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Selective Action of Human Sera Differing in Fatty Acids and Cholesterol Content on In Vitro Gene Expression

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ABSTRACT

Serum constituents might directly affect metabolic diseases pathogenesis and are commonly used as diagnostic tool. The aim of this study was to investigate the human serum effect on in vitro gene expression, related to nutrients action and involved in lipid metabolism. In detail, 40 human sera were firstly analyzed in fatty acids profile by gas-chromatography. Then samples were tested through direct addition within culture medium on Hep G2 human hepatoma cells, comparing samples from hypercholesterolemic (average 273 mg/dl) versus normocholesterolemic male subjects (average 155 mg/dl), since this condition is a relevant disease risk factor and is typically consequent to nutritional style. Hypercholesterolemic sera produced a 0.4-fold reduction of sterol regulatory element binding protein 1c (SREBP-1c) mRNA ($P < 0.05$) and a 1.5-fold increase of UDP-glucuronosyltransferase 1A1 (UGT1A1) mRNA ($P < 0.01$). Samples with higher concentrations of n-6 fatty acids produced a higher expression of UGT1A1 mRNA. Total fatty acids [docosahexaenoic, eicosopentanoic, arachidonic, linolenic, and linoleic acid (DHA, EPA, AA, LNA, and LA, respectively)] in each serum resulted roughly inverse with trend of SREBP-1c mRNA expression. Serum AA, LA, and *trans* fatty acids were more abundant in hypercholesterolemic subjects ($P < 0.01$) while DHA as quota of detected fatty acids was significantly higher in normocholesterolemic subjects ($P < 0.05$). While it is not possible to indicate which component was responsible for the observed gene modulations, our data indicate that sera differing in lipid profiles, mainly associated with dietary behavior, differentially affect gene expression known to be involved in metabolic and nutritional related conditions. J. Cell. Biochem. 113: 815–823, 2012.

KEY WORDS: CHOLESTEROL; GENE EXPRESSION; POLYUNSATURATED FATTY ACIDS; SERUM LIPIDS; DOCOSAHEXANEOIC ACID; EICOSOPENTANOIC ACID

Both in physiological and pathological conditions, human serum composition results from a combined action of endogenous metabolism and nutritional intake. Lipid component quality and quantity of human serum are relevant reference markers of cardiovascular disease risk and mortality [Warensjö et al., 2008]. Dietary fat intake strictly affects serum lipid profile and cardiovascular disease epidemiology. In particular, fatty acids derived from diet have different effects on disease risk: higher intakes of long chain n-3 fatty acids from fish oil has been consistently reported as lowering disease incidence while *trans* fatty acids had negative influence [Erkkilä et al., 2008]. Molecular mechanisms accounting for these effects are numerous and partially known; n-3 fatty acids have distinct capacities, selectively influencing both cellular metabolic functions and gene transcription and expression [Deckelbaum et al., 2006]. Serum composition and more particularly its lipids, including fatty acids, might then exert a direct role specifically affecting tissue gene expression both within physiological and pathological states.

Abbreviations used: DHA, docosahexaenoic acid; EPA, eicosopentanoic acid; AA, arachidonic acid; LNA, linolenic acid; LA, linoleic acid; FAME, fatty acid methyl esters; FBS, fetal bovine serum; FID, flame ionization detection; LXR, liver X receptor; RXR, retinol X receptor; SREBP-1c, sterol regulatory element binding protein 1c; SCD, stearoyl-CoA desaturase; UGT1A1, UDP-glucuronosyltransferase 1A1; UCP2, uncoupling protein 2.

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Since fatty acids composition of human serum is also related to diet quality [Bethune et al., 2006], we decided to evaluate if serum samples differing in lipid content could differently affect the expression of genes involved in mechanisms related to dyslipidemic diseases development. We have previously identified a number of genes which are differentially modulated from diet lipid content [Eletto et al., 2005]. Diet content in polyunsaturated fatty acids (PUFA) resulted to modulate numerous mRNA within rat liver. Among these genes we selected sterol regulatory element binding protein 1c (SREBP-1c), stearoyl-CoA desaturase (SCD), and UDP-glucuronosyltransferase 1A1 (UGT1A1), which we also found synergistically regulated from docosahexaenoic acid (DHA) and *Vitamin E* [Caputo et al., 2008]. We also analyzed uncoupling protein 2 (UCP2), which is known to be specifically affected by fatty acids [Thompson and Kim, 2004].

SREBP-1c, SCD, UGT1A1, and UCP2 genes are also known to be regulated through mechanisms involving heterodimeric nuclear receptors including peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), and retinoid X receptor (RXR) [Chawla et al., 2001; Ide et al., 2003; Xie et al., 2003; Frank et al., 2005]. The activity of SREBP-1c as transcriptional factor is generally considered a fundamental process within metabolic effects of dietary lipid intake and also in atherogenesis development [Lin et al., 2005]. SREBP-1c expression is controlled by hormonal and nutritional conditions and it is known to be reduced by PUFA enriched diets [Sampath and Ntambi, 2006]. UGT1A1 is an enzyme of phase II drug metabolism which has been already described as modulated by xenobiotics and dietary derived molecules [Sugatani et al., 2004]. SCD protein product is directly involved in fatty acids metabolism for its desaturase enzymatic activity and its expression is directly regulated by SREBP-1c. UCP2 is a carrier protein that lowers the efficiency of mitochondrial oxidative phosphorylation and was reported as increased in adipose, muscle, and heart tissues within physiological and pathological states with elevated plasma fatty acids [Thompson and Kim, 2004; Yonezawa et al., 2009].

