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RESEARCH PAPER

Influence of genotype on the modulation of gene and protein expression by n-3 LC-PUFA in rats

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Abstract It is becoming increasingly apparent that responsiveness to dietary fat composition is heterogeneous and dependent on the genetic make-up of the individual. The aim of this study was to evidence a genotype-related differential effect of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) on the modulation of hepatic genes involved in cholesterol metabolism. Fourteen spontaneously hypertensive (SH) rats, which present a naturally occurring variation in the gene encoding for sterol responsive element binding protein 1 (SREBP-1), contributing to their inherited variation in lipid metabolism, and 14 Wistar-Kyoto (WK) rats were fed a control diet or an n-3 LC-PUFA enriched diet for 90 days. Plasma lipid profile, total lipid fatty acid composition in plasma and liver, and the expression of SREBP-1 and 2, 3-hydroxy-3-methyl-glutaryl-CoA reductase, low-density lipoprotein receptor, and acyl-CoA:cholesterol acyltransferase 2 encoding genes and proteins were determined. The positive effect of the enriched diet on the serum lipid profile, particularly on total cholesterol and triglyceride level, was clearly evidenced in both WK and SH rats, but n-3 LC-PUFA acted through a different modulation of gene and protein expression that appeared related to the genetic background. Our study evidences a different transcriptional effect of specific nutrients related to genetic variants.

Keywords n-3 LC-PUFA · Gene expression · Protein expression · Genotype · Cholesterol · Spontaneously hypertensive rats

Abbreviations

AA	Arachidonic acid
ACAT-2	Acyl-CoA:cholesterol acyltransferase 2
AI	Atherogenic index
ALA	α-Linolenic acid
CI	Confidence interval
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
HDL-C	High-density lipoprotein cholesterol
HMGCR	3-Hydroxy-3-methyl-glutaryl-CoA reductase
HNF1α	Hepatocyte nuclear factor 1α
LA	Linoleic acid
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
n-3 LC-PUFA	n-3 long-chain polyunsaturated fatty acid
LXR	Sterol-responsive liver X receptor
n.s.	Not significant
PCSK9	Proprotein convertase subtilisin/kexin type-9
qPCR	Quantitative PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

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SH	Spontaneously hypertensive
Srebf	Sterol regulatory element binding transcription factor
SREBP	Sterol responsive element binding protein
St	Standard
TBST	Tris-buffered saline tween-20
TG	Triglyceride
TOT-C	Total cholesterol
WB	Western blotting
WK	Wistar-Kyoto

Introduction

n-3 long-chain polyunsaturated fatty acid (n-3 LC-PUFA) dietary intake and tissue content are closely related to numerous health outcomes including cardiovascular disease morbidity and mortality (Leaf 2006), immunological and inflammatory responses (Trak-Fellermeier et al. 2004; Kompauer et al. 2005), and mental health and psychiatric disorders (Muskiet and Kemperman 2006).

The response to eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) intervention is highly heterogeneous and supposed to be related to genetic variations; evidence existing on whether the effects of n-3 PUFA on health can be modulated by genetic variation in humans has been recently reviewed by Corella and Ordovás (2012) and Madden et al. (2011). Despite the fact that multiple observational studies have shown statistically significant interactions between n-3 PUFA and certain genetic variants, particularly related to the desaturase and the peroxisome proliferator-activated receptor-α encoding genes, firm conclusions cannot be established from intervention studies. To date, the most of the intervention studies were not basically designed to investigate the effect of a genetic variant or previously selected genetic variants, but to first undertake the intervention and then genotype the individuals. To do a priori, the choice of the main genetic variants to be studied and select individuals for their genotype could improve our knowledge on genotype influence on n-3 LC-PUFA effects.

The aim of this study was to verify whether the response to an n-3 LC-PUFA supplementation is different in spontaneously hypertensive (SH) and in Wistar-Kyoto (WK) rats, considered as SH control strain (Okamoto and Aoki 1963). Both SH and WK were inbred strains; since each inbred strain has a unique genotype, all individuals in each group were isogenic (genetically identical). Although every genetic component that plays a role in the development of systemic hypertension has not been identified, the main characteristics of SH rat genotype have been already reported in details (Greenhouse et al. 1990; Johnson et al. 1992). Among them, the variation in the sterol regulatory binding protein 1 (SREBP-1) encoding gene (*Srebf-1*) could contribute to the inherited variation in lipid metabolism observed in SH rats.

The SREBP family consists of the isoforms (SREBP-1 and SREBP-2) that are encoded by two different genes (*Srebf-1* and *Srebf-2)* (Shimomura et al. 1997). In contrast to SREBP-2, SREBP-1 is transcribed into two splice variants (Yokoyama et al. 1993): SREBP-1a, controlling cholesterol and lipid synthesis, and SREBP-1c, solely regulating the synthesis of fatty acids (Horton et al. 2003; Raghow et al. 2008). The relative activities of the two SREBP-1 isoforms differ: SREBP-1a is a potent activator of all SREBP-responsive genes, owing to its long transactivation domain encoded by its first exon, while in SREBP-1c, this exon encodes a shorter transactivation domain that is less potent than SREBP-1a (Horton et al. 2002). SH rats carry a valine to methionine substitution in the COOH terminal regulatory domain of SREBP-1; since SREBP-1a and SREBP-1c are derived from a different splicing of the same gene, with a nearly identical COOH terminal domain, the substitution is expected to be present in both isoforms (Pravenec et al. 2001).

When expressed at physiologic levels, the nuclear forms of all three SREBPs activate genes encoding multiple enzymes in the cholesterol and fatty acid biosynthetic pathways as well as the low-density lipoprotein receptor (LDLR) (Horton et al. 2003). Pravenec et al. (2008) evidenced that the variant *Srebf-1* form carried by SH rats underlies a quantitative trait locus influencing hepatic cholesterol levels in response to a high cholesterol diet, in agreement with the results of association studies indicating that common polymorphisms affecting SREBP-1 may influence cholesterol synthesis in humans (Laaksonen et al. 2006).

