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ORIGINAL ARTICLE

Inflammatory serum proteome pattern in mice fed a high-fat diet

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Abstract To investigate the influence of diet on serum protein pattern, mice were fed for 8 weeks either control chow or a high-fat diet (containing 21 % w/w milk fat and 0.2 % w/w cholesterol); sera were collected and analyzed by 2-DE. The main positive acute-phase reactant proteins, haptoglobin and hemopexin, were significantly up-regulated in animals receiving the high-fat diet. Data on all other proteins also pointed to an inflammatory condition in these animals. The largest change in concentration was observed for carboxylesterase N, a circulating enzyme seldom connected with lipid metabolism in earlier reports. These observations agree with the notion of a link between diet-induced hyperlipidemia and the inflammatory component of its cardiovascular sequels in humans, but the effects in the experimental animals are massive and obviously affect most of the major serum proteins.

Keywords Acute-phase reaction · Acute-phase reactant · Carboxylesterase · High-fat diet · Mouse

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Abbreviations

apo	Apolipoprotein
APR	Acute-phase reactant (protein)
CRP	C-reactive protein
h	Human
KO	Knock-out
m	Mouse
MUP	Major urinary protein-1
PPARα	Peroxisome proliferator-activated receptor α
TG	Transgenic

Introduction

Diets with a high lipid content, specifically saturated fatty acids, are linked with increased risk of disease in humans, the main target being the cardiovascular system (Djousse and Gaziano 2009; Lichtenstein et al. 1998; Sanders 2009; Siri-Tarino et al. 2010). Similar diets are used to induce, or enhance, in wild-type or genetically engineered animals a sequence of events that mimics the human pathology (murine models of vascular lesions are surveyed in Daugherty 2002; Temel and Rudel 2007; Zadelaar et al. 2007).

Animal models are useful both for the definition of dietary regimes that induce and for the assessment of treatments that prevent or revert vascular lesions. For a thorough evaluation of such effects it is useful to make reference to a large panel of biological markers (Daugherty et al. 2009). Proteomic investigations on whole serum (murine data reviewed in Gianazza et al. 2002, 2012a, b) provide quantitative data on several highly expressed proteins. Serum may be repeatedly sampled from the same animals in a time-course mode and treatment progression may then be

monitored with repeated measurements statistics. Changes in serum pattern brought about by specific experimental treatments may be compared with a growing database of already characterized physiological and pathological conditions. The main protein components may be easily recognized on the basis of p*I* and M_r data provided by reference maps as first introduced for the human samples (see at the SWISS-2DPAGE database of the ExPASy Bioinformatics Resource Portal, http://swissdock.vital-it.ch/) and extensively applied by our group in previous work on murine models (a dozen papers on rat biological fluids from Haynes et al. 1998 to Sironi et al. 2005).

These considerations suggested investigating the effect of a classical high-fat diet (also named "Western-type diet" Plump et al. 1992) on mouse serum proteome as the first step of a study aimed at elucidating the influence, and the interaction, of genetic background and drug treatment. Proteomic investigations entirely rely on observations, without having any specific hypothesis as framework. However, for liver secretion it is firmly established that for the main proteins essentially two alternative patterns are observed, one in the absence the other in the presence of an acute-phase reaction (Wait et al. 2005b). This term summarizes a number of complex endocrine, metabolic and neurological changes observed in an organism, either locally or systemically, a short time after an injury or at the onset of infections, immunological reactions, and inflammatory processes (Cray et al. 2009). On this basis one of the questions this investigation is meant to answer is whether, or better to which extent, a high-fat diet shifts the mouse serum proteome toward an inflammatory pattern—inflammation being one of the main issues on focus in connection with human cardiovascular disease (Athyros et al. 2008).

Materials and methods

Animals and treatments

Procedures involving animals and their care were conducted at Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, in conformity with the institution's guidelines, which are in compliance with national and international rules and policies.

Male mice, strain C57Bl/6J, 20–30 weeks old, average body weight 30 g at the beginning of the experiment, were enrolled and divided into two groups, each fed for 8 weeks either standard chow (Teklad, 2018) (control; $N = 21$) or high-fat diet (Teklad TD.88137) ("Western-type"; $N = 25$). The composition of the two diets is shown in Table 1, together with the USDA recommendations for a balanced human nutrition. In the high-fat diet, proteins are provided by casein, and lipids by anhydrous milk fat; the abundance of the various fatty acids in standard and high-fat diet for rodents is listed in Supplementary Table 1.

