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Divergent mechanisms of *cis*9, *trans*11- and *trans*10, *cis*12-conjugated linoleic acid affecting insulin resistance and inflammation in apolipoprotein E knockout mice: a proteomics approach

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ABSTRACT

Conjugated linoleic acids (CLA) affect atherogenesis, but mechanisms are not well understood. We explored how two isomers of CLA, *cis*9, *trans*11-CLA and *trans*10, *cis*12-CLA, affected lipid and glucose metabolism, as well as hepatic protein expression, in apolipoprotein E knockout mice. After 12 wk of intervention, plasma triglyceride, NEFA, and glucose concentrations were significantly higher in the *trans*10, *cis*12-CLA group, whereas plasma triglyceride, NEFA, glucose, and insulin concentrations were significantly lower in the *cis*9, *trans*11-CLA group, compared with control mice consuming linoleic acid. Proteomics identified significant up- or down-regulation of 113 liver cytosolic proteins by either CLA isomer. Principal component analysis revealed that the treatment effect of *cis*9, *trans*11-CLA was mainly explained by the up-regulation of different posttranslational forms of heat shock protein 70 kD. In contrast, the treatment effect of *trans*10, *cis*12-CLA was mainly explained by up-regulation of key enzymes in the gluconeogenic, β-oxidation, and ketogenesis pathways. Correlation analysis again emphasized the divergent effects of both CLA isomers on different pathways, but also revealed a linkage between insulin resistance and increased levels of hepatic serotransferrin. Thus, our systems biology approach provided novel insights into the mechanisms by which individual CLA isomers differentially affect pathways related to atherogenesis, such as insulin resistance and inflammation.

Key words: dietary fatty acids • atherosclerosis • glucose metabolism • systems biology

Conjugated linoleic acids (CLA) refer to a group of conjugated dieonic isomers of linoleic acid that are present as minor constituents of the lipid fraction of meat, milk, and dairy products or other foods derived from ruminant animals. CLA can protect against the development of atherosclerosis in rabbits (1, 2), hamsters (3–5), and transgenic mice (6). The mechanisms for this are not well understood but might involve modification of the production of atherogenic lipoproteins by the liver or modification of inflammatory pathways.

The regulatory effects of CLA on lipid metabolism may be produced largely by the *trans*10, *cis*12 isomer of CLA (5). This isomer significantly decreased apolipoprotein B secretion from HepG2 cells (7) as well as hepatic stearoyl-CoA desaturase expression (8) and activity (9). In addition, a CLA mixture increased both fatty acid oxidation and decreased esterification to triacylglycerol in OLETF rats (10). These results suggest a triglyceride lowering effect of *trans*10, *cis*12-CLA by reducing very low density lipoprotein (VLDL) triglyceride secretion, fatty acid synthesis, and fatty acid oxidation. However, in vivo data on the hypolipidaemic effects of CLA are not always convincing (5, 11), and even an increase in the production rate of VLDL by *trans*10, *cis*12-CLA has been reported in C57BL/6J mice (12), as well as a substantial increase in fasting plasma levels of triglycerides in apoE*3-Leiden transgenic mice (13). In addition, the *trans*10, *cis*12-CLA isomer caused hyperinsulinaemia with or without an increase in liver weight in several mouse studies (13–17). Hyperinsulinaemia could potentially contribute to the increase in the hepatic neutral lipid stores by way of an increased fatty acid uptake and lipogenesis.

Low-grade inflammation may contribute to insulin resistance, but so far very few studies have been published on the effects of CLA on inflammation in humans. Supplementation with *trans*10, *cis*12-CLA, compared with a CLA mixture or placebo treatment, caused significant increases in plasma concentrations of the proinflammatory marker C-reactive protein (CRP) and urinary levels of 15-ketodihydro $PGF_{2\alpha}$ in men with metabolic syndrome (18). Additionally, the *trans*10, *cis*12-CLA isomer also was mainly responsible for an increase in urinary levels of 15-ketodihydro $PGF_{2\alpha}$ in healthy volunteers (19), although one study reports that *cis*9, *trans*11-CLA was also able to modestly increase levels of 15-ketodihydro $PGF_{2\alpha}$ in obese men (20). However, different doses of individual *cis*9, *trans*11- and *trans*10, *cis*12-CLA isomers did not affect serum concentrations of CRP and did not have any consistent effects on ex vivo cytokine production in healthy subjects (21). In addition, a mixture of CLA had no effect on inflammatory markers interleukin (IL)-6 and CRP in patients with type 2 diabetes mellitus (22) or on the production of cytokines in healthy volunteers (23). The in vivo effect of *trans*10, *cis*12-CLA appears to be a unique example of how aggravated insulin resistance could increase inflammation by mechanisms that are as yet unknown. It cannot be excluded that consumption of this specific CLA isomer on a long-term basis might have pro-atherogenic effects in humans with metabolic syndrome (24).