At present, no data are reported in the literature about a possible differential response to n-3 LC-PUFA due to SH genotype. In the present study, WK and SH rats were fed a standard diet or a diet supplemented with n-3 LC-PUFA, EPA (20:5 n-3) and DHA (22:6 n-3). At the end of the dietary treatment, the plasma lipid profile and the plasma and liver total lipid fatty acid composition were determined. Furthermore, since the modulation of gene transcription is one of the n-3 LC-PUFA main mechanisms of actions (Bordoni et al. 2007), the expressions of some genes as well of the corresponding encoded proteins were determined in the liver. We focused on genes encoding for SREBP-1 and SREBP-2 since the reported genetic variation of *Srebf-1* could interfere with the cholesterol-lowering effect of n-3-LC-PUFA, and on genes encoding for hydroxymethyl-glutaryl-coenzyme A reductase (HMGCR), LDLR, and acyl-CoA:cholesterol acyltransferase 2

(ACAT-2) since they have a central role in cholesterol metabolism and trafficking, and their transcriptional regulation is mediated by SREBP.

Materials and methods

Materials

Diets were from Mucedola (Milan, Italy). Chloroform, n-hexane, sodium chloride and Tris (hydroxymethyl) aminomethane were from Carlo Erba reagenti (Milan, Italy). Methanol, potassium chloride, sodium sulphate anhydrous, Tris-borate EDTA buffer, methanolic-HCl, Triton X-100, glycine, N,N,N′,N′-tetramethylethylenediamine, primary antibody anti-actin and secondary antibody anti-mouse were purchased from Sigma Chemical Co. (Milan, Italy). DNA Ladder, SYBR Safe DNA gel stain and Ultrapure Agarose were from Invitrogen (Paisley, UK). 40 % acrylamide/bis solution, ammonium persulfate, blotting-grade milk and sodium dodecyl sulphate (SDS) were from Bio-Rad Laboratories (Hercules, CA, USA). Premade primers QuantiTect Primer Assay 200 (*Srebf-1*, QT00432684; *Srebf-2*, QT00403305; *Acat-2*, QT00412461; *Ldlr*, QT00177744; *Hmgcr*, QT00182861; *Gapdh*, QT00199633) were from Qiagen (Hamburg, Germany). β-*Actin* was a custom primer purchased from Integrated DNA Technologies (Leuven, Belgium). Primary antibodies anti SREBP-1 (ab3259), SREBP-2 (ab30682), LDLR (ab30532), HMGCR (ab98018), ACAT-2 (ab123934) and Goat pAb secondary Ab to Rabbit IgG (ab6721) were from AbCam (Cambridge, UK).

Methods

Animals and diet

The n-3 LC-PUFA solution used for diet preparation contained EPA and DHA as ethyl esters, in the ratio 0.9:1.5, and was added in appropriate amount (0.1 % w/w) to the standard diet during its preparation. Protein (about 21 g/100 g), lipid (about 8 g/100 g) and carbohydrate content (about 61.5 g/100 g) was in the normal range of adequacy for rats in the standard diet, which contained appropriate amounts of vitamins and minerals.

Fourteen male WK and 14 male SH rats aged 14 weeks were used. Animals were housed in individual cages under strictly controlled condition of temperature (20 ± 2 °C) and humidity (60–70 %) with a 12-h dark–light cycle and were weighted each week. Water and food were provided ad libitum, and food consumption was registered daily. After 15 days of standard (St) diet, both WK and SH rats were randomly divided into two subgroups, one kept at the standard diet (WK–St and SH–St) and the other fed the n-3 LC-PUFA supplemented diet (WK-PUFA and SH-PUFA). The dietary treatment lasted 3 months; then, after an 8 h fasting, rats were weighted, anesthetized and killed. Blood samples were collected in heparinized test tubes, and livers were quickly excised and frozen at −80 °C in RNALater (Ambion Inc., Austin, TX, USA). Plasma was obtained by centrifugation at 800*g* for 5 min, and stored at −20 °C until analysis.

The Animal Care Committee of the University of Bologna approved the study (Prot. 49264-X/6).

Lipid profile and fatty acid (FA) composition

Plasma lipid profile was evaluated using commercial kits (Roche Diagnostics SpA, Milan, Italy) according to the manufacturer's instruction and determined spectrophotometrically using a Hitachi 911 auto analyser. LDL cholesterol (LDL-C) level and the atherogenic index (AI) were estimated indirectly using the following formulas:

$$\text{LDL-C} = \text{TOT-C} - [\text{HDL-C} + (\text{TG}/5)]$$

(Friedewald etal. 1972)

$$\text{AI} = (\text{TOT-C} - \text{HDL-C})/\text{HDL-C} \quad \text{(Choi et al. 1991)}$$

where HDL-C: high-density lipoprotein cholesterol, TG: triglyceride, and TOT-C: total cholesterol.

Total lipid FA composition was determined in plasma and liver by gas chromatographic analysis. Lipids were extracted from 300 µl plasma or 0.3 g hepatic tissue according to Folch et al. (1957), and methyl esterified according to Stoffel et al. (1959). Methyl esters dissolved in n-hexane were gas chromatographed on a Carlo Erba model 4160 (Milan, Italy) equipped with a capillary column (30 m × 0.25 mm i.d.) filled with a thermo stable stationary phase (SP 2340, 0.10–0.15 µm film thickness), at a programmed temperature (160–210 °C, with a 8 °C/min gradient), with He as carrier gas at a flow rate of 2 ml/min as previously reported (Righi et al. 2011). Gas chromatographic traces and quantitative evaluations were obtained using a Chrom Card Software (Thermo Electron Scientific, Milan, Italy) computing integrator.

