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APOLIPOPROTEIN E4 ALLELE IS ASSOCIATED WITH SUBSTANTIAL CHANGES IN THE PLASMA LIPIDS AND HYALURONIC ACID CONTENT IN PATIENTS WITH NONALCOHOLIC FATTY LIVER DISEASE

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Fat may affect progression of liver damage in patients with non-alcoholic fatty liver disease (NAFLD). In this study we characterize the state of lipid metabolism in 22 patients with NAFLD and different *Apo-E* variants. Total concentration of plasma total fatty acids was quantified by gas chromatography, while their derivatives by liquid chromatography/tandem mass spectrometry (LC ESI MS/MS). The ratio of plasma saturated fatty acid to monounsaturated fatty acid increased, whereas the ratio of polyunsaturated fatty acids to saturated fatty acids was reduced in *Apo-E4* carriers. Simultaneously, the levels of individual plasma linoleic, arachidonic, and alpha linolenic acids significantly increased in subjects with the *Apo-E4* allele. The 15-lipoxygenase metabolite, 13-hydroxyoctadecadienoic acid, was significantly higher in *Apo-E3* carriers ($p<0.006$). 5-oxo-6,8,11,14-eicosatetraenoic acid was significantly elevated in *Apo-E4* carriers ($p<0.009$). A significant difference in hyaluronic acid concentration ($p<0.0016$) as well as predicted advanced fibrosis (using the BARD scoring system) was found in *Apo-E4* carriers ($p<0.01$). We suggest that a distinct mechanism of fibrosis between *Apo E* alleles. In *Apo-E4* carriers, an elevation in 5-oxo-6,8,11,14-eicosatetraenoic acid synthesis and fatty acid dysfunction may induce fibrosis, while an inflammatory process may be the main cause of fibrosis in *Apo-E3* carriers.

Key words: *non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, apolipoprotein E, fatty acids, 5-oxo-6,8,11,14-eicosatetraenoic acid, liquid chromatography/tandem mass spectrometry, gas chromatography*

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in developed countries (1). NAFLD is characterized by fatty infiltration of the liver and increased endogenous lipogenesis in the absence of chronic alcohol consumption (2). NAFLD is closely associated with visceral adiposity, insulin resistance and dyslipidemia, and is described as the hepatic component of metabolic syndrome (3). It is estimated that 20% of patients with NAFLD develop non-alcoholic steatohepatitis (NASH) involving steatosis combined with inflammation, that can progress into fibrosis and cirrhosis (4).

Historically, NAFLD progression was interpreted with "two-hit" hypothesis where the first hit is related to lipid accumulation (1) and the following one with inflammation, fibrosis and cellular death (1, 2). In contrast, a novel multifactor etiology model, with a central role for Kupffler cells and oxidized forms of LDL uptake has been recently postulated (5). In this model, lipid-induced cell toxicity and apoptosis are specific to fatty acids present in the circulation and liver (5). There are mainly saturated fatty acids: palmitic, stearic that promote liver damage *via* activation of apoptosis *via* a caspase-dependent mechanism (2). Both palmitic and stearic acid activate endoplasmic reticulum stress, which leads to activation of proapoptotic and proinflammatory factors such as C-Jun NH2 terminal kinase, nuclear factor κB, and pro-apoptotic Bcl-2 family members (*e.g.* Bax) (2, 6). It has been noticed, that patients with NASH experience significant changes (qualitative and quantitative) in the lipid composition of plasma and cell membranes compared to NAFLD patients (4).

5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) is a strong chemoattractant synthesized by proinflammatory cells (7-11). The biological actions of 5-oxo-ETE are mediated by the highly selective OXE receptors, which are expressed on variety of inflammatory cells: eosinophil, neutrophils, myeloid cells (8). Synthesis of 5-oxo-ETE (by 5 lipoxygenase and hydroxyeicosanoid dehydrogenase) is regulated by intracellular NADP(+) levels and is dramatically increased under oxidative stress and the respiratory burst in phagocytic cells (resting neutrophils metabolize 5-HETE principally by ω-oxidation to 5,20-diHETE) (9, 11).