Blood was drawn by retroorbital puncture at the end of the treatment, from overnight fasted mice for determination of serum cholesterol, glucose and triglycerides, from fed animals for analysis of serum proteins. After clotting at 37 °C for 30 min serum was collected by centrifugation at 2,900 rpm for 15 min. Serum samples were stored at −80 °C. Individual sera were pooled on a volume-to-volume basis for 2-DE analysis of serum proteins, 7–9 samples/pool to obtain 3 pools/group.

Blood chemistry

Total cholesterol, triglycerides and glucose were determined in mouse sera with standard commercial kits (for cholesterol, Horiba ABX, Roma, Italy; for triglycerides and glucose, Sentinel Diagnostics, Milano, Italy).

Electrophoresis

Two-dimensional electrophoresis IPG-DALT was performed as described (Gianazza et al. 2002). The first dimension was run on pH 4–10 NL IPG (Gianazza 1985, 2009), the second dimension on polyacrylamide gradient gels (7.5–17.5 % T; C % = 4), using the discontinuous buffer system of Laemmli (Laemmli 1970). Gel size was $0.15 \times 14 \times 16$ cm^3. Serum (10 µL) was diluted with an equal volume of water and loaded at the cathodic end of the strips, without and with reduction with 2 % 2-mercaptoethanol. Proteins were visualized with 0.3 % coomassie blue in 30 % ethanol:10 % acetic acid (v:v).

The scanned gel patterns were analyzed with Progenesis SameSpots.

Proteins were identified by comparison with published reference maps (Wait et al. 2005a, b).

Statistical analysis

Statistical analysis was performed with unpaired Student's *t* test, using GraphPad Prism version 5.0d for Mac OSX (GraphPad Software, San Diego California USA, http://www.graphpad.com).

Results

As shown in Fig. 1, body weight (panel a) as well as serum total cholesterol (panel b) and triglycerides (panel c) were significantly increased in mice fed a high-fat diet for 8 weeks. Conversely, in this setting, although serum glucose levels (panel d) increased with high-fat feeding, they

Table 1 Diet composition

Nutrient type	USDA recommendations for human nutrition % of calories	Standard chow for rodents % of calories	High-fat diet for rodents	
			% of calories	% by weight
Carbohydrates	55	58	43	48.5
Proteins	18	24	15	19
Lipids	29	18	42	21
Cholesterol	–	–	–	0.2

did not reach statistical significance in mice on high-fat versus control diet ($p = 0.0554$).

Figure 2 compares the 2-DE pattern obtained under non-reducing (top row) and reducing conditions (bottom row) for sera from mice fed standard chow (left panels) with that from mice fed a high-fat diet (middle panels). The right panels feature the up-regulated (dark gray) and down-regulated spots (light gray) (high-fat vs. control, see Table 2), and mark the affected proteins [names are abbreviated as in UniProt database entries (http://www.uniprot.org/)]. Identifications rest on published mouse serum maps (Wait et al. 2005a, b). Table 1 summarizes the significant changes in spot abundance pointed out by the statistical treatment of the 2-DE densitometric data. The extent of up- or down-regulation is reported as fold change in the high-fat versus control comparison. When several protein isoforms migrate in a spot row, the change is averaged over all the affected spots. This amounts to the whole row in most cases, but sometimes only a few isoforms are involved (size isoforms for α_1-antitrypsin and contrapsin aggregates, or proteolytic fragments of albumin and apolipoprotein A-I; charge isoforms for two of the glycoforms of clusterin). The gray background highlights those changes that are consistent with the occurrence of an acute-phase reaction: they obviously involve most of the proteins whose expression is regulated by a high-fat diet. Runs under non-reducing conditions (Miller et al. 2010; Wait et al. 2005a) are instrumental to the detection of statistically significant changes. All raw data for the individual spots whose level is found to change with a $p < 0.05$ significance are reported in supplementary Table 2a and b and supplementary Figs. 1a and b.

Discussion

The chief goal of this study was to assess the changes of serum protein expression after a dietary challenge. As a preliminary check, the high-fat diet used in this study resulted in the expected increase of body weight, serum cholesterol and triglycerides. High-fat feeding is typically used to induce glucose intolerance and insulin resistance (Petro et al. 2004); the lack of significant changes of serum glucose observed in this study may be explained with the duration of the treatment with the high-fat diet, not long enough to bring about hyperglycemia.