RNA extraction, reverse transcription and gene expression analysis

Thirty micrograms of hepatic tissue were mechanically disrupted by sterile scissors and homogenized using QIAshredder (Qiagen, Hamburg, Germany) according to manufacturer's instructions. RNA was extracted using RNeasy mini kit (Qiagen, Hamburg, Germany) and eluted in a final volume of 50 µl. Samples purity was assessed by NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription was performed on 200 ng of extracted RNA in a 20 µl total

reaction volume using QuantiTect reverse transcription kit (Qiagen, Hamburg, Germany) as follows: samples were incubated for 2 min at 42 °C with gDNA Wipeout buffer to eliminate possible genomic DNA contamination, and then quickly put in ice, adding the reverse transcription mix containing also RNAse inhibitors. Optimized blend of oligo-dT and random primers were used as primers. Reaction conditions were as follows: at 45 °C for 15 min, at 95 °C for 3 min and at 4 °C for 5 min. Obtained cDNA was quickly stored at −20 °C, and relative gene expression analysis was performed on RotorGene 6000 (Corbett Research, Mortlake, Australia) by two-step quantitative PCR (qPCR) assay using SYBR green detection (Qiagen, Hamburg, Germany). Amplification was done in a 25 µl final volume including 1 µl of cDNA as template. The PCR master mix was prepared according to Qiagen protocol, and amplification conditions were as follows: 15 min at 95 °C followed by 45 cycles (94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s). Melt curves were obtained at 95 °C for 15 s. Amplicon length was assessed using 2 % agarose gel electrophoresis using SYBR safe DNA gel stain (Invitrogen, Paisley, UK). The primer pairs used were from Qiagen (Hamburg, Germany). β-*Actin* primer was a custom primer: forward GGGAAATCGTGCGTGACATT (20 bp) and reverse GCGGCAGTGGCCATCTC (17 bp). Amplicon length was 76 bp on the recognized sequence NM_031144. Glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*) and β-*actin* were chosen as reference genes given that it is reported that fish oil supplementation has no effect on GAPDH expression (Xu et al. 2002); moreover, β-*actin* was previously used in gene expression study involving PUFA supplementation (Caplan et al. 2001; Vara Prasad et al. 2010). Primer specificity was evaluated using a melt curve that showed unique specific peak in all cases. qPCR validation was carried out by standard curve. Reaction efficiency and *R* value were in the range of 80–100 % and 0.99, respectively, for all examined genes. Three expression technical replicates were performed on reverse transcription duplicates. Seven biological replicates for each experimental group were used. Relative quantification of the mRNA levels of all examined genes was determined using the RotorGene 6000 software 1.7 comparative quantification analysis, in which reaction efficiency is calculated on each reaction tube. All samples used had >85 % efficiency.

Protein extraction and western blotting (WB)

Total proteins were extracted from 200 mg liver using T-PER tissue protein extraction reagent in presence of 1 % halt protease inhibitor cocktail according to the manufacturer (Thermo Fisher Scientific, Pittsburgh, PA, USA). The amount and purity of proteins were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Fifty micrograms of proteins from each sample was fractionated by 8 % SDS-PAGE gel (90 mV for 20 min and then 120 mV for 80 min), and transferred (100 mV, 120 min) onto a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). The membrane was blocked with Tris-buffered saline tween-20 (TBST) containing 5 % fat-free milk for 1 h at room temperature, and incubated overnight at 4 °C with primary antibodies directed against SREBP-1 (1:500), SREBP-2 (1:250), LDLR (1:500), HMGCR (1:1,000) and ACAT-2 (1:2,000), respectively. After washing 3 times with TBST, the membrane was incubated with horseradish-peroxidase conjugated secondary anti-mouse (Sigma Chemical Co., Milan, Italy) or anti-rabbit (AbCam, Cambridge, UK) antibodies at room temperature for 1 h. Immunoreactive bands were visualized with an enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK) on ChemiDoc MP System and quantified by Image Lab software version 4.0 (Bio-Rad Laboratories, Hercules, CA, USA). The intensities of bands were expressed relative to the actin intensities (antibody incubation overnight 4 °C at the concentration of 1:1,000) from the same membrane restored using restore plus western blot stripping buffer (Thermo Fisher Scientific, Pittsburgh, PA, USA) for 15 min in soft shaking.

Statistical analysis

Statistical analysis for body weight, lipid profile, FA composition and protein expression data was performed by the one-way ANOVA with Tukey's as post-test. Statistical analysis of gene expression data was performed by REST 2009 software in RotorGene (RG) mode that uses Taylor's series to find statistical differences, as shown by Pfaffl et al. (2002). Gene expression was normalized on the reference genes *Gapdh* and *β-actin*, and all sample groups were referred to WK–St rats (calibrator group). This normalization underlines fold-changes between sample and the calibrator group (WK–St) that becomes the baseline.

Results

Although in the beginning of the trial SH rats appeared smaller in size than WK counterparts, and their increase in body weight along the dietary intervention was lower, within each strain no differences ascribable to the n-3 LC-PUFA supplementation in body weight gain and food consumption were detected (data not shown).

The FA composition of plasma total lipid (Fig. 1a) showed in SH–St rats a higher content of arachidonic acid (20:4 n-6, AA), and a lower content of linoleic (18:2 n-6,

LA) and α-linolenic acid (C 18:3 n-3, ALA) than in WK–St. EPA and DHA plasma concentration increased in both PUFA-fed groups, confirming the absorption of the supplemented FA. In WK rats this increase was through a decrease in LA and ALA content, while in SH ones through a decrease in AA content.

No differences in liver FA composition were detected between standard diets fed animals (Fig. 1b); in both groups, dietary EPA and DHA were incorporated also in the hepatic tissue, mainly at the expense of AA.

When fed the standard diet, SH rats evidenced lower TOT-C, HDL-C, and triglyceride (TG) plasma levels, a higher LDL-C plasma concentration and an almost doubled AI compared to WK animals (Table 1). The PUFA diet decreased TOT-C and TG concentration in both groups; in SH rats, a decrease in LDL-C and an increase in HDL-C were also observed. Consequently, the AI, extremely higher in SH–St rats than in the WK counterparts, was significantly reduced by the PUFA diet.

Srebf-1 gene expression (Fig. 2a) was similar in WK and SH rats fed the standard diet, and it was down-regulated in SH-PUFA animals compared to both standard fed groups and WK-PUFA. In standard condition, the expression of the uncleaved form of SREBP-1 was significantly lower in the SH strain (Fig. 2b), while the level of the cleaved protein was similar (Fig. 2c); for both the inactive and active form, no modification ascribable to the PUFA diet were detected.

No differences ascribable to the strain or the diet were detected in *Srebf-2* gene expression (Fig. 3a). In the two strains of standard diet-fed animals, the expression of both uncleaved and cleaved SREBP-2 protein was similar, and appeared decreased by the PUFA diet in the WK one only (Fig. 3b, c).

