Elevated concentrations of circulating vitamin E in carriers of the apolipoprotein A5 gene -1131T>C variant and associations with plasma lipids and lipid peroxidation

Isabella Sundl¹*, Montse Guardiola²*, Gholamali Khoschsorur³, Rosa Solà², Joan C. Vallvé², Gemma Godàs², Lluís Masana², Michaela Maritschnegg¹, Andreas Meinitzer³, Nicolas Cardinault⁴, Johannes M. Roob⁵, Edmond Rock⁴, Brigitte M. Winklhofer-Roob¹, Josep Ribalta².

¹ Human Nutrition & Metabolism Research and Training Center, Institute of Molecular Biosciences, Karl-Franzens University, Graz, Austria
² Unitat de Recerca en Lípids i Arteriosclerosi, Facultat de Medicina, Hospital Universitari de Sant Joan de Reus, Universitat Rovira i Virgili, Institut de Recerca en Ciències de la Salut, Reus, Spain
³ Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University, Graz, Austria
⁴ UMMM, INRA-Theix, St. Genes Champanelle, France
⁵ Division of Clinical Nephrology and Hemodialysis, Department of Internal Medicine, Medical University, Graz, Austria.

* Isabella Sundl and Montse Guardiola have contributed equally to this paper.

Running head: APOA5 gene and vitamin E status

Correspondence:
Josep Ribalta, PhD
Unitat de Recerca de Lípids i Arteriosclerosi,
Facultat de Medicina, Universitat Rovira i Virgili
Sant Llorenç, 21, 43201 Reus (Spain)
Tel.+34 977 759367, Fax.+34 977 759322
E-mail. josep.ribalta@urv.cat
List of abbreviations:

apo, apolipoprotein
apo A-V, apolipoprotein A5 (as referred to the protein)
APOA5, apolipoprotein A5 (as referred to the gene)
α-TTP, α-tocopherol transfer protein
BMC, buccal mucosa cells
BMI, body mass index
EWP, Ernährungswissenschaftliches Programm
IDL, intermediate density lipoprotein
LPL, lipoprotein lipase
MDA, malondialdehyde
MUFA, monounsaturated fatty acids
PLTP, Phospholipid Transfer Protein
SFA, saturated fatty acids
TG, triglyceride
ABSTRACT

The aim of this study was to investigate the effects of the apolipoprotein A5 (APOA5) 1131T>C gene variant on vitamin E status and lipid profile. The gene variant was determined in 297 healthy non-smoking men aged 20-75 years and recruited in the VITAGE-project. Effects of the genotype on vitamin E in plasma, low density lipoproteins (LDL) and buccal mucosal cells (BMC) as well as on cholesterol and triglyceride concentrations in plasma and lipoproteins, apo A-I, B, E, C-III and plasma fatty acids were determined. Plasma malondialdehyde concentrations as a marker of \textit{in vivo} lipid peroxidation were determined. C allele carriers showed significantly higher triglycerides, VLDL and LDL in plasma, higher cholesterol in VLDL and intermediate density lipoprotein (IDL), as well as higher plasma fatty acids. Plasma \(\alpha\)-tocopherol (but neither \(\gamma\)-tocopherol, LDL \(\alpha\)- and \(\gamma\)-tocopherol, nor BMC total vitamin E) was significantly increased in C allele carriers as compared to homozygote T allele carriers (\(P=0.02\)), but not after adjustment for cholesterol or triglycerides. Plasma malondialdehyde concentrations did not differ between genotypes. In conclusion, higher plasma lipids in the TC+CC genotype are efficiently protected against lipid peroxidation by higher \(\alpha\)-tocopherol concentrations. Lipid-standardized vitamin E should be used for reliably assessing vitamin E status in genetic association studies.

Key words: APOA5 – polymorphism – SNP – \(\alpha\)-tocopherol – \(\gamma\)-tocopherol – triglycerides – cholesterol – plasma lipids – plasma fatty acids – lipoproteins
INTRODUCTION

Apolipoprotein A5 (APOA5) is located near the region of the APOA1/C3/A4 gene cluster in chromosome 11 involved in the regulation of triglyceride (TG) metabolism. The role APOA5 plays in such regulation has been extensively demonstrated in genetically modified animal models (1-3) and in a large number of association studies (4-10). Mice overexpressing the APOA5 gene have 65% lower plasma TG whereas the APOA5 knock-out mouse develops about 4-fold higher TG concentrations (1). Association studies using different APOA5 markers have clearly shown that apolipoprotein (apo) A-V, despite its low plasma concentration (11), is probably the strongest determinant of circulating TG concentrations (4-10). The most frequently analyzed variant is -1131T>C and the C allele has been consistently associated with higher TG levels (4-10). The exact function of apo A-V is not known although in vitro evidence shows that it may control plasma TG by down-regulating hepatic VLDL synthesis and stimulating lipoprotein lipase (LPL) activity (1,12).