Our data show that feeding mice with a high-fat diet results in statistically significant changes in the expression levels of several among the high-abundance serum proteins: in the vast majority of cases the direction of such changes corresponds to what is expected during an acute-phase reaction. For haptoglobin and hemopexin, the main

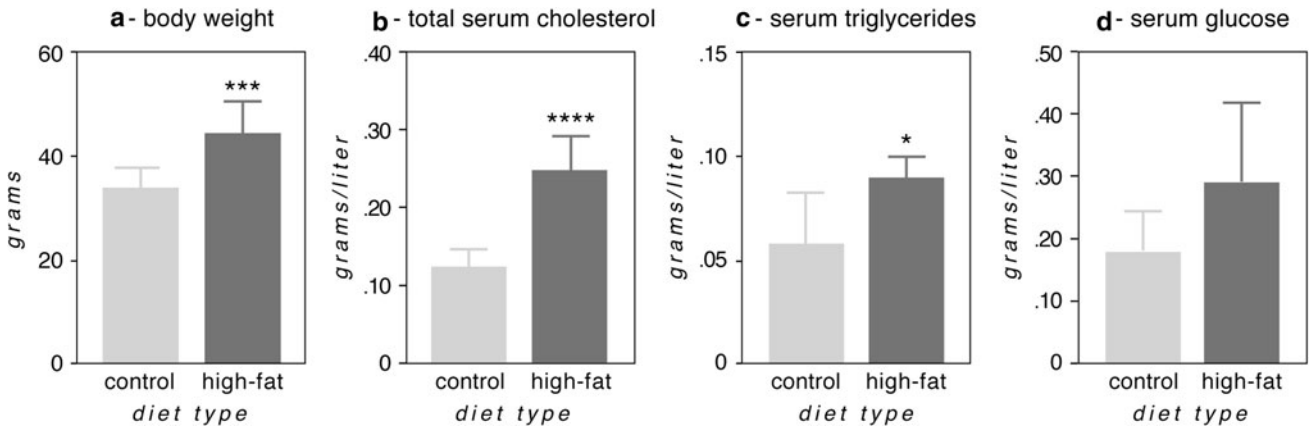

Fig. 1 Body weight and serum levels of total cholesterol, triglycerides and glucose in mice on control or on high-fat diet. Results are expressed as mean ± SD (21 and 25 mice/group). *, *** and **** indicate statistical significance at $p < 0.05$, $p < 0.001$ and $p < 0.0001$, respectively (unpaired Student's t test)