Although in standard diet-fed animals no difference was observed in *Hmgcr* gene expression between the WK and SH strains (Fig. 4a), HMGCR protein level was significantly lower in the latter than in the former (Fig. 4b). The

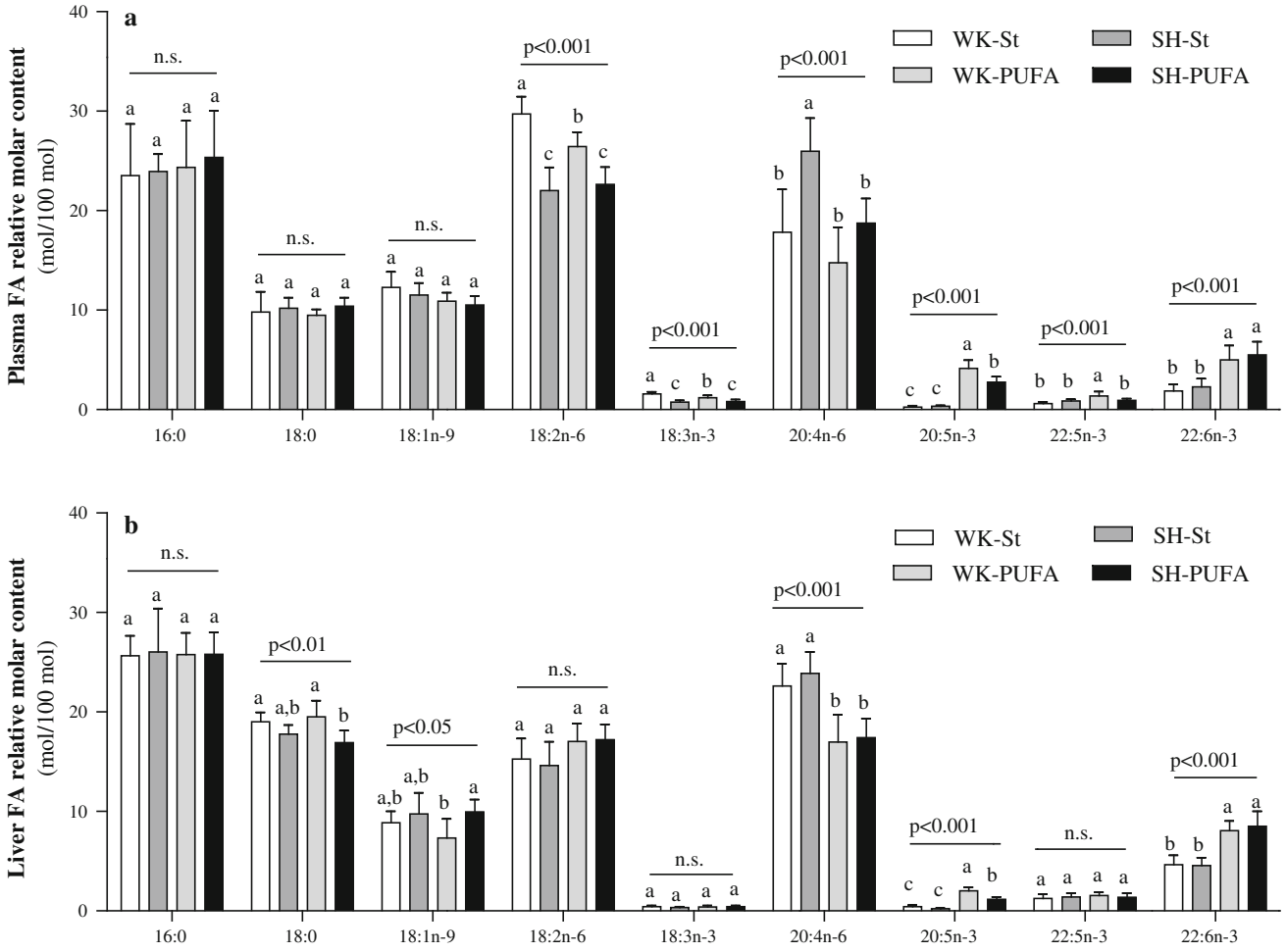
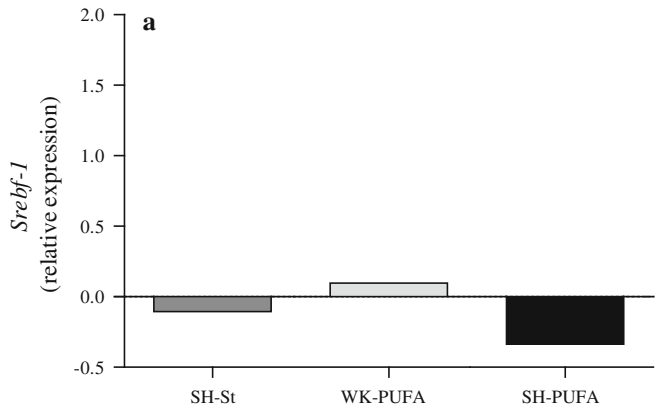


Fig. 1 Plasma (**a**) and liver (**b**) total lipid fatty acid composition in WK and SH rats fed the different diets. Data are mean ± SD of seven animals per group. Statistical analysis was performed by the one-way ANOVA using Tukey's as post-test. For each fatty acid, *bars* with different *superscript letters* are significantly different (at least $p < 0.05$)

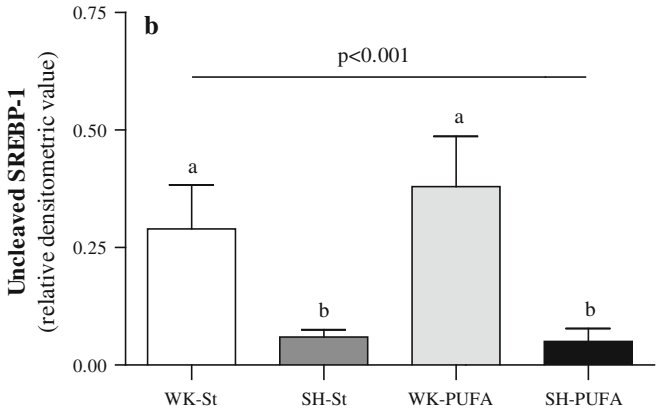
Table 1 Plasma lipid profile in WK and SH rats after the dietary treatment