Since SREBP-1c, SCD, UGT1A1, and UCP2 genes are modulated from nutrients as PUFA [Hun et al., 1999; Caputo et al., 2008; Di Nunzio et al., 2010], it is also conceivable that clinical serum samples from different individuals, containing different lipid profiles, could differentially exert expression modulations of these genes when directly added to cultured human liver cells. Because cholesterol concentration is the mostly diet-related and disease-related blood parameter, two groups of serum samples with very different concentrations of this steroid were selected to be compared.

MATERIALS AND METHODS

SERUM SAMPLES

Sixty-two serum samples were collected within the Clinical Pathology Laboratory, Santa Maria Goretti Hospital, AUSL Latina, Italy, from informed subjects undergoing periodical routine controls. The study was approved by the institution in accordance with its ethical standards and with the current revision of the Helsinki Declaration. All subjects were apparently healthy males aged between 40 and 50 years. Exclusion criteria eliminated samples from subjects with actual diseases or with abnormal values, beside those from hypercholesterolemic subjects, when tested for total cholesterol, HDL, total triacylglycerols (TAG), glucose, urea nitrogen, bilirubins, measured by an Architect c8000 (Abbott Laboratories, IL) analyzer, and complete blood cell count and ESR, performed with a XE-2100 (Sysmex Corporation, Kobe, Japan) and a BD SEDI-15 (Becton Dickinson, Plymouth, UK) instruments, respectively.

ASSAY OF SERUM FATTY ACIDS

Lipid standards [methyl esters of linoleic acid (LA, 18:2 n-6); linolenic acid (LNA, 18:3 n-3); arachidonic acid (AA, 20:4 n-6); eicosopentanoic acid (EPA, 20:5 n-3); DHA (22:6 n-3); elaidic acid (*trans*-9 18:1)] were from Sigma–Aldrich (St. Louis, MO).

For serum fatty acids determination 50 μg of tridecanoic acid (13:0), as internal standard, were added to a solution of 100 μl of serum in 2 ml of methanol-benzene 4:1 (v/v) [Lepage et al., 1989]. The mixture was slowly added with 200 μl of acetyl chloride, under magnetic stirring, over a period of 1 min, and tightly closed with teflon-lined caps. After stirring at 100°C for 1 h, the mixture was cooled to room temperature. A 6% K_2CO_3 solution (5 ml) was slowly added with stirring to stop reaction and neutralize mixture, which was then shaken and centrifuged. An aliquot of benzene upper phase was used for gas-chromatographic (GC) analysis.

Fatty acids methyl esters (FAME) analyses were performed by a gas-chromatograph (Shimadzu GC-2010) equipped with an AOC-20i autosampler, split/splitless injector, and a flame ionization detector (FID). Conventional GC analyses were performed at the following experimental conditions: used column was a fused-silica capillary column (Supelcowax^{TM} 10) (30 m × 0.32 mm id × 0.25 μm); oven temperature was held at 80°C for 5 min, increased from 80 to 230°C with a rate of 3.0°C/min and kept at 230°C for additional 30 min; injection volume was 1.0 μl, in split mode (17:1). Carrier gas was helium with a linear velocity (u) of 37.5 cm/s. FID temperature was 280°C. Gas flow were 47.0 ml/min for hydrogen, 400 ml/min for air, and 30 ml/min for make up gas (N_2/Air); sampling rate was 40 ms. Peak identification of FAME in analyzed serum samples was carried out by comparison with retention time of the FAME standards, and was confirmed by gas chromatography-mass spectrometry (GC-MS). Quantitative determination was carried out, at four different concentrations, using a calibration curve for each fatty acids that was obtained by the external standard method. Four replications were performed for each calibration point and mean values of three replicates for each sample were given.

CELL CULTURES AND TREATMENTS

Human hepatoma Hep G2 cell line was from GMP-IST cell bank (Genova, Italy). Hep G2 cells were maintained in Eagle's minimum essential medium (Bio-Whittaker, Frederick, MD), supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) nonessential amino acid, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

For experiments, cells were seeded at a density of 2.5×10^6 cell/dish and allowed to adhere for 24 h. Medium was then replaced with fresh serum-free medium with 10% (v/v) human serum sample, each in biologic duplicate, and cultured for 48 h. As negative control,

reference cell cultures were maintained in 10% FBS standard condition.

ISOLATION OF RNA, REVERSE-TRANSCRIPTION, AND QUANTITATIVE PCR

Total RNA was isolated from Hep G2 cells using Trizol Reagent (Gibco-BRL, Gaithersburg, MD), according to the manufacturer's instructions. Extracted RNA was dissolved in water treated with 1% diethylpyrocarbonate (Sigma–Aldrich) and contaminating genomic DNA was digested with 10 U of DNase I (Roche Applied Science, Mannheim, Germany). The solution was extracted with Tris-equilibrated phenol and RNA precipitated with ethanol. Finally, RNA pellet was dissolved in RNase-free water and stored at −80°C. RNA was then quantified by spectrophotometry and its quality was assessed by electrophoresis through 1.2% agarose gel.

Approximately 3 μg of RNA were reverse transcribed in a total volume of 20 μl with 20 U Superscript II RNAse H Reverse Transcriptase enzyme, 5× RT buffer, 0.5 μg/μl *Random primers*, dNTP mix (each 10 mM), 0.1 M dithiothreitol (Invitrogen Co., Carlsbad, CA). Thermal conditions for reverse-transcription were 42°C for 5 min, 70°C for 15 min, and 37°C for 20 min. In the last step RNAse H (Invitrogen Co.) was added.