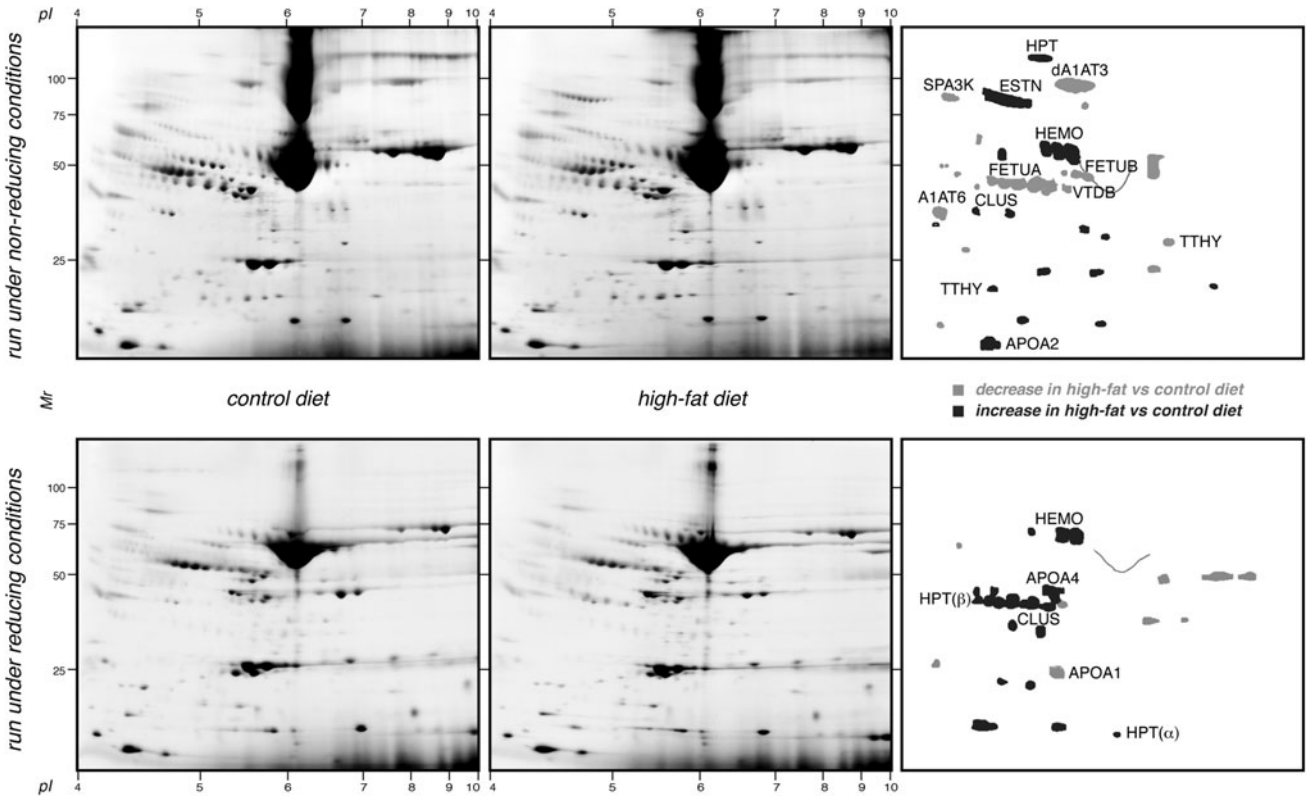

Fig. 2 2-DE patterns of sera from mice fed either standard (*left panels*) or high-fat diet (*middle panels*). *Right panels* color-code the protein spots for which a significant decrease (*light gray*) or a significant increase (*dark gray*) has been evaluated by statistical analysis of the quantization data. The albumin spot is outlined as a positional reference. *Top row* sera were run without treatment with thiolic reagents; *bottom row* sera were run after treatment with 2-mercaptoethanol. Experimental: sample load: 10 µL; 1 day = IPG4-10NL in 8 M urea; 2 day = SDS-PAGE on 7.5–17.5 % T PAA; coomassie stain

positive acute-phase reactant proteins in mice, up-regulation is massive. The acute-phase reaction is thought to have evolved as a response to acute injury and to infection; responses that are adaptive to such harms become ineffective and even dysfunctional when induced by chronic conditions. Such a concern has drawn considerable attention on the inflammatory component associated with degenerative diseases.

When dealing with both human and animal studies, it is worth reminding that in all species an acute-phase reaction is characterized, among other features, by the coordinated synthesis by hepatocytes of some positive acute-phase reactants (APR) and by a decreased synthesis of some negative reactants. However, nature of APR, time-course of the effects and extent of regulation vary among species (see Miller et al. 1998 for rats, Wait et al. 2005b for mice, Wait et al. 2002 for bovines, Miller et al. 2009 for swine). While the identification of individual proteins is of course essential, the most relevant aspect in cross-comparisons is then often protein function(s).

There is general consensus about atherosclerosis being "an inflammatory disease" as first provocatively summarized by Ross (1999). Thousands of scientific reports and review articles have dealt with the connection between cardiovascular disease and inflammation in human patients, and many of them have investigated the link with APRs. The interpretation of the role of APRs has evolved (Devaraj et al. 2009) with time, and at least three alternatives are considered: biomarkers, risk factors and/or therapeutic targets (Genest 2010). For instance, a recent paper has identified HP, the gene coding for haptoglobin, as a locus influencing serum cholesterol levels (Igl et al. 2010); a review collects evidence about the anti-atherogenic potential of serum amyloid A peptides (Kisilevsky et al. 2008). A large body of data and commentaries in the literature is devoted to C-reactive protein (CRP), the serum protein whose levels change most rapidly and most extensively during the human acute-phase reaction and which may be quantified with very high sensitivity. For instance, CRP has been connected with the prediction of first cardiovascular events (Ridker et al. 2002) and investigated as a determinant of mortality (Koenig et al. 2008). Various aspects have been discussed about the mechanisms by which CRP may mediate atherosclerotic damage (e.g. Cirillo et al. 2005; Eisenhardt et al. 2009). However, due to the stimulus-independent character of the acute-phase

Table 2 Effect of diet on mouse serum proteome (high-fat vs. control)