Circulating TG are transported within lipoproteins together with other lipophilic compounds such as vitamin E (13). Consequently triglyceride concentrations are closely related to vitamin E concentrations resulting in higher plasma vitamin E concentrations in hyperlipidemic subjects compared to normolipidemic subjects when not corrected for plasma lipids (14).

Vitamin E is an essential fat-soluble nutrient comprising 8 different isoforms including α-, β-, γ- and δ-tocopherols as well as the respective tocotrienols which differ both in number and position of methyl groups on the chroman ring and in having a saturated (tocopherols) or unsaturated side chain (tocotrienols). Together with dietary lipids all of these vitamin E isoforms are absorbed equally from the gastrointestinal tract and secreted into chylomicrons. In the liver the α-tocopherol transfer protein (α-TTP) preferentially recognizes RRR-α-tocopherol for incorporation into lipoproteins while the other vitamin E forms are excreted into bile and metabolized and excreted
into urine (15,16). Consequently α-tocopherol which also shows the highest biologic activity is the major form of vitamin E in human plasma and tissue.

The best known function of vitamin E relates to its capacity to scavenge reactive oxygen species and thus acts as a chain breaking antioxidant inhibiting lipid peroxidation. As an example, α-tocopherol inhibits LDL oxidation initiated by copper ions in an ex vivo model (17). Beside these antioxidant actions vitamin E also possesses anti-inflammatory functions such as inhibition of platelet adhesion, inhibition of monocyte proatherogenic activity and improvement of endothelial functions (18).

Transfer of circulating vitamin E from lipoproteins into tissue is regulated by mechanisms also controlling lipid metabolism. The enzyme LPL is able to transfer tocopherols during triglyceride hydrolysis to extrahepatic tissues (19), while the Phospholipid Transfer Protein (PLTP) is exchanging α-tocopherol between different lipoprotein classes (20). Furthermore vitamin E transported in LDL is delivered to cells via a receptor-mediated uptake of LDL (21). Consequently, lipid, lipoprotein and vitamin E concentrations correlate strongly with each other, indicating that their metabolic pathways are tightly linked. Therefore, it is conceivable that environmental and genetic factors affecting lipid metabolism would affect vitamin E status.

The hypothesis of the present study was that the APOA5 genotype not only influences lipid metabolism but, due to the mechanisms described above, also alters vitamin E metabolism in humans. To test this hypothesis we investigated the effects of the APOA5 variant -1131T>C on lipid status in a population of 297 healthy male non-smoking subjects aged 20 to 75 and whether these changes have repercussion on vitamin E status. A detailed lipoprotein profile including total lipids and apolipoproteins plus the lipid and apolipoprotein contents of all lipoprotein fractions were determined along with vitamin E concentrations in plasma, LDL and buccal mucosa cells (BMC). In addition, we studied the effects of the APOA5 variant-associated differences in vitamin E and lipid
status on malondialdehyde (MDA), a biomarker of in vivo lipid peroxidation, which has been used successfully in patients with elevated plasma lipids (22).

METHODS

Study subjects

In this cross-sectional study 299 healthy male non-smoking subjects (0 cigarette/day for more than 6 months), aged 20-75 y (stratified by ages) were recruited in Clermont-Ferrand, France (n=99), Graz, Austria (n=100), and Reus, Spain (n=100) as part of the European Commission-funded RTD project of the 5th Framework Program, specific RTD Program “Quality of Life and Management of Living Resources”, Key action #1 “Food, Nutrition and Health”, entitled “Vitamin A, Vitamin E and Carotenoid Status and Metabolism during Ageing: Functional and Nutritional Consequences”, acronym VITAGE (contract QLK1-CT-1999-00830) (23). After informative sessions a trained medical doctor conducted a personal interview to obtain information on anthropometric measurements, personal history, lifestyle, use of medication, physical activity, smoking habits and use of dietary supplements containing vitamins or trace elements. Exclusion criteria were familial hypercholesterolemia, chronic diseases (including diabetes, cancer, cardiac insufficiency, neurological diseases, inflammatory diseases and chronic diseases of the liver, lung or thyroid, non-stable hypertension, dementia and infectious diseases known to affect the immune system such as HIV and hepatitis C), vaccination during the past 2 months, alcoholism or drug addiction, competitive sports activities and the consumption of special diets or dietary supplements in the past 3 months. The study protocol was approved by the Ethics Committees of the 3 recruiting centers and written informed consent was obtained from all participants.