	WK–St	SH–St	WK-PUFA	SH-PUFA
TOT-C (mg/dl)	107.60 ± 2.70[a]	91.60 ± 4.83[b]	88.40 ± 2.30[b]	73.40 ± 3.65[c]
HDL-C (mg/dl)	41.80 ± 2.49[a]	26.00 ± 3.74[c]	37.20 ± 2.28[a,b]	31.20 ± 5.85[b]
LDL-C (mg/dl)	26.24 ± 6.19[b]	41.48 ± 9.57[a]	25.48 ± 3.04[b]	23.88 ± 6.14[b]
TG (mg/dl)	197.80 ± 14.20[a]	120.60 ± 12.50[b]	128.60 ± 12.44[b]	91.60 ± 11.28[c]
AI	1.44 ± 0.29[b]	2.59 ± 0.60[a]	1.38 ± 0.14[b]	1.41 ± 0.41[b]

Data are mean ± SD of seven animals per group. Statistical analysis was performed by the one-way ANOVA ($p < 0.001$ for all tested parameters) using Tukey's as post-test, which allowed a multiple comparison among the four groups. In each row, values with different superscript letters are significantly different (at least $p < 0.05$)



	median expression	range (min - max)	95% C.I.
WK-St	1	-	-
SH-St	0.894	0.654 - 1.321	0.563 - 2.051
WK-PUFA	1.097	0.671 - 1.506	0.559 - 2.920
SH-PUFA	0.663	0.471 - 0.988	0.421 - 1.522
statistic pairs		**p-value**	**result**
WK-St	SH-St	0.440	not changed
WK-St	WK-PUFA	0.602	not changed
WK-St	SH-PUFA	0.023	DOWN
SH-St	SH-PUFA	0.021	DOWN
WK-PUFA	SH-PUFA	0.008	DOWN
SH-St	WK-PUFA	0.158	not changed



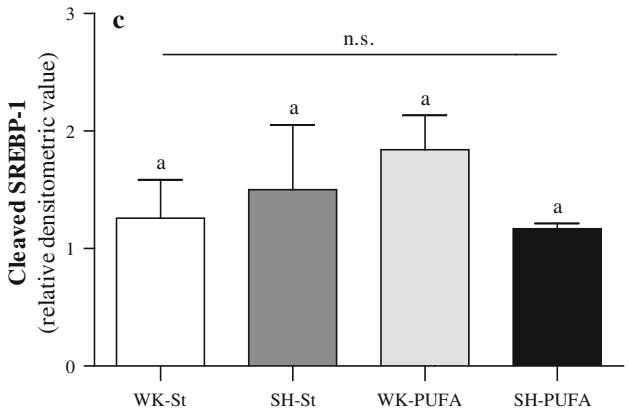
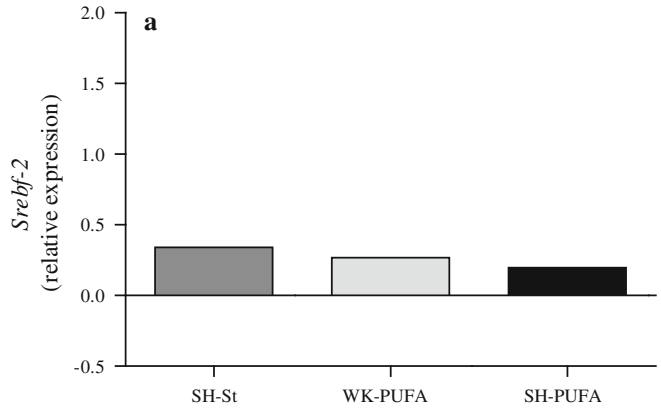


Fig. 2 SREBP-1 gene (**a**) and protein (**b**, **c**) expression in the liver of WK and SH rats fed the different diets. *Srebpf-1* expression (**a**). Data in the graph are represented as [(sample ratio) − (unitary calibrator group ratio)]; significant differences were evaluated with Taylor's series statistical analysis. Gene expression in each group ($n = 7$) is reported as median, range (min–max), and 95 % CI in the corresponding table, where statistic pairs, p value and resulting differences are also indicated. Uncleaved (**b**) and cleaved (**c**) SREBP-1 protein expression. Data in the graphs are mean ± SD of four animals per group and are represented as densitometric value normalized on actin. Statistical analysis was performed by the one-way ANOVA using Tukey's as post-test; different superscript letters indicate statistical significance (at least $p < 0.05$)

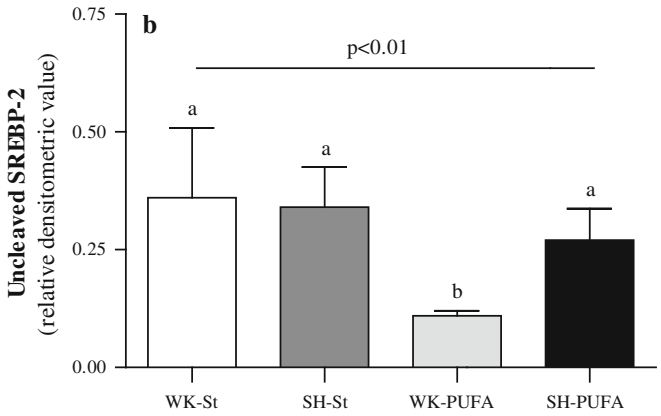
n-3 LC-PUFA supplementation decreased *Hmgcr* transcription and the level of the encoded protein in both WK and SH animals compared to their standard diet-fed counterparts; *Hmgcr* transcription was similar in WK-PUFA and SH-PUFA rats, while HMGCR protein level was significantly higher in the former than in the latter group.

In standard diet-fed animals, *Ldlr* gene (Fig. 5a) and LDLR protein (Fig. 5b) expression were higher in the SH than in the WK strain. In the latter, they were not modified by the PUFA diet, while they were down-regulated in the former.