The present study determined the isomer-specific effect of CLA on changes in lipid and glucose metabolism in apolipoprotein E knockout mice. A wide range of hepatic proteins that are up- or down-regulated by either the *cis*9, *trans*11 isomer of CLA or the *trans*10, *cis*12 isomer of CLA were identified by proteomics. The effects of the individual CLA isomers on lipid and glucose metabolism, inflammation, atherogenesis, and other pathways could be mediated through multiple mechanisms, stressing the need to extend the availability of relevant biomarkers to properly assess the physiological and biochemical effects of both individual CLA isomers.

MATERIALS AND METHODS

Animals and diet

The animal study was carried out in accordance with good animal welfare protocols of the University of Zaragoza, Spain, complying with European legislation governing the use of animals in research. Twenty-nine, 3-month-old male apolipoprotein E knockout mice were kept in groups of three to four animals per cage under standard conditions with free access to food and water. They were randomly assigned to three treatment groups that were matched for initial fasting plasma cholesterol concentrations. All animals consumed a semisynthetic high-fat (30% energy) and cholesterol-containing (0.15% w/w) diet that provided equal amounts of saturated fatty acids, monounsaturated fatty acids, and PUFAs, as described previously (15), for 12 wk. Ten animals received this diet containing 7% (w/w) of fat as linoleic acid (control group), 10 animals received this diet containing 7% (w/w) of fat as *cis*9, *trans*11-CLA, and 9 animals received this diet containing 7% (w/w) of fat as *trans*10, *cis*12-CLA. The PUFA fraction of the diets contained the linoleic acid and the CLA isomers as free fatty acids. The percentage of energy provided by linoleic acid or each CLA isomer was 2.1%. Diets were stored at –20°C under nitrogen, and fresh supplies of the diets were provided daily. Food intake and body weight were monitored throughout the experiment. After the 12-wk intervention period, food was removed at 6 p.m. and the animals were killed between 8 and 10 a.m. the following morning by CO_2 suffocation.

Plasma analysis and tissue isolation

Blood samples were obtained by cardiac puncture after the CO_2 suffocation. The blood was centrifuged at 3000 rpm for 10 min, and plasma was collected for the measurement of nonesterified fatty acids (NEFA) and triglycerides by standard commercial kits (Waku, Madrid, Spain; Sigma, Madrid, Spain), according to the manufacturers' instructions. Plasma insulin and glucose concentrations were measured using standard commercial kits (rat/mouse insulin ELISA kit, Linco Research, St. Charles, MO; Glucose RTU, BioMerieux, Lyon, France) and validated with standard controls (Calimat, BioMerieux). Fasting plasma insulin and glucose concentrations were used to calculate insulin resistance from the homeostasis model assessment for insulin resistance (HOMA) [($glucose_0$ * $insulin_0$)/22.5] (25), although it should be acknowledged that the HOMA model has not been validated for use in animal models (26). Insulin sensitivity was calculated with use of the revised quantitative insulin sensitivity check index (QUICKI) [1/(log $insulin_0$ + log $glucose_0$ + log $NEFA_0$)] (27). Liver, white adipose tissue (epididimal fat pad), brown adipose tissue, and skeletal muscle were removed and weighed. Liver tissue was subsequently used for proteomic analysis.

Proteomics

Cytosolic protein homogenates were prepared from each individual animal liver as described previously (13). Proteins were separated by two-dimensional gel electrophoresis, and the gels were analyzed using PDQuest software (Bio-Rad, Hemel Hampstead, UK). Spots with densities that significantly differed between treatments were excised from the SDS-PAGE gels using the robotic Bio-Rad spot cutter. These proteins were trypsinised using a protocol of the MassPrep

Station (Waters, Micromass, Manchester, UK) and analyzed by MALDI-TOF and electrospray LC mass spectrometric methods as previously described (13).

Western blot analysis

Liver protein homogenates and prestained protein markers (Precision Plus Protein Standards, Bio-Rad) were separated by SDS-PAGE and blotted onto 0.45 uM Biotrace polyvinylidene fluoride transfer membrane (Pall Life Sciences, Dublin, Ireland). Membranes were blocked using 10% nonfat dried milk in phosphate-buffered saline (PBS) with 0.05% Tween (PBS-T) and incubated overnight at 4°C with either anti-nuclear factor (NF)-κBp65 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500) or anti-IκBα (Santa Cruz Biotechnology; 1:500) antibodies. Membranes were washed with PBS-T and incubated for 2 h at room temperature with either peroxidase conjugate anti-rabbit IgG (Sigma, Dorset UK; 1:1000). After further washing, protein complexes were visualized with Supersignal (Pierce, Rockford, IL). Membranes were exposed to film for 1–10 min and processed using an Agfa X-ray processor. Protein bands were quantified using the GeneSnap acquisition and GeneTools analysis software (GeneGenius Gel Documentation and Analysis System, Cambridge, UK). NF-κBp65 and IκBα were expressed as arbitrary units.