Protein (abbreviation[c])	Fold change	
	Without sample reduction[a]	With sample reduction[b]
α_1-Antitrypsin (A1AT3), dimer	−1.2	
α_1-Antitrypsin (A1AT6)	−1.3	
Albumin (fSA), fragments		−1.3[d]
Apolipoprotein A-I (APOA1), large fragment		−1.1
Apolipoprotein A-II (APOA2)	+1.2	
Apolipoprotein A-IV (APOA4)[e]		+1.2
Carboxylesterase (ESTN)	+3.0	
Clusterin (apoJ; CLUS), glycoforms	+1.3	+1.3
Contrapsin (SPA3 K), isoforms	−1.6	
Fetuin A (α_2-HS-glycoprotein; FETUA)	−1.4	
Fetuin B (FETUB)	−1.5	
Haptoglobin (HPT)	+1.6	+1.6
Hemopexin (HEMO)	+1.4	+1.4
Vitamin D binding protein (VTDB)	−1.4	

[a] Compare with panels in upper row of Fig. 1

[b] Compare with panels in lower row of Fig. 1

[c] According to UniProt database entries (http://www.uniprot.org/)

[d] A gray background highlights the proteins for which found up- or down-regulation fits with the expected effects of an ongoing acute-phase reaction

[e] For apoAIV as a mouse APR see Khovidhunkit et al. (2004)

reaction, CRP lacks specificity for cardiovascular disease and may be associated as well to several clinical and subclinical conditions (e.g., colon and rectal cancer Aleksandrova et al. 2010). While various food components/food additives have been shown to influence CRP levels, epidemiological studies and clinical interventions have reported contradictory findings (reviewed in Puglisi and Fernandez 2008).

A few mouse models exist for dyslipidemias and atherosclerosis (Zadelaar et al. 2007). Genetically modified animals are the apolipoprotein E-deficient (ApoE−/−) and the LDL receptor-deficient (LDLr−/−) mice, the ApoE*3Leiden (E3L) transgenic (TG) mouse and the HcB-19 strain (Castellani et al. 1998). An alternative approach is dietary induction in differentially susceptible strains (Bird et al. 1985; Fazio and Linton 2001).

Inflammation in such mouse models has been often assessed by measuring cytokine levels (Cole et al. 2010; Lutgens et al. 2005; Nunemaker et al. 2008; Tabibiazar et al. 2006; Wan et al. 2010). Proteomic investigations have dealt with liver (de Roos et al. 2005; Feng et al. 2005; Kirpich et al. 2010; Park et al. 2004; Schmid et al. 2004; Van Greevenbroek et al. 2004) and intestinal mucosa (Calpe-Berdiel et al. 2007) but not with plasma/serum. In contrast with the standard use of biological fluids in clinical biochemistry, serum proteins are seldom quantified in laboratory animals, including mice (Gianazza et al. 2012a), despite the demonstration of their value as biological endpoints (Eberini et al. 2000). A single example of whole serum protein analysis in connection with metabolic disorders sought biomarkers for the development of diet-induced type 2 diabetes in mice (Okada et al. 2010). Significant changes were detected not only for apolipoprotein A-I, but also for plasma retinol binding protein, transthyretin, and kininogen—the last being one of the acute-phase reactant proteins (Wait et al. 2005b).

Our previous investigation (Wait et al. 2005b) has dealt with three TG mice (i.e., *h*apoA-I TG mice, *h*apoA-II TG mice, double *h*apoA-I/*h*apoA-II TG mice); contrary to the above models, *h*apoA-I TG mice feature a lower susceptibility to atherosclerosis (Castro et al. 1997). We found altered levels of expression for α_1-acid glycoprotein, α_1-macroglobulin, esterase, kininogen and contrapsin in *h*apoA-I TG mice in comparison with knockout mice lacking apolipoprotein A-I (*m*apoA-I KO), but no statistically significant difference in *h*apoA-II TG mice. From these data the cross-talk between proteins definitely connected and proteins not directly connected with lipid metabolism is evident, together with the involvement of some acute-phase reactants.

Another high-abundance serum protein that has been associated with metabolic regulation in mice is major urinary protein-1 (MUP) (Hui et al. 2009; Zhou et al. 2009). MUP/pheromone complexes mediate chemical communication in rodents and regulate their social and sexual behavior. However, both when over-expressed in vivo and when added to in vitro cultures, it also inhibits the transcription of gluconeogenic and lipogenic genes via a paracrine/autocrine mechanism.