Real-time RT-PCR was carried out using a thermocycler (iQ5 Multicolor Real-Time PCR Detection System version 2 Bio-Rad) according to manufacturer's recommendations, using SYBR Green detection in a total volume of 20 μl with 1 μl of forward and reverse primers (10 μM) and 10 μl of IQ SYBR Green Supermix (BIO-RAD).

Expression of target genes was normalized with housekeeping gene 18S. The following primers were used for RT-PCR to assay specific mRNAs: UGT1A1 (GenBank NM_000463) 5′-GATCACATGACCTT-CCTGCAG-3′ and 5′-CCACAATTCCATGTTCTCCAG-3′; SREBP-1c (NM_004176) 5′-AGATCGCGGAGCCATGGATTG-3′ and 5′-CACT-GACTCTTCCTTGATACC-3′; SCD (NM_005063) 5′-GCTGATCCTCA-TAATTCCCGA-3′ and 5′-TTAAGCACCACAGCATATCGC-3′; UCP2 (NM_003355) 5'-TTGGGATTGACTGTCCACGC-3' and 5'-CCTCCAA-GATCAAGCTTCTCT-3'; 18S (NR_003286) 5′-CGATGCTCTTAGCT-GAGTGT-3′ and 5′-GGTCCAAGAATTTCACCTCT-3′. Reactions included an initial cycle at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 5 s, extension at 72°C for 15 s. Cycle numbers obtained at the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted control samples. Fold change of induction were determined by calculating ratios between control and treatment normalized signals. Experimental data are means of three values.

STATISTICAL ANALYSIS

In fatty acid content determinations, each calibration point was performed in four replications. Then each serum sample was assayed in triplicate and mean values were obtained. Statistical analyses were performed by the Mann–Whitney *U*-test.

In vitro experiments were performed in biologic duplicates (cell cultures) and technical triplicates (mRNA determinations). Differences between normo and hypercholesterolemic groups were analyzed using *t*-test. Inter-group differences were analyzed by *t*-test. A *P*-value <0.05 was considered statistically significant.

RESULTS

SELECTION OF SERUM SAMPLES

Out of 62 serum samples, two groups including a total of 40 were selected for further experiments among healthy male subjects aged between 40 and 50 years. Group of normocholesterolemic subjects included 21 samples with cholesterolemia below 170 mg/dl (median 155 mg/dl). Group of hypercholesterolemic subjects included 19 samples with cholesterolemia above 260 mg/dl (median 273 mg/dl). Beside cholesterolemia, detected TAG and all other parameters were within normal clinical values.

Table I shows detected total cholesterol, TAG, and HDL cholesterol for each serum group.

ASSAY OF SERUM FATTY ACIDS

Table II shows detected fatty acids levels for each serum sample. Assayed fatty acids included LA, LNA, DHA, EPA, AA, and *trans*-9 18:1 species. Firstly, it can be noted that there is a wide variability of amount of each fatty acid, although TAG levels are within normal clinical ranges, and hypercholesterolemic subjects have higher

TABLE I. Representation of Blood Parameters for Each Group

	Total cholesterol	HDL cholesterol	TAG
N samples			
1	154	44	125
2	158	45	55
3	133	41	65
4	160	53	100
7	169	55	60
8	170	50	99
14	170	53	54
16	169	49	139
20	170	41	99
23	149	42	66
27	134	40	83
28	169	51	73
38	152	42	87
42	169	44	81
43	144	43	65
44	138	39	92
47	146	42	76
48	151	41	123
50	158	43	59
54	128	41	130
62	156	35	70
Average	154,61	44,48	85,76
H samples			
10	260	65	170
11	296	51	177
18	260	71	141
19	261	38	114
22	261	55	153
25	275	55	85
29	300	53	174
30	276	57	140
34	268	60	134
36	268	71	73
37	269	61	105
39	304	55	122
40	297	33	161
49	267	52	151
51	260	59	99
53	265	56	89
56	260	45	130
60	283	54	172
61	261	39	181
Average	273,21	54,21	135,31

N samples are normocholesterolemic group. H samples are hypercholesterolemic group. Values are expressed as mg/dl.

TABLE II. Detected Fatty Acids Concentrations of Serum Samples From Normo (N) and Hypercholesterolemic (H) Subjects (Values Are Expressed as mg/100 ml of Serum*)