Amongst other causes, NAFLD can develop (into NASH) due to specific gene variants associated with key elements of lipid metabolism (4), *e.g.* the *Apo-E* gene that encodes apolipoprotein

E (Apo-E) - a glycoprotein essential for the metabolism of triglyceride-rich lipoproteins. An interaction between Apo-E and receptors determines the removal of Apo-E rich lipoproteins, and homeostasis of cholesterol and triglycerides (12, 13). There are three major isoforms of human ApoE (ApoE2, ApoE3, ApoE4) encoded by 3 allels (epsilon 2, 3, and 4) (12). ApoE status is defined by combination of two SNPs (rs7412 and rs429358). *ApoE*-ε3 carriers contains rs7412(C) and rs429358 (T) while *ApoE*-ε4 has both rs7412(C) and rs429358 (C) (14, 15). In various combinations, *Apo-E* alleles are associated with neurodegenerative, coronary and vascular diseases (16-20) as well as correspond to an altered lipid profile (21, 22).

It was reported that the response of plasma lipids was different in subjects with *Apo-E* genotypes (12), but still is unknown whether variants of *Apo-E* may change plasma lipid components thus predisposing patients to NAFLD progression. Therefore, in this study a lipidomic approach was chosen to characterize the state of lipid metabolism in patients with *Apo-E* variants. Individuals with *Apo-E4* allele were compared to those with *Apo-E3* - as most frequently reported, wild-type allele. We quantified the total concentration of plasma total fatty acids by gas chromatography, fatty acids derivatives by liquid chromatography/tandem mass spectrometry (LC ESI MS/MS). *ApoE* genotype we estimated by real-time PCR, whereas liver fibrosis parameters by ELISA method and BARD scoring system. Although all examined subjects in our study were diagnosed with NAFLD, their lipid profiles differed in the composition of fatty acids and some of their metabolites.

MATERIALS AND METHODS

Chemicals and materials

Chloroform, methanol and acetic acid were sourced from Merck (USA). Boron trifluoride in methanol, NaCl and 2,6-di-tert-butyl-4-methylphenol (BHT) were supplied by Sigma-Aldrich (Poland). Double-distilled water was obtained from a Milli-Q Water System (Millipore, Billerica, MA, USA). Fatty acid standards were obtained from Sigma-Aldrich, Neochema (Germany) and Cayman (USA).

Patients

A group of 23 Caucasian individuals diagnosed with NAFLD were prospectively enrolled in the study. The degree of liver steatosis was assessed by a trained physician according to the Hamaguchi score (23) using an abdominal ultrasound high-resolution B-mode scanner (Acuson X300). All NAFLD patients included in the study were negative for HBV (hepatitis B virus) and anti-HCV (hepatitis C virus) and had a negative history of alcohol intake (less than 20 g/d).

All participants underwent *Apo-E* genotyping for three isoforms: *Apo-E2*, *ApoE-3*, and *Apo-E4*. Based on *Apo-E* allele sets, the individuals were divided into two subgroups *i.e*. *Apo-E3* (rs429358 [T] and rs 7412 [C]; n=11) or *Apo-E4* (rs429358 [C or CC] and rs 7412 [C or CC]; n=11). In *ApoE 3* group there are 3 woman and 9 man, in *Apo E4* group 5 woman 6 man.

After an overnight fasting venous blood for lipid analyses was collected into tubes with anticoagulant. Whole blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes. Blood was immediately placed on ice or in a refrigerator, and samples centrifuged at 3500 rpm for 10 min at 4°C within 2 hours of collection. Plasma was then immediately stored under conditions to minimize artificial oxidation (*i.e*., with an antioxidant cocktail under inert atmosphere). Standard blood biochemical analyses were carried out at the University Hospital Laboratory. Clinical and laboratory data on the analyzed patients are summarized in *Table 1*.

BMI was based on an individual's mass and height. A signed informed consent was obtained from each patient. The study protocol was approved by the ethics committee of Pomeranian Medical University and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

ApoE genotyping using real-time PCR

DNA from peripheral blood mononuclear cells was isolated using a DNeasy Blood&Tissue Kit (Qiagen). Genotypes were determined by real-time polymerase chain reaction (PCR) using Taqman® SNP genotyping assays for two Apo-E polymorphisms including rs7412 and rs429358 (Applied Biosystems, Assay ID: C_904973_10, C_3084793_20, respectively). The fluorescence data were analyzed with allelic discrimination 7500 Software v.2.0.2.

Fibrosis parameters

In order to predict advanced fibrosis, the BARD scoring system developed and validated by Harrison *et al*. was used (24). An easily calculated composite score was based on the results of forced entry logistic regression analysis and included a BMI ≥ 28 = 1 point, AAR ≥ 0.8 = 2 points (AAR: aminotransferase ratio of AST/ALT), and the presence of DM = 1 point (DM: diabetes mellitus). BARD was able to predict advanced fibrosis: a score of 2–4 was associated with an OR for advanced fibrosis (F3-F4) (confidence interval 9.2 to 31.9) and a negative predictive value of 96%. BARD reliably identified patients without significant fibrosis (19, 20) and was also validated in a Polish NAFLD population (25). Hyaluronic acid, a marker of liver fibrosis in subjects with NAFLD (26) was estimated ELISA kit (Wuhan EIAab Science).