Evaluation of plaque size

The heart and the arterial tree were perfused with PBS under physiological pressure, after which the hearts and aortas were dissected out, cleaned, and stored in neutral formaldehyde. The aortic base of the hearts was taken, transferred to liquid OCT (Bayer Diagnostic, Leverkusen, Germany) and frozen in liquid N_2-cooled isopentane. Serial cryosections were made, and average lesion sizes were used for morphometric evaluations based on the method of Paigen et al. (28). Images were captured and digitized using a Nikon microscope equipped with a Canon digital camera. Morphometric analyses were performed using Scion Image software.

Statistical analysis

Data are presented as means ± SD. The unpaired *t* test was used to determine differences in plasma levels at the end of the intervention period. Data were log-transformed before analysis when not normally distributed. Principal component analysis and PLS discriminant analysis were performed after centering and unit variance (UV) scaling of the data in SIGMA P+ (Umetrics Ltd., Windsor, UK). Analysis of correlations was done with Pearson correlation coefficients. The analysis of multiple hypotheses testing for many combinations of variables was done with by determining the *q* values (29) within Genstat. *q* values estimate the probability that a correlation that is called significant is false positive. For example, $q = 0.05$ would mean that we should expect that 5 out of 100 associations that were tested significant are in fact false positive.

RESULTS

Food intake, animal, and tissue weights

Food intake did not differ between the groups during the 12-wk intervention period (data not shown). Body weight gain in both the *cis*9, *trans*11-CLA group (9.29±2.20 g) and in the *trans*10,

*cis*12 CLA-group (8.01±1.17 g) was slightly but significantly lower ($P<0.05$ and $P<0.01$, respectively) than the body weight gain in the control group (11.14±2.73 g). After 12 wk of intervention, liver weight (expressed as percentage of total final body weight) was significantly higher in the *trans*10, *cis*12 CLA-group (11.9±2.6%) compared with the control group (4.2±0.5%) ($P<0.001$). White and brown adipose tissue weight (expressed as percentage of total final body weight) were significantly lower in both the *cis*9, *trans*11-CLA group (0.6±0.1% and 0.5±0.1%, respectively, both $P<0.001$) and in the *trans*10, *cis*12-CLA group (0.6±0.2% and 0.1±0.0%, respectively, both $P<0.001$), compared with the control group (3.0±0.7% and 1.3±0.3%, respectively). No significant difference in skeletal muscle weight was observed between both CLA groups and the control group.

Triglycerides, NEFA, insulin, and glucose

Fasting plasma triglyceride concentrations were >50% lower, and fasting NEFA concentrations were almost 35% lower (both $P<0.05$) upon intervention with *cis*9, *trans*11-CLA compared with the control group. In marked contrast, fasting triglyceride concentrations were >300% higher, and fasting NEFA concentrations were almost 170% higher (both $P<0.01$) upon intervention with *trans*10, *cis*12-CLA compared with the control group (Table 1). Plasma glucose concentrations were 16% lower after intervention with *cis*9, *trans*11-CLA but >30% higher after intervention with *trans*10, *cis*12-CLA, compared with the control group ($P<0.05$). Plasma insulin concentrations were 26% lower after intervention with *cis*9, *trans*11-CLA compared with the control group. Intervention with *trans*10, *cis*12-CLA did not change plasma insulin concentrations compared with the control group (Table 1). Intervention with *cis*9, *trans*11-CLA significantly decreased the index of insulin resistance (HOMA) and increased the index of insulin sensitivity (revised QUICKI) compared with the control group. Intervention with *trans*10, *cis*12-CLA significantly decreased the index of insulin sensitivity but had no effect on the index of insulin resistance, compared with the control group (Table 1).