As summarized in Table 1, our data on the effect of diet on mouse serum demonstrate the involvement of several among the major proteins. Most of them are the same as found affected in the *h*apoA-I TG animals (Wait et al. 2005b). The fold change for protein level in the high-fat versus control diet comparison typically ranges between 1.2 and 1.6. Carboxylesterase shows, instead, a threefold

increase; carboxylesterase is also the protein whose level was found to change the most in the *h*apoA-I TG versus *m*apoA-I KO comparison (Wait et al. 2005b).

Liver carboxylesterase N is one of a number of enzymes catalyzing the hydrolysis of carboxylic esters (EC = 3.1.1); their genes are evolutionarily related and cluster in a recombination unit on mouse chromosome 8. It is a serine esterase that belongs to the type-B carboxylesterase/lipase family (catalytic triad *S*203, *E*325, *H*438) and is able to hydrolyze ester- and amide bonds of substrates ranging from small molecule esters such as phenyl ester to long-chain fatty acid esters and thioesters (reviewed in Satoh and Hosokawa 1998). The enzyme is secreted from liver, lung and kidney, and is involved in the detoxification of xenobiotics; nonetheless the endogenous role of this enzyme in mammals remains largely unknown and is an important area of investigation.

Literature data are indeed scanty, and in some cases confuse when enzymes catalyzing the same main reaction receive generic or synonymous designations. Carboxylesterases is not a very abundant protein in human serum (Hortin et al. 2008).

While other carboxylesterases are directly connected with lipid metabolism (e.g. carboxylesterase 1 and 2 are inhibited by oxysterols and fatty acids Passweg and Tyndall 2007, carboxylesterase 3 is a major adipocyte lipase Boin and Rosen 2007), the only link between carboxylesterase N and lipids seems to be in an old report of decreased levels in the serum of clofibrate-treated mice (El-Badri et al. 2004). Clofibrate, a ligand of the nuclear receptor peroxisome proliferator-activated receptor α (PPARα), is a lipid-lowering agent once used for controlling high triacylglyceride levels in the blood. In humans it increases lipoprotein lipase activity to promote the conversion of VLDL to LDL, and hence reduce the level of VLDL; it also increases the level of HDL via enhanced expression of apoA-I and apoA-II (Schoonjans et al. 1996). While somewhat anecdotic, this old finding on carboxylesterase down-regulation with hypocholesterolemia actually complements our data on its up-regulation with hypercholesterolemia.

Only a handful of papers deal with any carboxylesterase in any species and inflammation. In our survey on mouse serum proteins we had evidence of a behavior as positive APR (Wait et al. 2005b). We mention that, on the contrary, in a model of cancer transplanted in an immuno-incompetent mouse (SJL mice colonized with RcsX lymphoma), esterase was down-regulated, together with paraoxonase and α_2-macroglobulin, whereas several other APR such as haptoglobin, hemopexin, serum amyloid P component, and ceruloplasmin underwent the typical up-regulation of the inflammatory reaction (Kristiansson et al. 2007). Also, in an in vitro setup, treatment of HepG2 cells in culture with lipopolysaccharide resulted in down-regulation and reduced activity of carboxylesterases (Oswald et al. 2004).

From the above, it seems not only possible but even likely that both generic inflammatory mediators and specific if uncharacterized stimuli induce the exceptionally large up-regulation of carboxylesterase in the serum of mice fed a high-fat diet.

As already mentioned above, due to species-specificity of the complement of proteins secreted by liver under reference conditions as well as during an acute-phase reaction, no direct connection may be made between differential regulation of individual proteins in animal sera and the same effects in humans. Instead, reference has to be made to the biological function of the affected proteins. With this proviso, our findings fully agree with human data about the connection between diet and inflammation (reviewed in Giugliano et al. 2006).

On a broader perspective, this investigation provides further evidence that major proteins in serum of laboratory rodents should not be neglected as markers of the physiopathological condition of the animals but, rather, exploited to monitor its onset and progression as well as to assess the outcome of any experimental intervention (Gianazza et al. 2012a, b).

If a general agreement could be reached on a link with dyslipidemia, the level of circulating carboxylesterase in mice could be monitored through an enzyme activity assay: as an abundant serum component, there would be no need to use immunological reagents to differentiate between this and other forms of the enzyme. A procedure tailored on the substrate specificity of the rat enzyme has long been reported (Clement and Erhardt 1990).

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