Eicosanoid analysis by liquid chromatography/tandem mass spectrometry (LC ESI MS/MS)

Isolation of fatty acid derivatives from plasma was carried out using a dual mobile phase gradient (30, 2007). The flow rate was 1.0 ml/min. The sample injection volume was 60 µl. The DAD detector monitored peaks by adsorption at 235 nm for 13S HODE, 9S HODE, 15S HETE, 12S HETE and 5S HETE, 280 nm for prostaglandin B_2 (PGB_2) (internal standard) and 5oxo HETE, 210 nm for 16RS HETE and 302 nm for 5 (S),6 (R),15 (R) lipoxin A4 and 5(S),6 (R) lipoxin A4.

Analyses were performed using a triple quadrupole mass spectrometer (Agilent 1290/Agilent 6400, Santa Clara, CA, USA). The liquid chromatograph was equipped with a binary pump (G4220A), diode array detector (G4212A), well plate sampler (G4226A) and thermostated column compartment (G1316C). The mass spectrometer was equipped with an ESI ion source (XESI with jet stream technology). The column used for compounds resolution was a Poroshell 120 SBC 18 (3.0×100 mm, 2.7 µM, PW. 68-975-302. Liquid chromatography parameters, in-lab developed, were as follows: flow – 0.8 ml/min, solvent A (H_2O/0.1% Acetonitrile), solvent B (methanol/0.1% Acetonitrile), solvent A ratio 90, solvent B ratio 10, time 12.5 – solvent ratio B – 85, time 20 – solvent B ratio – 100; DAD monitoring wavelengths: 235, 280, 210, 320, 280 nm; injection volume 20 µl; column temperature 25°C.

Spectra were acquired in a negative polarity using MRM. Compounds were detected at the retention times provided below after optimization of CE energy and two transitions were monitored for each compound, where the first was used for quantitation, the other for confirmative purposes: 12HETE

(retention time (Rt) 13.34 min; CE(V) 5; 319.2 › 301.2, 319.2 › 179.2); 13HODE (Rt 13.13 min; CE(V) 9; 295.2 › 277.3, 295.2 › 195.2); 15HETE (Rt 13.21 min; CE(V) 5; 319.2 › 301.3, 319.2 › 257.3); 16HETE (Rt 13.08 min; CE(V) 5; 319.2 › 301.2, 319.2 › 257.3); 5HETE (Rt 13.53 min; CE(V); 319.2 › 301.2, 319.2 › 115.0); 5oxoHETE (Rt 13.63 min; CE(V) 9; 317.2 › 203.2, 317.2 › 59.1); 5S,6R,15R lipoxin (Rt 10.85 min; CE(V) 9; 351, 2 › 217.2, 351 › 115.1); 5S,6R lipoxin (Rt 10.75 min; CE(V) 9; 351.2 › 217.2, 351.2 › 115.1); 9HODE (Rt 13.14 min; CE(V) 9; 295.2 › 277.3, 295.2 › 171.1); PGB_2 (internal standard; Rt 11.28 min; CE(V) 9; 333.2 › 235.2, 333.2 › 175.1). All transitions were optimized using an infusion of standards. The operating parameters for MS were as follows: gas temp – 300°C; gas flow – 5 l/min; nebulizer – 45 psi; sheath gas heater – 250°C; sheath gas flow — 11 ml/min; capillary voltage – 3500 V.

Acquired data were processed using Mass Hunter software (Agilent Technologies, Santa Clara, CA, USA). Quantitation was carried out based on MRM with internal standard (PGB_2). Calibration curves were determined for a concentration range of 0.05–1.00. Linearity for analyzed compounds exceeded 0.993 (this value was observed for 16HETE). For the majority of compounds linearity was 0.999.

Total fatty acids extraction and gas chromatography analysis

Total fatty acids (FFA) were extracted according to the Folch method (27). 1 ml of plasma extract was saponified with 1 ml of 2M KOH methanolic solution at 70°C for 20 min and then methylated with 2 ml 14% solution of boron trifluoride in methanol under the same conditions. In the next step, 2 ml of n-hexane and 10 ml saturated NaCl solution were added. The samples were allowed to completely separate into an upper (n-hexane phase) and lower layer. 1 ml of the n-hexane phase was collected.