	18:2 n-6		18:3 n-3		DHA		EPA		AA		*trans*-9 18:1	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N samples												
1	115.59	9.76	4.13	0.20	8.66	0.41	1.91	0.04	14.10	0.70	7.46	0.31
2	41.29	3.21	/	/	5.47	0.26	1.21	0.03	11.75	0.51	2.43	0.12
3	87.12	5.20	3.16	0.14	13.53	0.90	5.95	0.31	18.75	1.22	6.53	0.28
4	96.29	7.16	1.48	0.07	9.94	0.56	1.91	0.05	18.48	1.31	4.30	0.18
7	95.27	7.21	2.04	0.09	12.70	0.86	1.74	0.04	19.76	1.72	5.80	0.20
8	124.34	9.78	1.58	0.09	6.53	0.33	3.78	0.19	29.92	2.01	7.94	0.35
14	74.05	5.10	6.99	0.35	10.41	0.74	1.83	0.04	20.00	1.47	4.41	0.20
16	47.38	2.21	0.98	0.01	10.57	0.76	6.65	0.30	12.74	0.77	3.62	0.17
20	90.20	6.13	3.58	0.22	11.51	0.81	3.03	0.12	16.36	0.98	6.04	0.28
23	54.84	3.28	2.68	0.10	9.21	0.61	1.61	0.06	15.61	0.88	6.44	0.27
27	81.50	5.34	0.94	0.01	15.14	1.01	1.54	0.05	34.05	2.78	8.40	0.39
28	95.93	6.67	2.28	0.12	9.79	0.66	4.00	0.21	25.16	1.75	7.38	0.29
38	189.51	9.91	2.52	0.14	8.77	0.54	6.04	0.27	29.09	1.81	16.03	1.10
42	76.34	5.11	2.87	0.25	15.63	1.06	4.10	0.20	25.97	1.77	10.79	0.94
43	81.02	6.43	0.73	0.01	6.11	0.30	1.38	0.02	16.72	1.05	6.44	0.29
44	79.35	5.19	1.87	0.08	23.64	1.60	14.11	0.95	20.06	1.28	6.18	0.27
47	104.89	8.45	1.06	0.06	5.33	0.24	1.45	0.02	19.88	1.23	6.51	0.34
48	73.09	5.00	2.95	0.10	6.13	0.41	2.81	0.09	28.55	1.90	11.29	1.07
50	77.32	5.71	1.59	0.08	4.86	0.15	1.61	0.04	16.20	0.86	5.15	0.16
54	70.32	4.31	2.00	0.09	22.65	1.35	12.52	1.01	19.91	1.23	6.85	0.26
62	96.33	6.12	1.70	0.06	4.33	0.21	2.79	0.11	18.38	0.96	5.34	0.20
Average	88.19	30.83	2.35	1.43	10.52	5.33	3.90	3.54	20.55	6.03	6.92	2.14
H samples												
10	236.23	14.20	2.67	0.09	21.56	1.61	3.19	0.15	28.40	1.78	8.34	0.51
11	130.83	8.25	2.68	0.08	14.56	1.12	2.58	0.09	40.78	2.34	10.60	0.78
18	127.96	7.12	3.34	0.10	12.53	0.76	3.55	0.17	25.79	1.66	8.97	0.45
19	115.55	5.11	1.41	0.02	11.42	0.60	1.21	0.01	28.81	1.67	5.54	0.22
22	578.73	26.20	9.31	0.31	45.00	2.44	1.87	0.02	121.17	7.41	33.45	2.10
25	102.59	7.25	3.22	0.11	11.75	0.78	4.34	0.18	33.68	1.71	9.35	0.77
29	162.06	10.35	2.84	0.12	17.07	1.26	6.68	0.45	41.24	2.91	10.03	0.79
30	150.15	9.77	2.53	0.10	8.01	0.41	7.98	0.44	39.86	1.86	13.83	1.06
34	105.53	5.15	2.40	0.08	10.38	0.56	3.55	0.15	36.53	2.01	9.31	0.57
36	149.66	9.81	4.18	0.22	11.01	0.61	7.40	0.39	43.68	2.45	7.96	0.34
37	157.56	10.11	3.86	0.19	18.79	1.11	8.83	0.43	33.94	2.11	12.98	0.87
39	102.53	6.55	6.23	0.41	20.25	1.40	5.51	0.20	33.85	2.54	9.27	0.53
40	163.71	10.66	6.35	0.39	16.07	1.24	8.19	0.56	27.37	1.45	10.44	0.93
49	162.99	10.10	3.54	0.12	22.46	1.43	5.16	0.32	40.26	2.76	9.18	0.66
51	139.67	6.59	2.35	0.07	6.81	0.35	5.91	0.28	29.62	1.69	8.12	0.48
53	110.16	5.20	2.31	0.07	6.17	0.41	5.68	0.21	24.19	1.67	7.34	0.43
56	138.81	6.54	5.07	0.16	16.88	1.17	6.02	0.32	30.18	1.89	10.23	0.88
60	159.60	7.33	3.20	0.13	11.14	0.54	4.05	0.19	42.38	2.53	14.73	1.02
61	108.44	7.01	2.40	0.06	8.24	0.49	5.30	0.22	35.06	2.00	14.07	0.98
Average	163.30	105.6	3.68	1.89	15.27	8.73	5.03	2.18	38.78	6.15	11.25	2.47

SD, standard deviation.
*Each number represents the mean of three determinations for each serum sample.

concentrations of all species with predominance of AA. Although DHA has an higher concentration in hypercholesterolemic serum samples, its quota within the whole sum of all assayed PUFA is more elevated in the normocholesterolemic group. This is shown in Fig. 1 (upper left) where DHA percentages of total fatty acids (sum of LA, LNA, DHA, EPA, AA, and *trans*-9 18:1) are compared between normo and hypercholesterolemic subjects. Values are significantly higher in normocholesterolemic subjects ($P < 0.05$). EPA quota of total fatty acids (LA, LNA, DHA, EPA, AA, and *trans*-9 18:1) is significantly higher in hypercholesterolemic subjects ($P < 0.01$), as shown in Figure 1 (upper right). Hypercholesterolemic subjects resulted to have higher concentrations of AA ($P < 0.01$) and *trans*-9 18:1 ($P < 0.01$), as shown in Figure 1, lower left and right, respectively. High levels of AA in hypercholesterolemic subjects coincide with significantly ($P < 0.01$) higher levels of LA as n-6 acids precursor and the n-3/n-6 fatty acid ratio is significantly higher in normocholesterolemic subjects ($P < 0.05$) (not shown in picture).

EFFECT OF SERUM SAMPLES ON mRNA EXPRESSION OF SREBP-1C, SCD, UGT1A1, AND UCP2 GENES

The expression of SREBP-1c, SCD, UGT1A1, and UCP2 genes was assessed after culturing each serum sample with Hep G2 cells and normalized referring to the values of control untreated cells. Observed modulations are reported for each gene in Table III, where values obtained treating with normo and hypercholesterolemic serum samples are reported. It can be noted that gene expression is markedly and differently affected from each sample of the two groups. Average SREBP-1c mRNA expression was reduced by 40% ($P < 0.05$) from hypercholesterolemic serum samples, as shown in Figure 2 (left). UGT1A1 mRNA expression was markedly modulated by hypercholesterolemic sera with a 1.5-fold increase ($P < 0.01$), as shown in Figure 2 (right).