Analytical methods

Sample collection

Collection of blood samples, preparation of plasma and buffy coats

After an overnight fast venous blood was drawn into plastic tubes containing EDTA to obtain EDTA plasma (Sarstedt Ltd., Nümbrecht, Germany), protected from light and centrifuged immediately at
1,500 x g at 8°C for 10 min. Plasma was separated and divided into aliquots. For LDL isolation, a 60 % sucrose solution was added to plasma to obtain a final concentration of 0.6 % (24). For genomic DNA isolation, buffy coat was collected from blood drawn on EDTA. All samples were stored at –80°C until determination of analytical variables.

**Collection of buccal mucosa cells (BMC)**

After rinsing the mouth with drinking water BMC were collected as described by Gilbert *et al.* (25). Briefly, study subjects were asked to brush the inside of their cheeks with a soft toothbrush 20 times on one side and rinse the mouth with 25 mL of isotonic table salt solution (0.9 % sodium chloride) and repeat the procedure at the other side. The two rinsing volumes were collected in a single tube and centrifuged at 1,400 x g for 10 min at 4°C. The supernatant was discarded and the cell pellet washed with 15 mL of cold phosphate buffered saline solution. After vortexing, the sample was centrifuged as above and the supernatant was removed. The cell pellet was resuspended in 1.2 mL of cold PBS, flushed with nitrogen and stored at –80°C until determination of vitamin E.

**Five-day food records**

For dietary assessment, subjects recorded all food items and drinks consumed during a 5-day period. On the basis of these food records intake of total fat, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and PUFA were calculated. For calculation of nutrients, the REGAL food composition tables (26,27) and the software package of the Austrian food composition table Ernährungswissenschaftliches Programm (EWP) (dato Denkwerkzeuge, Vienna 1997) were used.

**Determination of plasma lipids**

* Determination of TG and cholesterol

TG and cholesterol concentrations in plasma and lipoprotein fractions were measured using enzymatic kits (F. Hoffmann-La Roche Ltd, Switzerland) adapted for a Cobas Mira centrifugal
analyzer (F. Hoffmann-La Roche Ltd, Switzerland) with Precilip EL® and Precinorm® (F. Hoffmann-La Roche Ltd, Switzerland) as quality controls. Immuno-turbidometry was employed for the measurement of the apolipoproteins using specific antiserum purchased from F. Hoffmann-La Roche Ltd, Switzerland (for apo A-I and apo B), Daiichi Chemicals, Japan (for apo E and apo C-III) and Incstar Corporation, U.S.A (for Lp(a)).

Sequential preparative ultracentrifugation

Lipoproteins were separated by sequential preparative ultracentrifugation, using a Kontron 45.6 fixed-angle rotor in a Centrikon 75 (Kontron Instruments, Italy). The following lipoprotein fractions were isolated: VLDL (d<1.006 g/mL), intermediate density lipoproteins (IDL, d=1.006-1.019 g/mL) and LDL (d=1.019-1.063 g/mL). Total HDL cholesterol was measured subsequently to the precipitation of the apo B-containing lipoproteins with polyethylene glycol (Immuno AG, Austria).

Determination of plasma total fatty acids

The determination of the fatty acid content in plasma was based on an esterification procedure and a subsequent GC analysis of the fatty acid methyl esters as described by Sattler et al. (28). Briefly, 450 μL of EDTA plasma and 100 μL of internal standard (10 mg heptadecanoic acid and 100 mg BHT in 10 mL methanol) were added to a teflon screw-capped tube, vortexed for 10 sec and then kept at –80°C for a minimum of 30 min. The deep-frozen suspension was freeze-dried on the lyophilisator (Virtis, Servo Lab, Graz, Austria) for about 15 hours. Thereafter 1 mL boron trifluoride-methanol-complex and 500 μL toluene were added and vortexed for 20 sec. Transesterification was performed at 110°C for 90 min. Following the transesterification step 2 mL aqua purificata was added and the fatty acid methyl esters were extracted 3 times with n-hexane. The hexane extracts were dried in a Speed Vac (Bachhofer, Servo Lab, Graz, Austria) at room temperature for 30 min, redissolved in 450 μL dichloromethane, and subjected to GC analysis (Hewlett Packard 5890 Serie II, Agilent, Vienna, Austria). Separation of fatty acid methyl esters was achieved on a DB-23 column (Agilent Technologies, Vienna, Austria) with a length of 30 m and a diameter of 0.250 mm. The mobile phase was a mixture of helium and hydrogen gas. The
oven temperature at injection was 150°C and was raised to 222°C (3°C/min) and kept at this temperature for 3 min, then raised further to 238°C (3°C/min) and kept at this temperature for 3 min and finally raised to 255°C (20°C/min). The areas under the GC peaks were quantified by integration and the internal standard described above was used for calculation of the amounts of fatty acids. Nineteen plasma samples of the patients were processed along with a control sample from the plasma pool for long-term quality control. Coefficients of variation for the different fatty acids were between 0.38 % and 8.3 % within-run and 1.7 % and 8.6 % between-run.