Gas chromatography was performed using an Agilent Technologies 7890A GC System. The instrument was equipped with a SUPELCOWAX™ 10 Capillary GC Column (15 m×0.10 mm, 0.10 µm) (Supelco, Bellefonte, PA, USA). Chromatographic conditions were as follows: the initial temperature was 60°C for 0 min, increased at a rate of 40°C/min to 160°C (0 min), increased at a rate of 30°C/min to 190°C (0.5 min) and then increased at a rate of 30°C/min to 230°C for 2.6 min, where it was maintained for 4.9 min. The total analysis time was 8 min and the gas flow rate was 0.8 ml/min with nitrogen as the carrier gas.

Fatty acids were identified by comparison of their retention times with those of commercially available standards. Quantitative analyses were made using ChemStation Software (Agilent Technologies, UK) and were based on peak areas. C17:0 was used as the internal standard for calculation. Results were expressed in ug/ml.

Analysis of diet

Diet composition and energy intake were ascertained using questionnaires (24-hour food diaries). Each subject was interviewed about their dietary pattern in the previous day. Data from questionnaires were analyzed using Food composition tables (IZZ, Poland) and DIETETYK 6.0 software (Jumar, Poland).

Statistical analysis

Statistica 7.1 software was used for the statistical analysis and all results are expressed as median and interquartile range (IQR), and mean ± standard deviation. As the distribution in most cases deviated from normal (Shapiro-Wilk test), non-parametric tests were used: Mann-Whitney test for comparisons between groups, where $p<0.05$ was considered significant.

RESULTS

Changes in anthropometry and plasma findings

The baseline characteristics of the study population as well as laboratory and anthropometry findings in *Apo-E* carriers are shown in *Table 1*. There were no significant differences in anthropometric parameters between both groups. In terms of biochemical parameters, we found a significant difference in the plasma concentration of postprandial glucose which was elevated in *Apo-E4* variant carriers (111.86 ± 36.36 mg/dl versus 88.89 ± 5.97, *Apo-E3* control, $p<0.02$) (*Table 1*). There were no differences in insulin, level of enzymes as well as cholesterol and its fractions HDL and LDL (in relation to patients with *Apo-E3*) (*Table 1*).

Changes in predicted advanced fibrosis and hyaluronic acid concentration

A significant difference in predicted advanced fibrosis calculated using the BARD scoring system was found between *Apo-E* variant carriers. *Apo-E4* carriers had a score of 2.36 ± 1.03 versus 1.17 ± 1.03 in *Apo-E3* carriers, $p<0.01$, associated with an increased OR for advanced fibrosis (*Table 2)*. We noted an elevation in plasma hyaluronic acid concentration in *Apo-E4* carriers compared to *Apo-E3* carriers (60.1 ± 11.9 ng/ml versus 40.25 ± 6.2 ng/ml). Interestingly, there were no differences in liver steatosis according to the Hamaguchi score (*Table 2*).

Changes in total fatty acid in plasma

Palmitic acid, stearic acid, oleic acid and linoleic acid were the major FFA in the circulating plasma pool in both *Apo-E* variant carriers (*Table 3*). Compared with *Apo-E3* carriers, the ratio of saturated fatty acids (SFA) to monounsaturated fatty acids increased, whereas the ratio of polyunsaturated fatty acids (PUFA) to SFA decreased in *Apo-E4* carriers.

Estimated fatty acids ratios:

a) ratio all SFA to all PUFA was: 1:2.3 (*Apo-E3* alleles) versus 1: 1.2 (*Apo-E4* alleles)
b) ratio all SFA to all MUFA was 1.5:1 (*Apo-E3* alleles) versus 2:1 (*Apo-E4* alleles)
c) ratio all PUFA to all MUFA was 3.25:1 (*Apo-E3* alleles) versus 2.4:1 (*Apo-E4* alleles)