Finally, *Acat-2* gene expression was higher in the SH-St than in the WK–St rats, and it was further up-regulated in



	median expression	range (min - max)	95% C.I.
WK-St	1	-	-
SH-St	1.340	0.916 - 1.965	0.694 - 2.758
WK-PUFA	1.268	0.827 - 1.806	0.619 - 2.898
SH-PUFA	1.197	0.814 - 1.543	0.719 - 2.066
statistic pairs		**p-value**	**result**
WK-St	SH-St	0.074	not changed
WK-St	WK-PUFA	0.218	not changed
WK-St	SH-PUFA	0.184	not changed
SH-St	SH-PUFA	0.180	not changed
WK-PUFA	SH-PUFA	0.729	not changed
SH-St	WK-PUFA	0.750	not changed



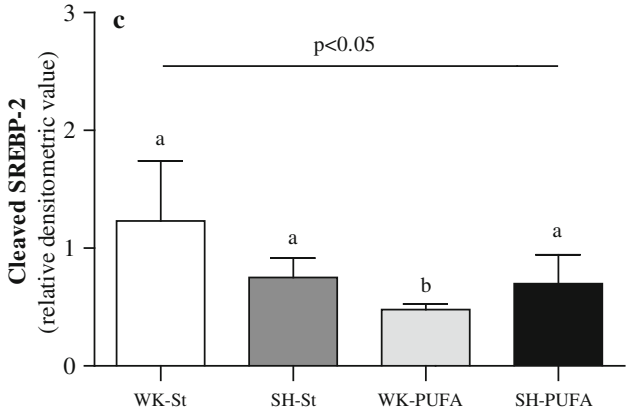


Fig. 3 SREBP-2 gene (**a**) and protein (**b,** c) expression in the liver of WK and SH rats fed the different diets. *Srebpf-2* expression (**a**). Data in the graph are represented as [(sample ratio) − (unitary calibrator group ratio)]; significant differences were evaluated with Taylor's series statistical analysis. Gene expression in each group ($n = 7$) is reported as median, range (min–max), and 95 % CI in the corresponding table, where statistic pairs, p value and resulting differences are also indicated. Uncleaved (**b**) and cleaved (**c**) SREBP-2 protein expression. Data in the graphs are mean ± SD of four animals per group and are represented as densitometric value normalized on actin. Statistical analysis was performed by the one-way ANOVA using Tukey's as post-test; different superscript letters indicate statistical significance (at least $p < 0.05$)

the SH-PUFA compared to both standard fed groups and WK-PUFA; notwithstanding no significant differences related to strain or diet were detected in the encoded protein level (Fig. 6a, b, respectively).

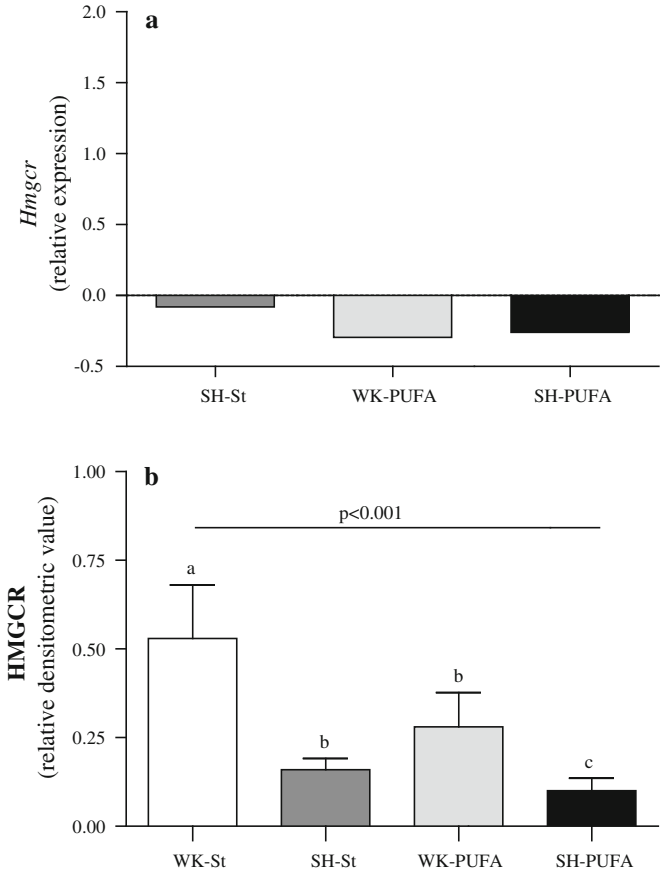
Discussion

It is becoming increasingly apparent that responsiveness to dietary fat composition is highly heterogeneous and dependent on the genetic make-up of the individual. The aim of this study was to verify the influence of different genotypes on the modulation of the expression of selected genes in response to EPA and DHA dietary supplementation, using WK and SH rats as a model.

As indicated by the plasma and liver FA composition, in both strains the supplemented FA were absorbed, and incorporated in liver mainly at the expense of AA, reflecting the previously reported metabolic competition between the two PUFA families (Mu et al. 2006; Simopoulos 2008).

The PUFA diet significantly and positively modified lipemic values in both strains, decreasing TOT-C and TG level. These modifications, as well as the decrease in LDL-C and the increase in HDL-C, significantly reduced the AI in SH rats. Dyslipidaemia is one of the principal causative factor contributing to the atherogenic processes (Steinberg 2004), and the AI is commonly used as the finest lipid parameter to predict human CVD risk (Vinson et al. 2001). The AI lowering effect of n-3 LC-PUFA has been previously reported by Deutch et al. (2000) and Kabir et al. (2007) in women, and our data confirm the importance of EPA and DHA in the prevention of dyslipidaemia and related diseases, emphasizing the need of a better understanding of the molecular process involved.