**Determination of vitamin E**

**LDL isolation**

LDL was isolated as described by Bergmann et al. (24). Briefly, 1.5264 g of solid potassium bromide was added to 4 mL of defrosted EDTA plasma which had been mixed with sucrose as described above. Separation of LDL was achieved in a single-step discontinuous gradient ultracentrifugation in a Beckman NVT65 rotor (Beckman Coulter, Servo Lab, Graz, Austria) at 60,000 rpm for 2 h at 10 °C (29). Thereafter, the LDL band was isolated and filtered through a 0.20 µm sterile filter (Corning, Inc., Corning, NY, USA) into an evacuated glass vial (BD Vacutainer, Belliver Industrial Estate, Plymouth, UK) and processed on the same day. LDL density was determined using an Anton Paar DMA 48 density meter (A. Paar Ltd., Graz, Austria). Cholesterol content of LDL was determined using a kit from Roche Diagnostics GmbH (F. Hoffmann-La Roche Ltd, Switzerland).

**Determination of α- and γ-tocopherols in plasma and in LDL**

The determination of tocopherols in plasma and in LDL was performed as described by Aebischer et al. (30). Briefly, EDTA plasma or isolated LDL were diluted with deionized distilled water, and deproteinized with 400 µl absolute ethanol. To extract lipophilic compounds, 800 µl of n-hexane/BHT (350 mg BHT in 1000 mL n-hexane) were added, centrifuged and the clear supernatant transferred by a dispenser/dilutor system (Micro Lab 500B Dilutor, Hamilton, Martinsried, Germany) to an Eppendorf tube to be dried on a Speed Vac (Savant, New York,
USA). The residue was then redissolved in a mixture of methanol and 1,4-dioxane (1:1), diluted with acetonitrile and injected into the HPLC system (Hewlett Packard 1100A, Agilent, Vienna, Austria). Separation was achieved on a reversed-phase column, the mobile phase was a mixture of acetonitrile, tetrahydrofuran, methanol, 1 % ammonium acetate solution and 10 mg L(+)-ascorbate, the flow rate was 1.6 mL/min. Vitamin E was detected using a fluorescence detector (Jasco, Model FP-920, Biolab, Vienna, Austria) at extinction 298 and emission 328 nm (α- and γ-tocopherol). The areas under the HPLC peaks were quantified on an HP Chemstation (Hewlett Packard 35900E, Agilent, Vienna, Austria). The coefficient of variation within-run was 1.26 % for α-tocopherol and 0.80 % for γ-tocopherol in plasma and 0.70 % for α-tocopherol and 1.4 % for γ-tocopherol in LDL. The coefficient of variation between-day was 1.81 % for α-tocopherol and 2.78 % for γ-tocopherol and 2.54 % for α-tocopherol and 2.98 % for γ-tocopherol in LDL. Six plasma and LDL samples, respectively, of the subjects were processed along with 2 control samples from a plasma and LDL pool, respectively, obtained from a number of healthy subjects to be used for long-term quality control along with a standard solution. The detection limit was 0.012 µmol/L for α- and γ-tocopherol. Tocopherol content of LDL was expressed as mol tocopherol per mol LDL.

**Determination of total vitamin E in BMC**

Vitamin E was extracted from a resuspension of BMC in PBS (1 mL) as described by Borel et al. (31). Briefly, after short sonication (15 seconds, Labsonic U – BBRAUN, USA), α-tocopheryl acetate (Fluka, L’Isle d’Abeau, France) was added to samples as an internal standard. The proteins were precipitated with ethanol precipitation. Then the vitamin E was extracted twice with hexane. The extract was evaporated to dryness under nitrogen, dissolved in methanol-dichloromethane (65:35, v/v) and injected into a C18 column 5 µm, 250 mm x 4.6 mm, Nucléosil (Interchim, Montluçon, France) and was assayed by reverse-phase HPLC (HPLC apparatus: Waters 996 UV-vis DAD; Waters SA, St-Quentin-en-Yvelines, France). Pure methanol, at a flow-rate of 2 mL/min eluted α-tocopherol in 5.0 min and tocopheryl acetate in 6.3 min. The compounds were detected by U.V. (292 nm), then quantified by internal and external calibration using daily-
controlled standard solutions. Vitamin E concentrations were standardized for protein, determined by Lowry et al. (32).

**Determination of malondialdehyde in plasma**
Plasma malondialdehyde concentrations were determined by HPLC as a biomarker of *in vivo* lipid peroxidation. The method described by Khoschsorur et al. was used (33).

**APOA5 genotyping**
According to the nomenclature and methodology used by Pennacchio and colleagues (1), single nucleotide polymorphism –1131T>C was used as the genetic marker. Genotyping was performed with primers AV-1-5’-GATTGATTCAAGATGCATTTAGGAC-3’ and AV-2-5’-CCCCAGGAAGTGGAGCGAAATT-3’, which forced a Msel (New England Biolabs, Beverly, MA, USA) site for enzymatic restriction.