There was a significant increase in palmitoleic acid (16:1, n=7) (11.5 ± 4.8 vesus 19.2 ± 13.6; $p=0.05$), vaccenic acid (18:1, n=7) (12.4 ± 2.8 versus 24.1 ± 14.4; $p<0.0004$) as well oleic acid (18:1, n=9) (87.2 ± 24.7 versus 151.1 ± 87.6; $p<0.006$) in *Apo-E4* carriers (*Table 3*). Linoleic acid (18:2, n–6) and α linolenic acid (18:3, n=3) are both essential fatty acids and are sources of n=6 and n=3 PUFA, respectively. There were increased amounts of the n=6 PUFA family: linoleic acid (18:2, n=6) (85.0 ± 15.6 versus 164.4 ± 64.5; $p<0.00001$), arachidonic acid (20:4, n=6) (0.4 ± 0.5 versus 4.5 ± 6.1, $p<0.01$) in subjects with the *Apo-E4* polymorphism (*Table 3*). The amount of α linolenic acid was also significantly increased in subjects with *Apo-E4* (3.0 ± 0.8 versus 6.1 ± 3.3, $p<0.004$), followed by docosahesaenoic acid 22:6, n=3 (10.6 ± 5.0 vesus 15.4 ± 6.9, $p<0.03$), a product of peroxisomal metabolism of PUFA (4) (*Table 3*).

Changes in plasma inflammatory metabolites of linoleic and arachidonic acid

5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-HETE) was significantly elevated in *Apo-E4* carriers (*Table 4*). Many products of the LOX pathway (5-, 12-, 15-HETE) were increased in *Apo-E4* carriers, however, a significantly higher content of 13-HODE was

Table 1. Baseline characteristics of the study population. *significant between *Apo-E3* versus *Apo-E4*; p<0.05 versus the control (*Apo-E3*); U Mann Whitney Test.

Parameters	*Apo E 3* allele	*Apo E 4* allele
	Median (IQR)	Median (IQR)
	Mean ± S.D.	Mean ± S.D.
Age (year)	51.00 (16.50)	59.00 (14.50)
	48.45 ± 11.14	53.36 ± 13.90
Body Mass Index (kg/m^2)	31.61 (11.00)	34.22 (6.90)
	33.26 ± 7.92	33.80 ± 4.56
Aspartate transaminase (U/l)	31.5 (14.75)	22.0 (7.25)
	27.14 ± 9.39	27.14 ± 12.03
Alanine transaminase (U/l)	54.0 (34)	28.5 (15.25)
	27.14 ± 9.39	34.86 ± 18.77
Gammaglutamyl trasferase (U/l)	53.5 (54.75)	25.0 (36.5)
	56.74 ± 41.55	48.71 ± 51.83
Triacyloglicerols (mg/dl)	106 (33.5)	169 (113)
	119.93 ± 90	226.60 ± 264.80
Cholesterol (mg/dl)	191 (43.5)	218 (45.25)
	186.34 ± 38.15	246.36 ± 148.38
High density lipoprotein (mg/dl)	50 (6.25)	45 (14.25)
	44.9 ± 11.56	47.86 ± 12.40
Low density lipoprotein (mg/dl)	115 (38)	120.5 (55.5)
	115.78 ± 41.83	118.4 3± 40.75
Glucose (mg/ml)	95 (4.5)	106.5 (16.75) *
	88.89 ± 5.97	111.86 ± 36.36
Insulin (U/ml)	8.8 (8.03)	7.75 (12.83)
	12.45 ± 18.91	53.42 ± 159.06

Table 2. Hepatic parameters of the study population. All values are expressed as the median (IQR) and mean ± S.D. * p<0.05 *Apo-E 4* versus the control (*Apo-E 3*); ** p<0.005; (U Mann Whitney Test).

Parameters	*Apo E 3* allele	*Apo E 4* allele
	Median (IQR)	Median (IQR)
	Mean ± S.D.	Mean ± S.D.
Liver steatosis (Hamaguchi score)	3 (2.25)	3 (3)
	2.67 ± 1.5	3.46 ± 2
Advanced fibrosis	1 (0.5)	3.00 (1.75)*
	1.17 ±1.0	2.36 ± 1.0
Hyaluronic acid (ng/ml)	40 (6)	59 (20)**
	40 ± 6	60 ± 12

noted in *Apo-E3* carriers. In humans, the conversion of linoleic acid leads to the production of linoleic acid derivatives -HODEs (9, 13-hydroxyoctadecadienoic acids), whereas oxidation of arachidonic acid is a source of HETEs (5, 12, 15-hydroxyeicosatetraenoic acids). These reactions are catalyzed by cellular lipoxygenases (LOXs) as well as reactive oxygen species (4).

Analysis of dietary energy intake among patients

Analysis showed no significant differences in energy intake and diet composition with regard to key nutrients among the *Apo-E* variants (*Table 5*).

