. Chan YM, Varady KA, Lin Y, et al. Plasma concentrations of plant sterols: physiology and relationship with coronary heart disease. *Nutr Rev*. 2006;64:385–402.
19. Weingartner O, Lutjohann D, Bohm M, Laufs U. Relationship between cholesterol synthesis and intestinal absorption is associated with cardiovascular risk. *Atherosclerosis*. 2010;210:362–365.
20. Weingartner O, Lutjohann D, Vanmierlo T, et al. Markers of enhanced cholesterol absorption are a strong predictor for cardiovascular diseases in patients without diabetes mellitus. *Chem Phys Lipids*. 2011; 164:451–456.
21. Mackay DS, Jones PJ. Plasma noncholesterol sterols: current uses, potential and need for standardization. *Curr Opin Lipidol*. 2012;23:241–247.
22. Mackay D, Jones PJ. Evaluation of methods for the determination of cholesterol absorption and synthesis in humans. *Atherosclerosis*. 2011;218:253–262.
23. Clayton PT. Disorders of cholesterol biosynthesis. *Arch Dis Child*. 1998;78:185–189.
24. Grundy SM, Mok HY. Determination of cholesterol absorption in man by intestinal perfusion. *J Lipid Res*. 1977;18:263–271.
25. Tilvis RS, Miettinen TA. Serum plant sterols and their relation to cholesterol absorption. *Am J Clin Nutr*. 1986;43:92–97.
26. Matthan NR, Lichtenstein AH. Approaches to measuring cholesterol absorption in humans. *Atherosclerosis*. 2004;174:197–205.
27. Honda A, Yamashita K, Miyazaki H, et al. Highly sensitive analysis of sterol profiles in human serum by LC-ESI-MS/MS. *J Lipid Res*. 2008; 49:2063–2073.
28. Hoenig MR, Rolfe BE, Campbell JH. Cholestanol: a serum marker to guide LDL cholesterol-lowering therapy. *Atherosclerosis*. 2006;184: 247–254.
29. Hoenig MR, Walker PJ, Gurnsey C, Beadle K, Johnson L. Markers of cholesterol absorption and synthesis predict the low-density lipoprotein cholesterol response to atorvastatin. *J Cardiovasc Pharmacol*. 2010;56:396–401.
30. Wu AH. Biomarkers for cholesterol absorption and synthesis in hyperlipidemic patients: role for therapeutic selection. *Clin Lab Med*. 2014; 34:157–166 viii.
31. Acimovic J, Rozman D. Steroidal triterpenes of cholesterol synthesis. *Molecules*. 2013;18:4002–4017.
32. van Himbergen TM, Matthan NR, Resteghini NA, et al. Comparison of the effects of maximal dose atorvastatin and rosuvastatin therapy on cholesterol synthesis and absorption markers. *J Lipid Res*. 2009; 50:730–739.
33. Andrade I, Santos L, Ramos F. Advances in analytical methods to study cholesterol metabolism: the determination of serum noncholesterol sterols. *Biomed Chromatogr*. 2013;27:1234–1242.
34. Kesaniemi YA, Ehnholm C, Miettinen TA. Intestinal cholesterol absorption efficiency in man is related to apoprotein E phenotype. *J Clin Invest*. 1987;80:578–581.
35. Nieminen T, Kahonen M, Viiri LE, Gronroos P, Lehtimaki T. Pharmacogenetics of apolipoprotein E gene during lipid-lowering therapy: lipid levels and prevention of coronary heart disease. *Pharmacogenomics*. 2008;9:1475–1486.
36. Berge KE, von Bergmann K, Lutjohann D, et al. Heritability of plasma noncholesterol sterols and relationship to DNA sequence polymorphism in ABCG5 and ABCG8. *J Lipid Res*. 2002;43:486–494.
37. Heinemann T, Axtmann G, von Bergmann K. Comparison of intestinal absorption of cholesterol with different plant sterols in man. *Eur J Clin Invest*. 1993;23:827–831.
38. Plat J, Mackay D, Baumgartner S, Clifton PM, Gylling H, Jones PJ. Progress and prospective of plant sterol and plant stanol research: report of the Maastricht meeting. *Atherosclerosis*. 2012;225: 521–533.
39. Jones ML, Martoni CJ, Prakash S. Cholesterol lowering and inhibition of sterol absorption by Lactobacillus reuteri NCIMB 30242: a randomized controlled trial. *Eur J Clin Nutr*. 2012;66:1234–1241.