The sum of n-3 and n-6 FA (DHA, EPA, AA, LA, and LNA) in each serum, also significantly higher in normocholesterolemic subjects, is roughly inverse with trend of SREBP-1c mRNA expression. Figure 3 (left) shows the values of the sums of n-3

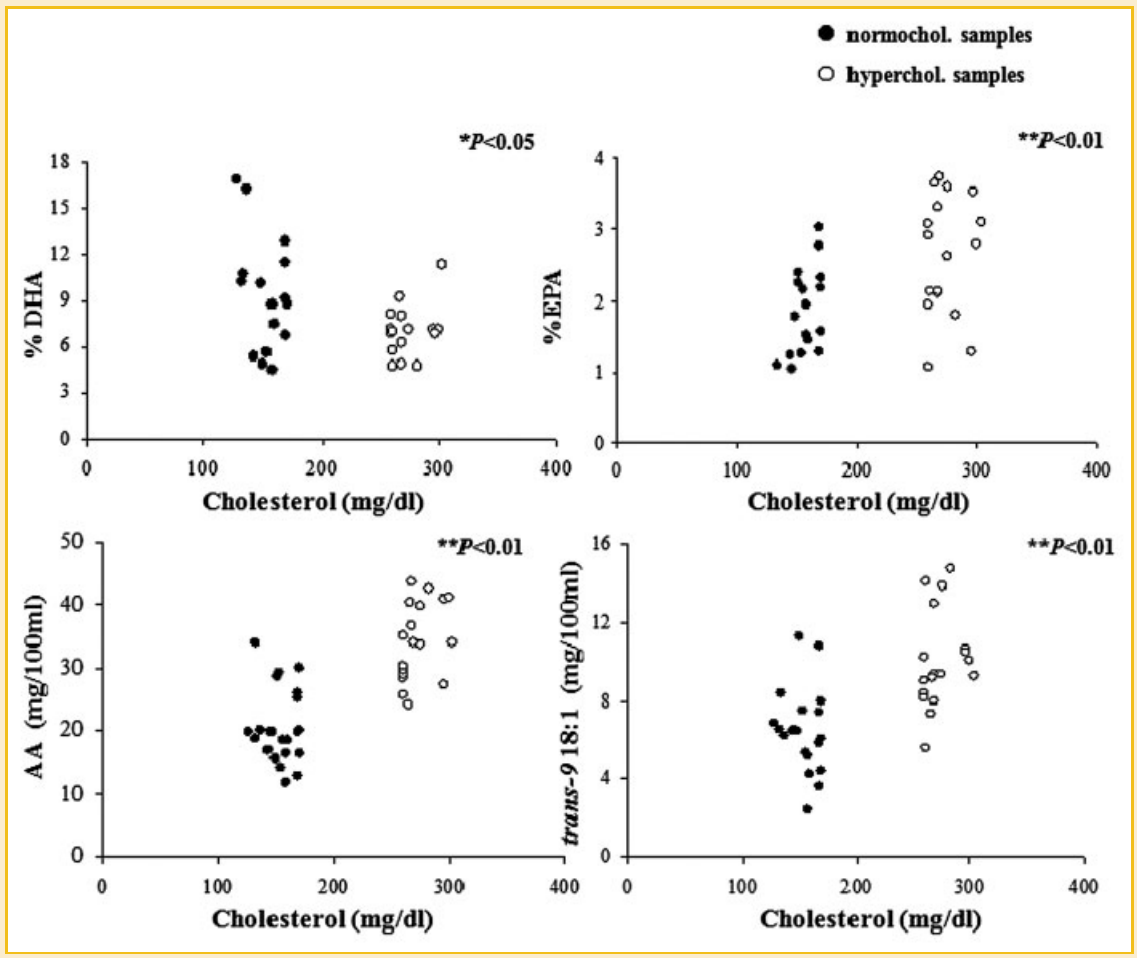

Fig. 1. Serum fatty acids and cholesterolemia. Values of DHA (upper left) and EPA quota (upper right) of total assayed fatty acids (LNA, LA, DHA, EPA, AA, *trans*-9 18:1) in selected sera of normo and hypercholesterolemic subjects. DHA quota is significantly higher in normocholesterolemic subjects ($P < 0.05$). EPA percentage is significantly higher in hypercholesterolemic sera ($P < 0.01$). Values of AA (lower left) and of *trans*-9 18:1 (lower right) in selected sera of normo and hypercholesterolemic subjects. AA and *trans*-9 18:1 species are significantly more abundant in hypercholesterolemic subjects ($P < 0.01$). Indicated cholesterol values are serum concentrations.

and n-6 fatty acids versus induced gene expression modulation of SREBP-1c in Hep G2 cells.

The sum of long PUFA (EPA, DHA, and AA) in each sample, that is also significantly lower in normocholesterolemic subjects, increases together with an higher trend of UGT1A1 mRNA expression. Figure 3 (right) shows the values of the sums of long PUFA versus the induced gene expression modulation of UGT1A1 in Hep G2 cells.

Concerning SCD and UCP2 genes expression, there are not considerable differences between the two selected sera groups. Nevertheless individual sera were able to induce gene modulations, but we found no correlation between observed effects and analyzed parameters.