The transcriptional response to dietary PUFA appeared greatly affected by genotype; in fact, the expression of 4 out of 5 genes (*Srebpf-1*, *Hmgcr*, *Ldlr*, and *Acat-2*)



	median expression	range (min - max)	95% C.I.
WK-St	1	-	-
SH-St	0.921	0.622 - 1.333	0.486 - 1.698
WK-PUFA	0.706	0.475 - 1.053	0.372 - 1.389
SH-PUFA	0.741	0.539 - 1.041	0.385 - 1.321
statistic pairs		**p-value**	**result**
WK-St	SH-St	0.566	not changed
WK-St	WK-PUFA	0.047	DOWN
WK-St	SH-PUFA	0.060	not changed
SH-St	SH-PUFA	0.040	DOWN
WK-PUFA	SH-PUFA	0.743	not changed
SH-St	WK-PUFA	0.034	DOWN

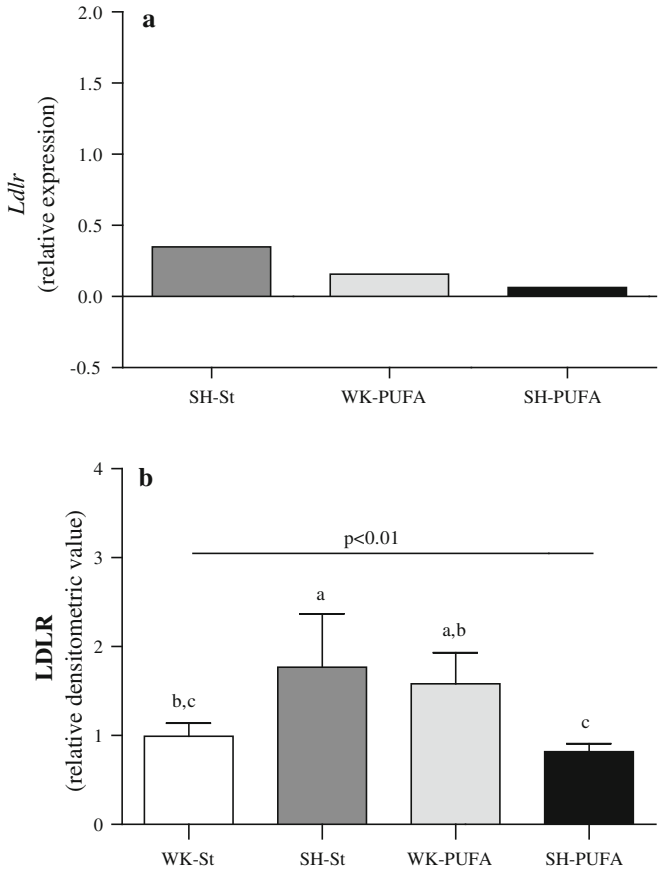
Fig. 4 HMGCR gene (**a**) and protein (**b**) expression in the liver of WK and SH rats fed the different diets. *Hmgcr* gene expression (**a**). Data in the graph are represented as [(sample ratio) − (unitary calibrator group ratio)]; significant differences were evaluated with Taylor's series statistical analysis. Gene expression in each group ($n = 7$) is reported as median, range (min–max), and 95 % CI in the corresponding table, where statistic pairs, p value and resulting differences are also indicated. HMGCR protein expression (**b**). Data in the graph are mean ± SD of four animals per group and are represented as densitometric value normalized on actin. Statistical analysis was performed by the one-way ANOVA using Tukey's as post-test; different superscript letters indicate statistical significance (at least $p < 0.05$)

appeared modulated in SH animals, while only *Hmgcr* was down-regulated in WK ones. The impact of the genetic variation was less evident considering the level of the corresponding proteins, confirming that gene expression at the mRNA level is generally informative but not predictive for that at the protein level.

Although EPA and DHA effect on *Srebf-1* expression was influenced by the genetic variation, no significant modifications in the level of both uncleaved and cleaved SREBP-1 were detected upon PUFA supplementation in either WK or SH rats. This discrepancy from previous studies could be dependent on the different experimental model, that is in vivo study versus cultured liver cells (Kohan et al. 2011), to the different FA concentration in the diet and to the different length of the dietary treatment (Lu et al. 2011), or to the use of purified EPA and DHA in spite of oils for dietary supplementation (Yang et al. 2011). Although the inability of PUFA in modifying active SREBP-1 level in our experimental condition, as well as the low level of uncleaved SREBP-1 observed in SH rats, deserves further attention to our aim, the most relevant data were the evidence of a different modulation of *Srebpf-1* expression by PUFA in the two strains.

It is well documented that n-3 LC-PUFA do not interfere with *Srebpf-2* transcription, and they inhibit the activating cleavage of SREBP-2 via indirect mechanisms involving increased flow of free cholesterol between the plasma membrane and intracellular membranes (Di Nunzio et al. 2010; Ma et al. 2004). Accordingly, *Srebpf-2* gene expression was not modified by PUFA in both strains; notably the modulation of SREBP-2 cleavage by EPA and DHA was detected in WK rats only.

According to Notarnicola et al. (2010) in both WK and SH strain, the PUFA diet caused a down-regulation of *Hmgcr* transcription and translation, which was consistent with the observed decrease in plasma TOT-C after the

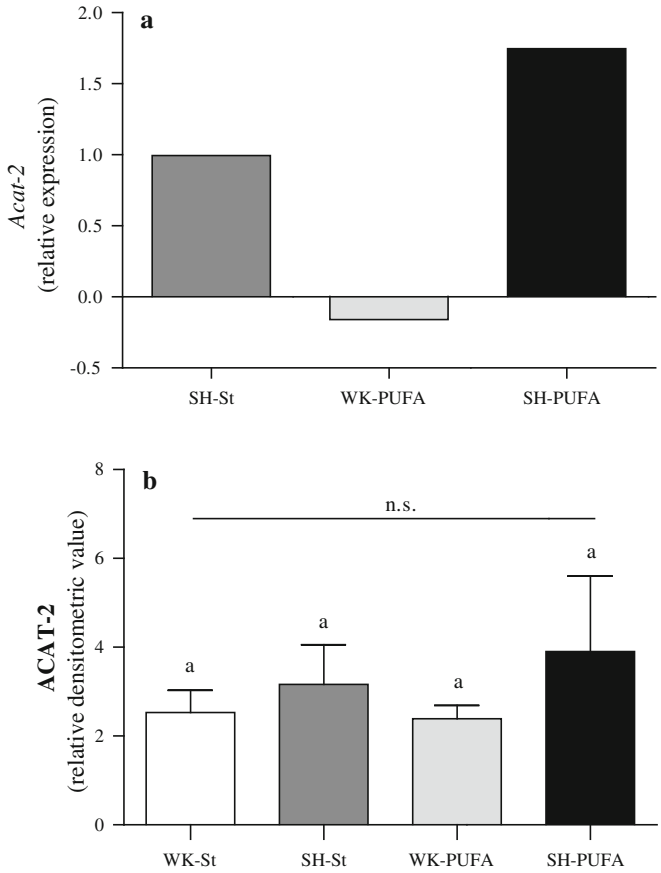


	median expression	range (min - max)	95% C.I.
WK-St	1	-	-
SH-St	1.349	1.157 - 1.603	0.895 - 1.828
WK-PUFA	1.157	0.927 - 1.438	0.706 - 2.031
SH-PUFA	1.063	0.852 - 1.306	0.694 - 1.487
statistic pairs		**p-value**	**result**
WK-St	SH-St	0.002	UP
WK-St	WK-PUFA	0.216	not changed
WK-St	SH-PUFA	0.511	not changed
SH-St	SH-PUFA	0.001	DOWN
WK-PUFA	SH-PUFA	0.393	not changed
SH-St	WK-PUFA	0.099	not changed