DISCUSSION

The role of *Apo-E* genotypes in NAFLD has been previously investigated with conflicting results. In the last year it was reported that the *Apo-E4* allele showed a statistically significant two-fold reduction in NAFLD risk compared with homozygotes Apo-*E3* (28). A protective effect of the *E4* allele towards NAFLD was related to its role in the regulation of hepatic very low-density lipoproteins (VLDL) secretion (28). Meanwhile VLDL secreted by the liver may contain different composition and concentration of fatty acids and their derivatives (it is depended on diet and liver function). Numerous studies have indicated that

Table 3. Fatty acid composition of the plasma fatty acids study population. All values are the Median (IQR) (μg/ml) mean ± S.D. * p<0.05, ** p <0.005, # p<0.0005, *Apo-E 4* versus the control (*Apo-E3*) (U Mann Whitney Test).

Fatty acids classification	*Apo E 3* allele Median (IQR) Mean ± S.D.	*Apo E 4* allele Median (IQR) Mean ± S.D.
Saturated fatty acids		
Lauric acid	0.9 (1.2)	0.0 (1.7)
	1.1 ± 1.4	1.1 ± 1.6
Myristic acid	6.2 (3.4)	9.7 (10.6)*
	6.6± 2.9	11.8± 6.7
Palmitic acid	117 (18.5)	156.3(115)**
	118.5± 25.5	206.3 ± 127.2
Stearic acid	36.0 (9.7)	52.8 (29.0)**
	38.9 ± 7.8	66.7± 37.0
Monounsaturated fatty acids		
Palmitoleic acid	10.0 (4.4)	14.5 (10.2)*
	11.5± 4.8	19.2± 13.6
Vaccenic acid	12.3 (3.0)	17.3 (19.0)#
	12.4 ± 2.8	24.1± 14.4
Oleic acid	75.4 (31.5)	116.5 (109.4)**
	87.2 ± 24.7	151.1
Polyunsaturated fatty acids		
Linoleic acid	81.4 (19.1)	149.4 (106.9) #
	85.0 ± 15.6	164.4 ± 64.5
Gamma linolenic acid	1.9 (1.2)	2.3 (2.3)
	1.9 ± 0.9	2.8 ± 2.4
Eicosadienoic acid	0.9 (1.2)	0.0 (0.7)
	0.9 ± 0.8	0.4 ± 0.7
Arachidonic acid	0.0 (0.6)	2.1 (5.9)*
	0.4 ± 0.5	4.5 ± 6.1
Docosapentaenoic acid	2.2 (1.3)	3.4 (3.6)
	2.2 ± 1.4	4.4 ± 4.1
Alpha linolenic acid	3.0 (1.2)	6.3 (5.7)**
	3.0 ± 0.8	6.1 ± 03.3
Eicosapentaenoic acid	3.7 (3.3)	6.4 (5.4)
	6.8 ± 6.3	7.6 ± 4.9
Docosapentaenoic acid	0.0 (0.9)	0.0 (1.4)
	0.4 ± 0.6	1.2 ± 2.2
Docosahesaenoic acid	8.9 (4.2)	13.7 (7.7)*
	10.6 ± 5.0	15.4 ± 6.9

Table 4. Plasma fatty acid derivatives of the study population. All values are expressed as the median (IQR) and mean ± S.D.; * p< 0.05, ** *p*<0.005, *Apo-E4* versus the control (*Apo-E3*) (U Mann Whitney Test).

Parameters	*Apo E 3* allele Median (IQR) Mean ± S.D.	*Apo E 4* allele Median (IQR) Mean ± S.D.
5S.6RLipoxin	0 (0)	0 (0)
	0 ± 0	0.004 ± 0.02
5S.6R.15R Lipoxin	0 (0)	0 (0)
	0 ± 0	0 ± 0
13 HODE	0.004 (0.003)	0.003 (0.001)**
	0.005 ± 0.002	0.002 ±0.001
9 HODE	0.005 (0.003)	0.004 (0.002)
	0.006 ± 0.004	0.004 ± 0.002
15 HETE	0.003 (0.003)	0.003 (0.002)
	0.003 ± 0.002	0.003 ± 0.001
12 HETE	0.017 (0.011)	0.013 (0.009)
	0.014 ± 0.007	0.014 ± 0.006
5 HETE	0 (0.007)	0.003 (0.005)
	0.003± 0.004	0.005 ± 0.006
5 oxoHETE	0 (0)	0.001 (0.003)*
	0.00 ± 0.00	0.002 ± 0.002

the content of saturated acids in plasma's VLDL are correlated with severity of disease and is more deleterious to hepatocyte functions than unsaturated fatty acids (2, 5, 29, 30). There is a concept that elevated fatty acids (and their derivatives) promote hepatotoxicity (4) - especially 18:0 and C16:0 (that were elevated in *Apo-E4* allele carriers in this study, *Fig. 1 A*) can have varied metabolic consequences on NAFLD progression (5).