**Statistical methods**
Statistical analyses were carried out with SPSS version 14.0. The Chi-square test was used to test for the Hardy-Weinberg equilibrium. Normal distribution of data was checked with the Kolmogorov-Smirnov test. Comparisons of age and body mass index (BMI) were made with the Student’s *t*-test. ANOVA was performed to compare the mean values of lipid, lipoprotein, apolipoprotein and vitamin E data adjusted for age and BMI, because of well-known associations between plasma lipids and age and BMI. Linear Regression Analysis was performed between plasma and LDL vitamin E concentrations. Log-transformation was performed when variables were not normally distributed. Results are expressed as mean ± standard deviation. *P* < 0.05 was considered statistically significant.
RESULTS

Demographic data of the study population

We studied a total of 297 non-smoking healthy males aged 20-75 years from France, Austria and Spain. Genetic material was missing for 2 subjects and thus 2 subjects had to be excluded from statistical analysis. There were no differences in mean age, age distribution and BMI among countries (Table 1) and, therefore, all subjects were pooled for genetic analyses of lipid and vitamin concentrations.

Frequencies of the -1131T>C polymorphism in the APOA5 gene

Among the 297 subjects, 251 had the common genotype (T/T), 45 were heterozygote (T/C) and one subject was homozygote (C/C). For association analyses all carriers of one or two copies of the C allele were pooled. The C allele was carried by 15.5 % of the subjects, resulting in an allele frequency of 0.08, which was similar to that found in the Caucasian general population (6). The observed frequencies of the –1131 T>C genotypes were not different from those predicted by the Hardy-Weinberg distribution. There were no differences in the allele frequencies between Spain, France and Austria (Table 1).

Characteristics of the study subjects according to APOA5 genotype

There were no differences in age between homozygote carriers of the T allele (45.8 ± 15.1 yrs) and carriers of the C allele (48.0 ± 17.1 yrs). BMI did not differ between the TT (25.0 ± 2.66 kg/m²) and the TC+CC genotypes (24.7 ± 2.65 kg/m²).

Effects of the APOA5 genotype on lipoprotein profile

Carriers of the C allele had 15.2 % higher plasma TG concentrations (P = 0.01) due to elevations in VLDL-TG (P = 0.001), while IDL-TG, LDL-TG and HDL-TG did not differ significantly between the genotypes (Table 2). This was accompanied by a 10 % increase in the lipoprotein lipase inhibitor, apo C-III in the TC+CC genotype (17.0 ± 3.27 mg/dL) compared to the TT genotype (15.4
± 3.30 mg/dL) (P = 0.002). Total cholesterol concentrations were not different between carriers and non-carriers of the C allele. However, carriers of the C allele showed significantly higher cholesterol concentrations in the VLDL (P = 0.002) and IDL fractions (P < 0.05), while there were no differences in the LDL and HDL fractions (Table 2). Circulating apoB-containing lipoproteins were also 11 % higher in carriers of the C allele (apo B100, P = 0.006) due to an accumulation of VLDL and LDL particles as assessed by their apo B content (VLDL, P = 0.008; LDL, P = 0.03). In contrast, the APOA5 genotype did not have an effect on HDL (Table 2). There was no difference between the TT and TC+CC genotype in plasma Lp(a) (23.5 ± 21.7 mg/dL versus 23.1 ± 20.0 mg/dL) and apo E (3.50 ± 0.872 mg/dL versus 3.48 ± 0.691 mg/dL).

**Effects of dietary intake and of the APOA5 genotype on plasma fatty acids**

There was neither a difference in total fat intake, nor in intake of SFA, MUFA and PUFA between the genotypes. After dichotomizing dietary PUFA intake according to the study population mean (16.2 ± 6.95 g/d), there was no effect of high or low PUFA intake on TG concentrations, plasma VLDL and VLDL-TG. Total plasma fatty acids were 8.6 % higher in the carriers of the C allele compared to the homozygote carriers of the T allele (10.4 ± 2.31 mmol/L versus 11.3 ± 2.32 mmol/L) (P = 0.007). The distribution of fatty acids among SFA, MUFA, n-3 or n-6 PUFA expressed as mol% did not differ between the genotypes.

**In vivo lipid peroxidation according to APOA5 genotype**

Plasma malondialdehyde concentrations did not show a significant difference between the TT and TC+CC genotypes, neither when the concentrations (TT: 0.68 ± 0.34 μmol/L versus TC+CC: 0.75 ± 0.38 μmol/L), nor when ratios malondialdehyde:cholesterol (TT: 0.144 ± 0.074 μmol/mmol versus 0.155 ± 0.086 μmol/mmol) were used.