Fig. 5 LDLR gene (**a**) and protein (**b**) expression in the liver of WK and SH rats fed the different diets. *Ldlr* gene expression (**a**). Data in the graph are represented as [(sample ratio) − (unitary calibrator group ratio)]; significant differences were evaluated with Taylor's series statistical analysis. Gene expression in each group ($n = 7$) is reported as median, range (min–max), and 95 % CI in the corresponding table, where statistic pairs, p value and resulting differences are also indicated. LDLR protein expression (**b**). Data in the graph are mean ± SD of four animals per group and are represented as densitometric value normalized on actin. Statistical analysis was performed by the one-way ANOVA using Tukey's as post-test; different superscript letters indicate statistical significance (at least $p < 0.05$)

dietary intervention. Although *Hmgcr* expression is considered to be controlled by SREBPs (Fuhrman et al. 2007), the different SREBP-2 level after the dietary treatment let us hypothesize different mechanisms and transcription factors for the PUFA-induced reduction in *Hmgcr* expression in the two strains. As a possibility, unsaturated FA have been recognized as ligands for the orphan nuclear receptor Nur77 (Vinayavekhin and Saghatelian 2011) that has been recently shown to regulate HMGCR expression in cultured liver cells (Zhang et al. 2012).

The differential effect of PUFA in the two genotypes was particularly evident for LDLR. In WK rats, the treatment caused an increase in LDLR protein expression, as already reported in HepG2 cells (Yu-Poth et al. 2005), without any modification in gene expression, while in the SH strain, EPA + DHA supplementation reduced both gene and protein expression. The decreased LDLR level in SH-PUFA rats could appear in contrast to the observed decrease in plasma LDL-C, but it is worth noting that in rats, most of the cholesterol taken up into the liver from serum is HDL-C rather than LDL-C, serum LDL-C being regulated more by cholesterol and LDL biosynthesis than by liver LDL uptake (Tsutsumi et al. 2001). The effect of PUFA on *Ldlr* transcription is controversial, and both down- and up-regulations have been observed (Lindsey et al. 1992; Zheng et al. 2002). Our data suggest that the modulation of LDLR gene and protein expression by PUFA is also related to genotypes, and involves mechanisms and transcription factors other than SREBP-1 and -2. A dual control system (on the synthesis and on the degradation end) of hepatic LDLR expression is currently recognized (Attie and Seidah 2005), and different observations support a SREBP-independent PUFA effect on the regulation of LDLR level (Bjermo et al. 2012; Kuang et al. 2012). Furthermore, Yu-Poth et al. (2005) suggested that PUFA may regulate LDLR expression via a pathway that is



	median expression	range (min - max)	95% C.I.
WK-St	1	-	-
SH-St	1.994	1.443 - 2.762	1.084 - 3.486
WK-PUFA	0.841	0.613 - 1.176	0.441 - 1.468
SH-PUFA	2.747	2.010 - 3.831	1.374 - 4.804
statistic pairs		**p-value**	**result**
WK-St	SH-St	0.002	UP
WK-St	WK-PUFA	0.212	not changed
WK-St	SH-PUFA	<0.001	UP
SH-St	SH-PUFA	0.021	UP
WK-PUFA	SH-PUFA	<0.001	UP
SH-St	WK-PUFA	<0.001	DOWN

Fig. 6 ACAT-2 gene (**a**) and protein (**b**) expression in the liver of WK and SH rats fed the different diets. *Acat-2* gene expression (**a**). Data in the graph are represented as [(sample ratio) − (unitary calibrator group ratio)]; significant differences were evaluated with Taylor's series statistical analysis. Gene expression in each group ($n = 7$) is reported as median, range (min–max), and 95 % CI in the corresponding table, where statistic pairs, p value and resulting differences are also indicated. ACAT-2 protein expression (**b**). Data in the graph are mean ± SD of 4 animals per group and are represented as densitometric value normalized on actin. Statistical analysis was performed by the one-way ANOVA using Tukey's as post-test; different superscript letters indicate statistical significance (at least $p < 0.05$)

independent of SREBP and involves ACAT activity. In this study, *Acat* gene expression was up-regulated by PUFA in SH animals, and the level of the encoded protein increased although not reaching significant value. Lin et al. (2004) evidenced in hamsters that the amount of dietary cholesterol is an important factor in determining the mode and extent of effects of dietary n-3 LC-PUFA on ACAT; our data indicate that also genotype has a role in the final response.

In conclusion, our data confirm the positive effect of EPA and DHA on the serum lipid profile in both WK and SH rats, but despite the similar final outcomes gene and protein expression analyses showed differential, genotype-related effect of n-3 LC-PUFA. In recent years, a more comprehensive understanding of the impact of PUFA intake on the regulation of genes involved in lipogenesis has emerged, and a number of common gene variants have been identified which may be important determinants of the blood lipid response to altered dietary fat composition. The authors are aware that the use of animals represents a limitation of the present study, but it allowed to strictly regulate the diet and to investigate defined genetic models in order to check diet–gene interaction evaluating the modulation of the effect of specific nutrients due to genetic variants.

The reported different influence of n-3 LC-PUFA on the expression of specific gene and protein cannot attributed to single gene variations but rather to the whole genotype, and improve our scientific awareness of genotype influence on the effects of bioactive molecules. Although further studies are needed, the reported observations could contribute to ongoing nutrigenetics and nutrigenomics research aimed at

the development of personalized dietary advice, optimized to suit the individual, that may also improve end-user motivation for dietary changes.

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