Interestingly, in *Apo-E4* carriers in this study an increased concentration of some monounsaturated fatty acids such as palmitoleic, vaccenic acid and oleic acid was noted. These results may connect *Apo-E4* polymorphism with with up-regulation (activation) of two main lipogenic enzymes: fatty acid synthase (FAS) and Δ9 stearoyl-CoA desaturase (Δ9 SCD). Expression of Δ9 SCD is regulated by several transcription factors: peroxisome proliferator activated receptors (PPAR), liver X receptor (LXR) and sterol regulatory element binding protein-1c (SREBP 1-c) which are up-regulated during inflammation (31) and by a number of nutritional factors (SFA, glucose, fructose) (5, 32). As noted previously, increased Δ9 SCD activity was critical in the regulation of the synthesis of fatty acids in a mouse model of NASH (31-33). Interestingly, although the level of the above-mentioned fatty acids increased in *Apo-E4* individuals, the intake of calories (originally from fats) was similar in both groups of *Apo-E* carriers. Analysis of 24-hour dietary diaries collected from the patients showed no significant differences in the intake of nutrients between individuals from both groups. In addition, *Apo-E4* allele carriers (similar to NAFLD individuals) have a negative ratio of SFA to PUFA (5). Furthermore, an increase in SFA concentration (stearic and palmitic acid) is accompanied by augmentation of plasma PUFA content: linoleic acid (LA - the precursor of n=6 PUFAs) and α linolenic acid (αALA a precursor of n=3 PUFAs). The metabolic fate of both LA and αALA are not the same - only carriers of *Apo-E4* showed an increased content of the selected products of LA and αALA elongation and desaturation - with greater synthesis of arachidonic acid and its derivatives (5-oxo-ETE) in individuals with *Apo-E4* (*Fig. 1B*).

In this study, also we showed clearly that *Apo-E 4* allele is related to the intensification of synthesis 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE). Earlier, elevation 5-oxo-ETE (as well as other biomerkers) among individuals with NASH have been noticed (29, 30). The increase 5-oxo-ETE content in NASH was associated with progression of inflammatory stress and liver fibrosis (4, 29). It is possible that similar phenomena has been observed in our study where *Apo-E4* carriers were characterized by a higher plasma content of docosahexaenoic acid, linoleic acid and alpha linolenoic acid as well as arachidonic acid (*Fig. 1B*). Both arachidonic acid and docosahexaenoic acid stimulate 5-oxo-ETE synthesis in neutrophils which triggers the synthesis of proinflammatory cytokines as well as cell proliferation (11). We suppose that plasma 5-oxo-ETE was elevated by circulating proinflammatory fatty acids elevated among *Apo-E4* allele carriers (*Fig. 1 A*).

Although all examined subjects in our study were diagnosed with NAFLD, their lipid profiles differed in the composition of fatty acids and some of their metabolites. It seems that *Apo-E4* favors lipid metabolism disorders and a tendency for fibrosis. In our study, fibrosis (predicted by the BARD scoring system) as

Table 5. Nutrient intake among patients. Nutrient analyses were carried out using the corresponding Polish food table and the nutrient database developed in Poland.

Diet analysis	*Apo E 3* allele Median (IQR) Mean ± S.D.	*Apo E 4* allele Median (IQR) Mean ± S.D.
Estimated caloric intake (kcal/day)	1556.00 (511.25) 1650.25 ± 435.42	1709.50 (962.25) 1804.00 ± 639.17
Estimated protein intake (g/day)	84.70 (35.88) 87.07 ± 35.60	76.00 (47.25) 93.55 ± 51.12
Estimated lipids intake (g/day)	46.60(19.85) 49.23 ± 15.58	66.50(36.48) 62.29 ± 30.58
Estimated carbohydrate intake (g/day)	215.70 (104.85) 220.95 ± 75.73	196.50 (89.75) 196.03 ± 82.60