**Effects of the APOA5 gene on vitamin E status**

Carriers of the C allele had significantly higher (9.1 %) plasma α-tocopherol concentrations compared to non-carriers (P = 0.02) (Figure 1). Because there was a significant relation between
plasma α-tocopherol concentrations and TG concentrations ($r = 0.50$, $P < 0.001$) and cholesterol concentrations ($r = 0.75$, $P < 0.001$), standardization was performed. The difference in α-tocopherol concentrations between the genotypes was not statistically significant when standardized for TG and cholesterol (both separately and in combination), nor when measured in the LDL fraction. γ-Tocopherol concentrations as well as total vitamin E concentrations measured in BMC did not differ significantly between carriers and non-carriers of the C allele (Table 3). There was a close relation between plasma and LDL α- and γ-tocopherol concentrations in both genotypes ($α$-tocopherol: $r = 0.45$, $P < 0.001$ in T/T; $r = 0.64$, $P < 0.001$ in C/T and CC; γ-tocopherol: $r = 0.93$, $P < 0.001$ in T/T; $r = 0.94$, $P < 0.001$ in C/T and CC).

**DISCUSSION**

The hypothesis of this study was that, through its action on lipid metabolism, the APOA5 gene would have an effect on the metabolism and distribution of the fat-soluble vitamin E and thus could alter the status of this most potent lipophilic antioxidant. In the study subjects, i.e. healthy male non-smoking volunteers, the APOA5 variant -1131T>C had a significant impact on plasma vitamin E concentrations such that the carriers of the C allele exhibited high α-tocopherol but not γ-tocopherol concentrations in association with elevated TG concentrations as compared to carriers of the T allele. TG concentrations are well known to be a major determinant of circulating vitamin E concentrations (14), therefore standardization for TG concentrations was performed. After standardization, the effect of the genotype disappeared, indicating that elevated vitamin E concentrations are a function of elevated TG concentrations. This relationship is further supported by the highly significant linear regression of α-tocopherol concentrations on TG concentrations in the present study.

**Apolipoprotein A5 and vitamin E**

The reason for this differential effect of the -1131 T>C variant of the APOA5 gene on plasma α-tocopherol compared to γ-tocopherol concentrations may relate to the fact that, while intestinal
absorption of the different vitamin E isoforms is similar, α-TTP in the liver preferentially binds the α-tocopherol, which is then incorporated into nascent VLDL particles (15,16). While this metabolic pathway is responsible for the enrichment of α-tocopherol in lipoproteins and, consequently, also in tissues, γ-tocopherol is either excreted via bile or metabolized in the liver to be excreted as the water-soluble γ-carboxyethyl-hydroxychroman in the urine (15,16). Since in vitro data suggests that apo A-V may influence hepatic VLDL synthesis (34), the incorporation of α-tocopherol into VLDL could be modulated by the −1131 T>C genotype. This does not only result in higher TG concentrations in the carriers of the C allele, but also in higher α-tocopherol concentrations. In contrast, due to lack of incorporation of γ-tocopherol into nascent VLDL, γ-tocopherol concentrations are not affected by the genotype. This could explain the absence of genotype-related differences both in plasma and LDL γ-tocopherol concentrations observed in the present study.

The extrahepatic tissue uptake and distribution of vitamin E is mainly directed by LPL (19). Since Apo A-V stimulates LPL activity (2,35) the APOA5 genotype could also influence the distribution of vitamin E between plasma and tissues. Our results indicate that elevated α-tocopherol concentrations are entirely due to elevated plasma lipid concentrations. Given that there was no difference in LDL TG content between the genotypes, it is not surprising that there was also no difference in LDL α-tocopherol concentrations. Because micronutrient concentrations in BMC have been suggested to reflect vitamin E status better than plasma concentrations (36), we determined vitamin E in BMC. There were no differences between the APOA5 genotypes in BMC vitamin E, which is in agreement with LDL vitamin E concentrations and vitamin E plasma concentrations standardized for lipid concentrations.

**Apolipoprotein A5 and lipids and lipoproteins**

The crucial role of apo A-V on TG metabolism has been demonstrated in several transgenic and knock-out animal models (1-3), as well as in epidemiological studies investigating single nucleotide polymorphisms at the human APOA5 locus (5,9,10). In the present study, we aimed to study its
effect on a population of subjects of different ages specifically recruited to qualify as healthy and with a complete lipid and lipoprotein profile. Carriers of the C allele had elevated TG concentrations compared to wild-type subjects which were mainly due to elevations of TG in the VLDL fraction. Carriers of the C allele also had higher VLDL and IDL cholesterol and higher apo B concentrations, indicating a significantly higher number of apo B containing lipoproteins. Similar observations have been made before (2,37). However, it remains to be determined whether increased synthesis, decreased clearance of TG-rich lipoproteins or both are the underlying mechanisms of the effects of apo A-V on lipid status. Our results would support both possibilities since carriers of the C allele had increased plasma concentrations of free fatty acids and apo C-III.