DISCUSSION

Human serum composition results from a wide number of substances, which are only partially known and have very different roles within physiological and pathological processes. Serum lipid components have been extensively studied for their implication in cardiovascular diseases (CVD) development [Erkkilä et al., 2008; Warensjö et al., 2008]. In particular, total cholesterol and LDL cholesterol levels are relevant factors of coronary heart disease incidence and their reduction is a major goal in preventing stroke recurrence [Manktelow and Potter, 2009]. While several other fat species have essential clinical relevance, cholesterol concentration is probably the most important lipid serum parameter in assessing cardiovascular risk and disease progression [van Wijk et al., 2009]. Hypercholesterolemia and all other dyslipidemic conditions are more frequently related to the quality and quantity of nutrition, even if individual genetic factors are also remarkable [Alwaili et al., 2009]. Cholesterol concentration is not only important at a diagnostic level, but seems to be causally related to the conditions affecting CVD risk. Therefore, we felt relevant, comparing serum samples from subjects mainly differentiated for normal and high levels of this steroid to investigate possible effects of whole serum on gene expression. No subject of the two groups had apparent disease signs with the exception of hypercholesterolemia. To obtain higher groups homogeneity, only male subjects between age of 40 and 50 were selected. While there is no direct information about the origin

TABLE III. Modulation of Gene Expression from Normo (N) and Hypercholesterolemic (H) Serum Samples

	UCP2		SCD		UGT1A1		SREBP-1c	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N samples								
1	0.99	0.1	0.43	0.2	0.54	0.0	0.23	0.0
2	0.96	0.0	0.36	0.0	0.54	0.0	0.31	0.2
3	0.91	0.0	0.26	0.0	0.34	0.2	0.25	0.3
4	0.35	0.0	1.29	0.1	0.12	0.0	1.25	0.1
7	0.20	0.1	0.41	0.1	0.29	0.0	0.39	0.0
8	0.85	0.3	0.69	0.1	0.90	0.1	0.38	0.0
14	1.13	0.2	0.97	0.0	0.46	0.1	0.77	0.2
16	0.25	0.0	0.20	0.4	0.34	0.0	2.04	0.3
20	1.19	0.1	1.10	0.2	0.72	0.0	0.54	0.0
23	1.93	0.2	1.40	0.1	2.42	0.2	2.31	0.0
27	1.02	0.0	0.93	0.0	2.23	0.1	3.32	0.0
28	1.48	0.1	1.34	0.0	2.36	0.0	0.91	0.0
38	0.26	0.1	0.52	0.0	1.17	0.0	0.18	0.1
42	0.11	0.0	0.65	0.1	/	/	1.04	0.0
43	0.30	0.1	0.39	0.0	/	/	1.04	0.1
44	0.23	0.0	0.40	0.1	/	/	0.80	0.1
47	0.31	0.0	0.36	0.0	1.92	0.1	0.90	0.0
48	0.10	0.0	0.43	0.0	2.41	0.0	0.89	0.0
50	0.19	0.0	0.50	0.2	2.63	0.0	1.06	0.1
54	0.38	0.0	1.11	0.0	2.54	0.0	0.59	0.0
62	0.54	0.2	0.63	0.2	/	/	0.74	0.0
Average	0.65	0.5	0.68	0.4	1.26	1.0	0.95	0.8
H samples								
10	2.06	0.0	0.16	0.1	/	/	0.19	0.0
11	2.04	0.0	0.27	0.1	/	/	0.25	0.1
18	0.74	0.0	0.56	0.0	1.20	0.0	0.92	0.1
19	1.06	0.0	1.10	0.1	3.08	0.0	0.71	0.0
22	1.52	0.0	0.44	0.0	0.70	0.1	0.15	0.0
25	1.81	0.0	0.64	0.2	0.84	0.2	0.35	0.0
29	1.04	0.2	0.66	0.0	1.92	0.0	0.40	0.2
30	0.87	0.0	0.73	0.0	1.74	0.3	/	/
34	0.75	0.0	1.08	0.2	2.66	0.0	0.56	0.1
36	0.70	0.1	0.94	0.0	1.41	0.2	1.00	0.0
37	0.90	0.0	0.53	0.0	1.26	0.1	0.63	0.0
39	0.29	0.2	0.28	0.0	1.14	0.1	0.18	0.0
40	0.35	0.3	0.91	0.0	1.25	0.1	0.27	0.1
49	0.06	0.0	0.40	0.1	3.96	0.0	0.40	0.0
51	0.75	0.1	0.73	0.2	4.97	0.0	1.25	0.4
53	0.38	0.0	0.50	0.1	7.79	0.0	0.66	0.1
56	0.45	0.2	0.37	0.0	4.61	0.3	0.32	0.0
60	0.32	0.1	0.49	0.4	10.21	0.2	0.48	0.0
61	0.95	0.0	0.57	0.0	5.54	0.1	0.60	0.0
Average	0.90	0.6	0.60	0.3	3.19	2.7	0.51	0.3

Each value (derived from six determinations from two independent in vitro treatments) represents the ratio of mRNA expression in treated versus control cells. 0.0 are SD values below 0.05.

of hypercholesterolemia, this cannot likely be genetic in most cases, since this does not occur very frequently. Nutrition status, however, is likely among the major causes of the difference, even considering the variable amounts of detected fatty acids in serum samples.

The quality of lipid quota of nutrition and, more specifically, its content in n-3 and n-6 PUFA is well known to be strictly connected to CVD risk and pathogenesis [Mensink et al., 2003; Breslow, 2006; Carpentier et al., 2006; Lichtenstein et al., 2006; Erkkilä et al., 2008; Li et al., 2008; Warensjö et al., 2008]. PUFA, nevertheless, have been found to have very specific biological and even pharmacological actions [Chawla et al., 2001]. The significance of clinical monitoring of DHA and EPA intake is progressively being considered more important [Carpentier et al., 2006] and daily intake of these fatty acids was reported to influence their serum concentration [Bethune et al., 2006].