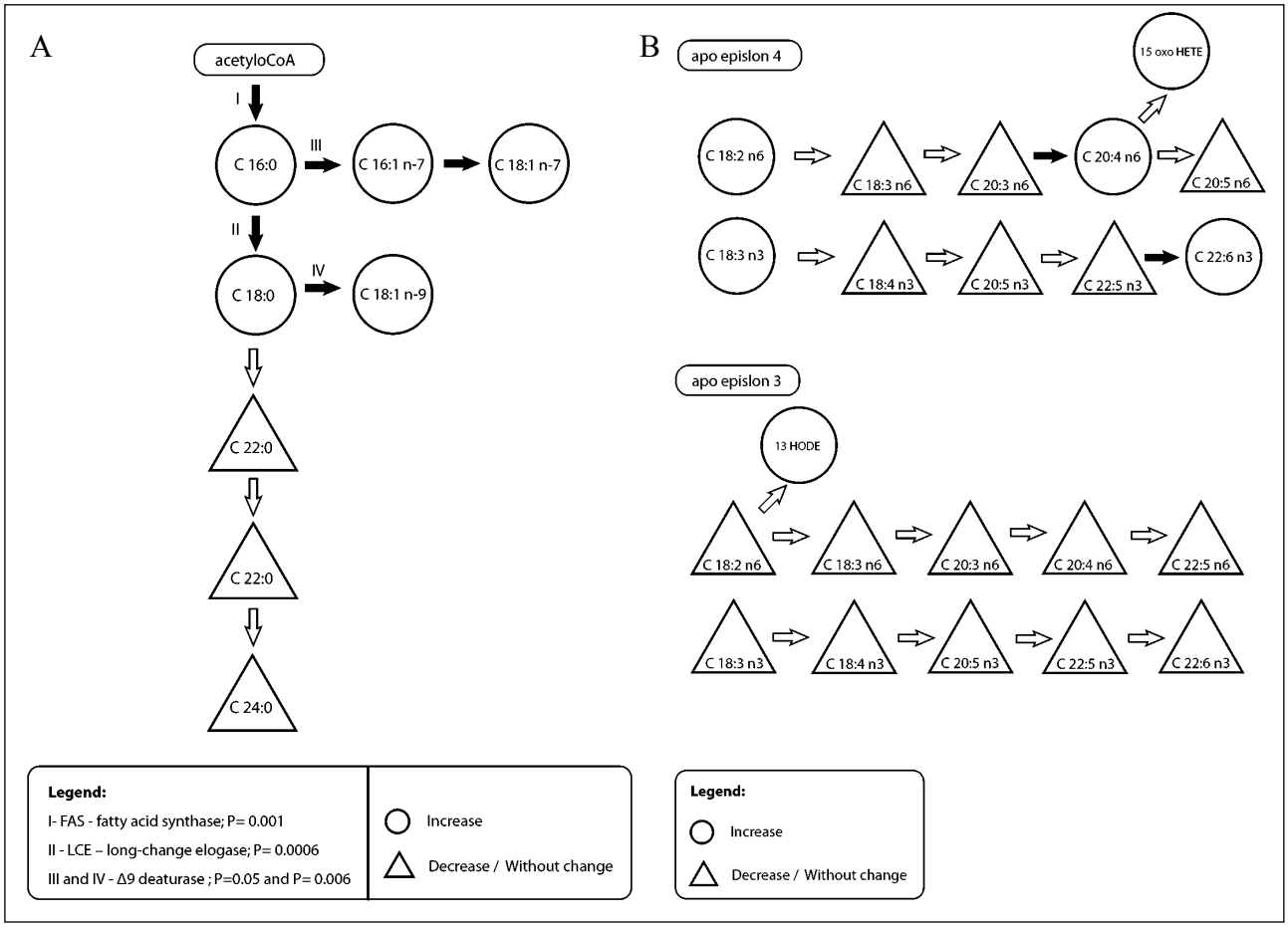

Fig. 1. Apo-E4 is associated with increased *de novo* lipogenesis of saturated fatty acids (A) and polyunsaturated fatty acids (B). A composite fatty acid methyl ester data from plasma is displayed as pathway maps. The activity of stearoyl CoA desaturase (Δ9 SCD) and other enzymes are displayed showing comparisons of *Apo-E4* versus *Apo-E3*.

well as plasma hyaluronic acid concentration increased significantly in patients with *Apo-E4* alleles, whereas 13-hydroxy-9,11-octadecadienoic acid (13HODE), a marker of lipid oxidation, only increased in the plasma of patients with *Apo-E3* alleles. In a previous study, the *Apo-E3* allele was strongly related to an increased risk of non-alcoholic steatohepatitis disease (which may be characterized by progression to liver fibrosis and cirrhosis) (4). It is probably premature to associate *Apo-E4* with a more intensive and more severe course of the disease. However, we hope that this work, which for the first time shows the significant differences in lipid composition between NAFLD patients as carriers of different *Apo-E* variants, will initiate a further research on the subject.

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