While elevated concentrations of free fatty acids are known to induce hepatic synthesis of VLDL, elevated concentrations of apo C-III can impair the ability to hydrolize TG as well as the removal of lipoprotein remnants. This could explain all the observed features associated with the -1131 T>C gene variant, namely, increased VLDL, increased TG and increased IDL (VLDL remnants).

Increased plasma fatty acid concentrations did not show a specific pattern of elevated fatty acids. Recently, a modulating effect of dietary n-6 PUFAs on plasma lipids in carriers of the C allele has been shown (38). However, in the present study, such an effect was not observed.

This study was not designed to test the hypothesis that differences in vitamin E status due to APOA5 variant -1131T>C would affect biomarkers of oxidative stress and inflammation. Only healthy volunteers were enrolled who had passed strict inclusion/exclusion criteria such that they did not have elevated markers of inflammation such as CRP, leukocyte count or bands. At the same time they had to be non-smokers, BMI<30 kg/m², and free of acute or chronic diseases, making an elevated oxidative stress status very unlikely. They were also in a fasted state such that postprandial lipid peroxidation would not be investigated. Furthermore, plasma α-tocopherol standardized for lipids, i.e. α-tocopherol:cholesterol of 5.96 ± 0.91 μmol/L (TT) compared to 6.15 ± 1.03 μmol/L (TC+CC) are not associated with significant differences in lipid peroxidation, because
higher lipids in the TC+CC genotype are associated with higher \(\alpha\)-tocopherol concentrations, resulting in comparable protection against lipid peroxidation.

In summary, the data presented indicate that the APOA5 gene significantly alters the lipoprotein profile even in healthy subjects. Such effect is accompanied by increased circulating vitamin E concentrations as a result of increased TG concentrations, while vitamin E metabolism does not seem to be affected. From a health perspective, the association of increased plasma lipids with an increase in \(\alpha\)-tocopherol concentrations is highly relevant for ensuring efficient protection against lipid peroxidation. These results should be taken into account when interpreting plasma vitamin E concentrations in humans. Given the fact that the -1131 T>C variant affects lipid status and, as a consequence, also alters plasma vitamin E concentrations, this study further supports the use of vitamin E standardized for lipids for reliably assessing vitamin E status, particularly in genetic association studies.
ACKNOWLEDGMENTS

This study has been carried out with financial support from the Commission of the European Communities, specific RTD programme "Quality of Life and Management of Living Resources", QLK1-CT-1999-00830, acronym VITAGE. It does not necessarily reflect its view and in no way anticipates the Commission's future policy in this area. Additional support by the Government of Austria (bm:bwk) and DSM Nutritional Products, Switzerland, is gratefully acknowledged. This study has also been supported by SAF 2002-02781 of the Spanish Ministry of Education and Science and the Thematic Network ISCIII-RETIC RD06. Isabella Sundl was supported by a KUWI Stipend (Kurzfristige Wissenschaftliche Arbeiten für Studierende und Graduierte) of the Austrian Government (bm:bwk) and a Research Stipend of the Karl-Franzens University, Graz, Austria. Montse Guardiola is a recipient of a pre-doctoral fellowship at the Spanish Ministry of Education and Science (BES-2003-1090).
REFERENCES


Table 1. Characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>France (n=97)</th>
<th>Austria (n=100)</th>
<th>Spain (n=100)</th>
<th>Total (n=297)</th>
<th>P value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anthropometric variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>46.6 ± 15.1²</td>
<td>45.5 ± 15.1²</td>
<td>46.3 ± 16.0²</td>
<td>46.1 ± 15.4²</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6 ± 2.58</td>
<td>25.2 ± 2.71</td>
<td>25.1 ± 2.66</td>
<td>25.0 ± 2.66</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Genotype</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT (%)</td>
<td>84.5</td>
<td>86.0</td>
<td>83.0</td>
<td>84.5</td>
<td>NS³</td>
</tr>
<tr>
<td>TC/ CC (%)</td>
<td>15.5</td>
<td>14.0</td>
<td>17.0</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>C allele frequency</td>
<td>0.08</td>
<td>0.07</td>
<td>0.09</td>
<td>0.08</td>
<td>Chi²-test</td>
</tr>
</tbody>
</table>

¹France vs. Austria vs. Spain
²Mean values (SD)
³Chi²-test
Table 2: Lipoprotein profiles according to APOA5 genotype.