For these reasons, we decided to analyze the effect of serum samples on gene expression together with the assay of their content in main PUFA. The two groups of normo and hypercholesterolemic subjects have very different serum fatty acids levels. Several differences between the two groups are also statistically significant. There is a substantial difference in AA concentration between the two groups. Similarly DHA level results more abundant in hypercholesterolemic samples, although its percentage within PUFA in normocholesterolemic samples is higher. Serum fatty acids concentrations are in fact becoming recognized biomarkers of fatty acids intake, also being related to cholesterol concentration [Crowe et al., 2006], but even nondietary factors such as sex and age may be important [Crowe et al., 2008]. The actual presence of these molecular species, separately from their origin, might be active part of the metabolic conditions leading to CVD pathogenesis. The finding that hypercholesterolemic subjects resulted to have higher levels of *trans* fatty acids is another point indicating different nutrition behaviors between the two groups. Serum *trans* fatty acids levels are in fact known to be only derived from diet intake, also increasing CVD risk [Mozaffarian and Willett, 2007]. Consistent with previous observations, indicating n-3/n-6 fatty acid ratio as nutritional biomarker of cardiovascular risk, our results show this with an higher value in normocholesterolemic sera ($P < 0.05$) [Mensink et al., 2003].

Gene regulation mechanisms are very complex and exerted at numerous levels from DNA binding of transcription factors and cofactors to translation of appropriate proteins [Foley, 2008]. Serum obtained from circulating blood is known to contain factors able to affect gene expression [Rollins and Stiles, 1989; Chai and Tarnawski, 2002], through direct binding to specific responsive elements [Groskopf and Linzer, 1994; Michaud et al., 2005; Kim et al., 2009; Poimenidi et al., 2009]. Our data, obtained adding serum to cultured Hep G2 cells and analyzing mRNA expression of four genes, show that each sample is able to induce significant and different effects. Nevertheless serum is a central crossroad of the whole metabolism where also nutritionally derived factors affecting gene expression are present. These factors certainly include PUFA which are well known to have specific biological effects [Chawla et al., 2001] and their serum concentrations affected from nutrition styles [Bethune et al., 2006; Erkkilä et al., 2008]. Also other serum components likely participate to produce observed effects since, at least in the case of analyzed genes, known regulation mechanisms are multifactorial. Additionally, nutrition derived molecules are able to affect gene expression both directly or through induced metabolism modifications.

The modulation of UGT1A1 and SREBP-1c mRNA expression is markedly different between serum samples from normo and hypercholesterolemic subjects. Additionally, the expression of these two mRNA is roughly associated with serum concentrations of PUFA. This could also derive from the synergic action of more factors. Although very different modulations of UCP2 and SCD genes are observed with all analyzed serum samples, they do not show any significant association with the assayed lipid parameters. The fact that we could not associate SCD and UCP2 expression to the species assayed in serum samples might be due to other serum factors, that differently contribute to the observed modulations.

SREBP-1c is a transcription factor governing lipid synthesis and data show that hypercholesterolemic sera are able to reduce its

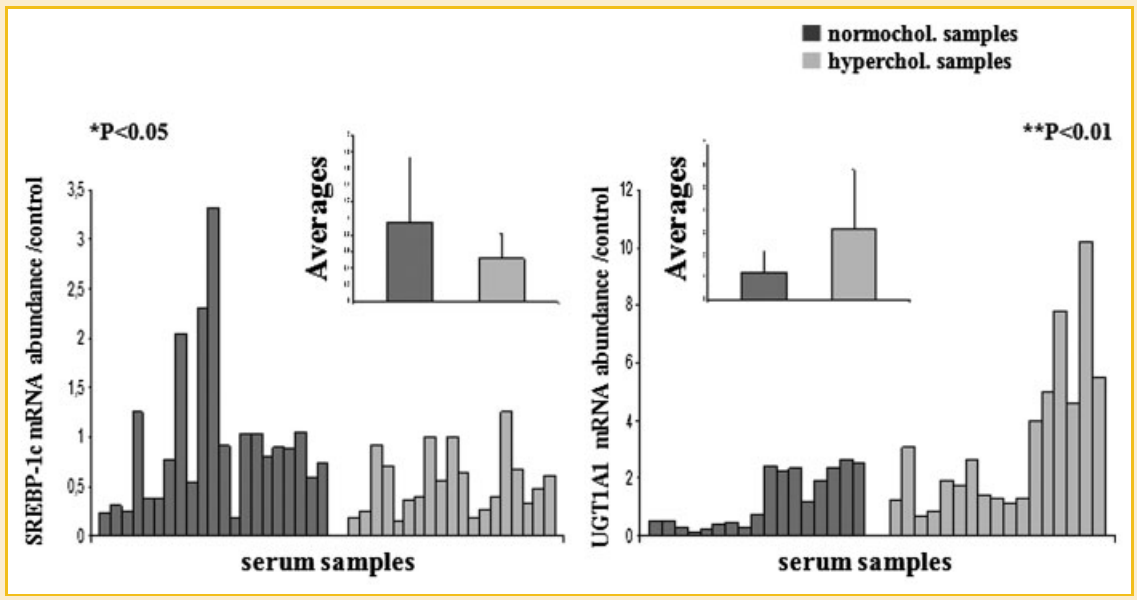

Fig. 2. Normo and hypercholesterolemic serum samples and gene regulation. Values of SREBP-1c (left) and UGT1A1 (right) mRNA expression in Hep G2 cells treated with selected sera of normo and hypercholesterolemic subjects. Average SREBP-1c expression is significantly higher ($^{*}P < 0.05$) in cells treated with sera from normocholesterolemic subjects, while average UGT1A1 expression is significantly higher ($^{**}P < 0.01$) in cells treated with sera from hypercholesterolemic subjects. Inserts show averages and standard deviation (SD) from each group in each experiment: hypercholesterolemic sera produced a 0.4-fold reduction of SREBP-1c mRNA and a 1.5-fold increase of UGT1A1 mRNA.