40. Wang Y, Yi X, Ghanam K, Zhang S, Zhao T, Zhu X. Berberine decreases cholesterol levels in rats through multiple mechanisms, including inhibition of cholesterol absorption. *Metabolism*. 2014;63: 1167–1177.
41. Hallikainen M, Simonen P, Gylling H. Cholesterol metabolism and serum non-cholesterol sterols: summary of 13 plant stanol ester interventions. *Lipids Health Dis*. 2014;13:72.
42. Cohen JC, Pertsemlidis A, Fahmi S, et al. Multiple rare variants in NPC1L1 associated with reduced sterol absorption and plasma low-density lipoprotein levels. *Proc Natl Acad Sci U S A*. 2006;103: 1810–1815.
43. Salen G, Shefer S, Nguyen L, Ness GC, Tint GS, Shore V. Sitosterolemia. *J Lipid Res*. 1992;33:945–955.
44. Escola-Gil JC, Quesada H, Julve J, Martin-Campos JM, Cedo L, Blanco-Vaca F. Sitosterolemia: diagnosis, investigation, and management. *Curr Atheroscler Rep*. 2014;16:424.
45. Brown AJ, Ikonen E, Olkkonen VM. Cholesterol precursors: more than mere markers of biosynthesis. *Curr Opin Lipidol*. 2014;25: 133–139.
46. Cederberg H, Gylling H, Miettinen TA, et al. Non-cholesterol sterol levels predict hyperglycemia and conversion to type 2 diabetes in Finnish men. *PLoS One*. 2013;8:e67406.
47. Burtis CA, Ashwood ER, Burns DE, editors. Tietz: Textbook of Clinical Chemistry and Molecular Diagnosis. USA: Elsevier, 2012.
48. Lupattelli G, Pirro M, Mannarino MR, et al. Visceral fat positively correlates with cholesterol synthesis in dyslipidaemic patients. *Eur J Clin Invest*. 2012;42:164–170.
49. Sato Y, Suzuki I, Nakamura T, Bernier F, Aoshima K, Oda Y. Identification of a new plasma biomarker of Alzheimer's disease using metabolomics technology. *J Lipid Res*. 2012;53:567–576.
50. Bertolotti M, Mussi C, Pellegrini E, et al. Age-associated alterations in cholesterol homeostasis: evidence from a cross-sectional study in a Northern Italy population. *Clin Interv Aging*. 2014;9: 425–432.
51. Matthan NR, Zhu L, Pencina M, D'Agostino RB, Schaefer EJ, Lichtenstein AH. Sex-specific differences in the predictive value of cholesterol homeostasis markers and 10-year cardiovascular disease event rate in framingham offspring study participants. *J Am Heart Assoc*. 2013;2:e005066.
52. Kempen HJ, de Knijff P, Boomsma DI, van der Voort HA, Gevers Leuven JA, Havekes L. Plasma levels of lathosterol and phytosterols

in relation to age, sex, anthropometric parameters, plasma lipids, and apolipoprotein E phenotype, in 160 Dutch families. *Metabolism*. 1991; 40:604–611.

53. Gylling H, Kuusi T, Vanhanen H, Miettinen TA. Apolipoprotein E phenotype and cholesterol metabolism in familial hypercholesterolemia. *Atherosclerosis*. 1989;80:27–32.
54. Santosa S, Varady KA, AbuMweis S, Jones PJ. Physiological and therapeutic factors affecting cholesterol metabolism: does a reciprocal relationship between cholesterol absorption and synthesis really exist? *Life Sci*. 2007;80:505–514.
55. Cofan M, Escurriol V, Garcia-Otin AL, et al. Association of plasma markers of cholesterol homeostasis with metabolic syndrome components. A cross-sectional study. *Nutr Metab Cardiovasc Dis*. 2011;21:651–657.
56. Escurriol V, Cofan M, Moreno-Iribas C, et al. Phytosterol plasma concentrations and coronary heart disease in the prospective Spanish EPIC cohort. *J Lipid Res*. 2010;51:618–624.
57. Harris WS, Pottala JV, Thiselton DL, et al. Does APOE genotype modify the relations between serum lipid and erythrocyte omega-3 fatty acid levels? *J Cardiovasc Transl Res*. 2014;7: 526–532.
58. Zintzaras E, Kitsios GD, Triposkiadis F, Lau J, Raman G. APOE gene polymorphisms and response to statin therapy. *Pharmacogenomics J*. 2009;9:248–257.
59. Bennet AM, Di Angelantonio E, Ye Z, et al. Association of apolipoprotein E genotypes with lipid levels and coronary risk. *JAMA*. 2007;298:1300–1311.