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
<th>Apo B / apo A-I (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT (n=251)</td>
<td>TC + CC (n=46)</td>
<td>TT (n=251)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P value&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.82 ± 0.93&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5.06 ± 0.87&lt;sup&gt;4&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.05 ± 0.52&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.21 ± 0.58&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;5,6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>70.6 ± 17.2&lt;sup&gt;7&lt;/sup&gt;</td>
<td>78.2 ± 17.4&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.006&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.298 ± 0.248</td>
<td>0.397 ± 0.290</td>
<td>0.002&lt;sup&gt;5,8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.568 ± 0.419</td>
<td>0.751 ± 0.505</td>
<td>0.001&lt;sup&gt;5,8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>3.16 ± 2.18&lt;sup&gt;9&lt;/sup&gt;</td>
<td>4.15 ± 2.58&lt;sup&gt;8&lt;/sup&gt;</td>
<td>0.008&lt;sup&gt;5,8&lt;/sup&gt;</td>
</tr>
<tr>
<td>IDL</td>
<td>0.193 ± 0.106</td>
<td>0.224 ± 0.113</td>
<td>0.048&lt;sup&gt;5,8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.093 ± 0.337</td>
<td>0.105 ± 0.442</td>
<td>NS&lt;sup&gt;5,8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2.73 ± 1.37&lt;sup&gt;9&lt;/sup&gt;</td>
<td>3.11 ± 1.57&lt;sup&gt;9&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;5,8&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL</td>
<td>2.68 ± 0.728</td>
<td>2.89 ± 0.652</td>
<td>NS&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.237 ± 1.00</td>
<td>0.188 ± 0.048</td>
<td>NS&lt;sup&gt;5,8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>54.4 ± 14.6&lt;sup&gt;10&lt;/sup&gt;</td>
<td>59.7 ± 13.4&lt;sup&gt;10&lt;/sup&gt;</td>
<td>0.03&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL</td>
<td>1.33 ± 0.308</td>
<td>1.33 ± 0.296</td>
<td>NS&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.094 ± 0.031</td>
<td>0.103 ± 0.041</td>
<td>NS&lt;sup&gt;5,8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>112.6 ± 17.4&lt;sup&gt;11&lt;/sup&gt;</td>
<td>114.8 ± 16.6&lt;sup&gt;2&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Apo B content, <sup>2</sup>apo A-I content, <sup>3</sup>ANOVA, <sup>4</sup>Mean values ± SD, <sup>5</sup>P value standardized for BMI and age, <sup>6</sup>On-log transformed data

Abbreviations: apo = apolipoprotein
Table 3: Vitamin E profile according to APOA5 genotype.

<table>
<thead>
<tr>
<th>Vitamin E status</th>
<th>APOA5 genotype</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>TC + CC</td>
<td>P value$^1$</td>
</tr>
<tr>
<td></td>
<td>(n=251)</td>
<td>(n=46)</td>
<td></td>
</tr>
<tr>
<td>Plasma α-tocopherol (μmol/L)</td>
<td>28.7 ± 6.34$^2$</td>
<td>31.3 ± 7.43$^2$</td>
<td>0.02$^{3,4}$</td>
</tr>
<tr>
<td>adjusted for cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adjusted for triglycerides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adjusted for cholesterol and triglycerides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adjusted for apo B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma α-tocopherol:cholesterol (μmol/mmol)</td>
<td>5.96 ± 0.907</td>
<td>6.15 ± 1.03</td>
<td>NS$^{3,4}$</td>
</tr>
<tr>
<td>Plasma γ-tocopherol (μmol/L)</td>
<td>1.38 ± 0.835</td>
<td>1.46 ± 0.689</td>
<td>NS$^{3,4}$</td>
</tr>
<tr>
<td>Plasma γ-tocopherol:cholesterol (μmol/mmol)</td>
<td>0.285 ± 0.154</td>
<td>0.290 ± 0.126</td>
<td>NS$^{3,4}$</td>
</tr>
<tr>
<td>LDL α-tocopherol (μmol/L)</td>
<td>8.28 ± 1.10</td>
<td>8.51 ± 1.25</td>
<td>NS$^3$</td>
</tr>
<tr>
<td>LDL γ-tocopherol (μmol/L)</td>
<td>0.329 ± 0.180</td>
<td>0.329 ± 0.138</td>
<td>NS$^{3,4}$</td>
</tr>
<tr>
<td>BMC total vitamin E (ng/mg protein)</td>
<td>119.0 ± 95.8</td>
<td>110.0 ± 55.6</td>
<td>NS$^{3,4}$</td>
</tr>
</tbody>
</table>

$^1$ANOVA, $^2$Mean values ± SD, $^3$P value standardized for BMI and age, $^4$On-log transformed data

Abbreviations: BMC = buccal mucosal cells, apo = apolipoprotein
Figure 1. Differences in plasma α-tocopherol concentrations (upper panel) and α-tocopherol standardized for triglyceride concentrations (lower panel) between APOA5 genotypes.
Figure 1

Plasma α-tocopherol : triglycerides (µmol/mmol)

TT                            CT+CC

Plasma α-tocopherol (µmol/L)

TT                            CT+CC