mRNA expression. The maturation of SREBP-1c was shown to be inhibited by high fat diets while cholesterol concentration resulted to have an inverse relation with its expression [Sampath and Ntambi, 2006; Sato, 2009; Zhang et al., 2009]. Our data show that hypercholesterolemic sera are able to reduce mRNA expression of this lipid-related transcription factor. This finding is in agreement with our previous data where individual pure fatty acids such as DHA, EPA, and AA directly administered on the same liver cell culture induced a down regulation of SREBP-1c [Caputo et al., 2011].

Our data in Figure 3 show that higher levels of AA and LA correspond to elevated gene expression of UGT1A1. This is in

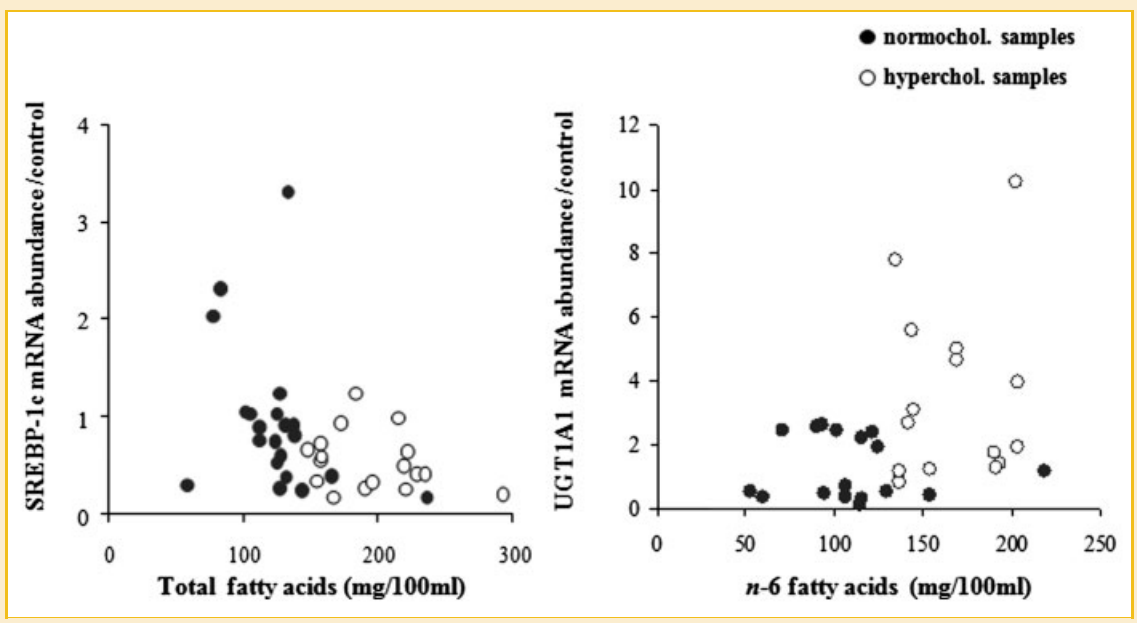

Fig. 3. Serum fatty acids and gene regulation. Total serum fatty acids (DHA, EPA, AA, LNA, and LA) versus expression of SREBP-1c mRNA (right). Sum is significantly higher in hypercholesterolemic subjects and is inverse with trend of SREBP-1c mRNA expression. Serum n-6 fatty acids in selected sera of normo and hypercholesterolemic subjects versus expression of UGT1A1 mRNA (left). Values are significantly higher in hypercholesterolemic subjects and coincide with an increasing trend of UGT1A1 expression.

agreement with the fact that AA was shown to be a substrate of UGT1A1 and members of UGT1A and UGT2B protein families are capable of converting AA and LA metabolites into glucuronide derivatives, as irreversible step of their elimination from the body [Turgeon et al., 2003]. Other data show that UGT1A1 induction by pro-inflammatory molecules (such as AA and LA) might represent a key adaptive response to cellular oxidative stress that defends against a variety of detrimental stimuli [Yueh and Tukey, 2007], as in our condition, for example, UGT1A1 expression could be influenced by hypercholesterolemic serum samples. The additional role of inflammatory factors, present in these sera within hypercholesterolemic status, could explain the different result of our previous data in which individual fatty acids reduced UGT1A1 mRNA [Caputo et al., 2011].

These findings give insights in the biochemistry of conditions which are part of dyslipidemic disease development. Detected lipid profiles within the complexity of serum composition are differentially associated to gene expression in our experimental model. The different concentrations of molecules like PUFA which are already known to specifically regulate genes of lipid metabolism, suggests that their presence in serum could also indicate a mechanism acting within disease pathogenesis. This point is also suggested from the fact that observed gene regulation regards genes like UGT1A1 and SREBP-1c, already known to be implicated in lipid metabolism and CVD development. Further investigation will have to identify in more details all other components responsible for the observed expression modulations. Nevertheless we believe that direct administration of serum on cultured cells shows evidence of a specific and functional relationship between its composition, also depending from nutritional status, and expression of genes linked with CVD development. This information may then result in improvements of assessment of nutritional status and therapy of cardiovascular and other dyslipidemic diseases.

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