Proteomics

Two-dimensional gel electrophoresis of individual liver cytoplasmic protein fractions revealed 113 cytosolic proteins of which levels were significantly up- or down-regulated by *cis*9, *trans*11-CLA and/or *trans*10, *cis*12-CLA, compared with linoleic acid. Of these proteins, we were able to identify 104 (92%) proteins with MALDI-TOF mass spectrometry. The proteins identified were categorized according to their major biochemical functions to facilitate the elucidation of pattern changes between treatments (Fig. 1). These classifications are arbitrary to some extent, but clearly effects could be seen on pathways that are related to lipid metabolism, glucose metabolism, protein metabolism, and iron metabolism and oxidation. *cis*9, *trans*11-CLA increased five posttranslationally modified forms of the proteins HSP 70 kD, of which identity was confirmed by peptide sequencing using electrospray LC-MS/MS technology (Fig. 2). This technology was also applied to confirm the identity of macrophage migrating inhibitory factor, fructose 1,6-bisphosphatase, phosphoenolpyruvate carboxykinase, acyl CoA thioester hydrolase, and hydroxymethylglutaryl CoA synthase.

Western blot analysis

Western blot analysis of liver homogenates revealed that IKBα protein was significantly up-regulated in the liver by *cis*9, *trans*11-CLA intervention (133.0±22.0) compared with the control group (93.5±10.87) ($P<0.01$). Hepatic levels of IKBα upon intervention with *trans*10, *cis*12-CLA were not significantly different from levels in the control group. NF-κBp65 protein levels in the liver were not significantly altered by both CLA interventions, as compared with the control group.

Principal component and PLS-discriminant analysis

Principal component analysis of the scaled and centered spot density values revealed that 35% of all variance in the dataset was accounted for by the first principal component (PC1), and an additional 12% was accounted for by the second principal component (PC2) (Fig. 3, upper panel). Adding a third principal component to the model did not significantly contribute toward the predictability of the model. The largest treatment effect on the first principal component (i.e., the largest distance between the spots representing the dietary intervention groups on the *x*-axis) was between the control group (blue spots) and the *trans*10, *cis*12-CLA intervention group (red spots). When considering the PC2 (which represents one or more other, independent variables), both CLA isomers initiated a specific treatment effect. The loadings plot (Fig. 3, lower panel) revealed the proteins that provided the largest positive and negative contribution to the dietary treatment effects in the principal component analysis (Table 2).

PLS-discriminant analysis provided a significant separation between the *cis*9, *trans*11-CLA and *trans*10, *cis*12-CLA intervention groups. The coefficients representing the following proteins were responsible for this separation: selenium binding protein, (glucose regulated) thioredoxin, carboxyl esterase, acyl CoA thioester hydrolase, α enolase, isocitrate dehydrogenase 2 (NAD+) α, NAD-dependent glycerol-3-phosphate dehydrogenase, serotransferrin precursor, ferritin, ATP-dependent 26S proteasome regulator unit, fructose 1,6-bisphophatase, ornithine-oxo-acid transaminase precursor, histidine ammonia-lyase, and apolipoprotein A4.

Pair-wise correlation analysis

In addition to PCA, we performed a pair-wise correlation analysis over the different treatments, including the physiological data measured in plasma lipid as well as the data on hepatic protein levels. Such an analysis shows which parameters vary in a similar way throughout the two CLA treatments, but also highlights which of the parameters are differentially affected by the two CLA isomers. Figure 4 shows a network of all pair-wise interactions for each of the CLA isomers with a Pearson correlation coefficient >0.66 and $q < 0.005$, using the software tool Cytoscape (30).

Plaque size evaluation

Mice fed *cis*9, *trans*11-CLA showed significantly smaller lesion areas compared with animals receiving the control diet (0.074±0.019 mm^2 and 0.109±0.044 mm^2, respectively, $P<0.05$). In contrast, the mice fed *trans*10, *cis*12-CLA showed a significant increase in lesion area compared

with the control mice (0.189±0.078 mm^2 and 0.109±0.044 mm^2, respectively, $P<0.05$), indicating a clear difference in lesion development between both CLA isomers.

DISCUSSION

This study compared the ability of two dietary CLA isomers, *cis*9, *trans*11-CLA and *trans*10, *cis*12-CLA, to regulate physiological parameters and protein expression in a single well-validated animal model for atherosclerosis. This approach enabled us to identify pathways involved in atherogenesis that may underlie the physiological and biochemical changes caused by these dietary treatments. The main pathways affected by both CLA isomers were related to insulin resistance and inflammation.

CLA and insulin resistance

In the apolipoprotein E knockout mice, we observed a simultaneous increase in fasting plasma concentrations of triglycerides, NEFA, and glucose upon consumption of *trans*10, *cis*12-CLA, whereas *cis*9, *trans*11-CLA significantly lowered fasting triglyceride, NEFA, glucose, and insulin concentrations. Although levels of insulin were not significantly affected by *trans*10, *cis*12-CLA, a combination of changes in hepatic protein levels that were involved in β-oxidation of fatty acids, gluconeogenesis, and ketone body formation suggested that this particular CLA isomer provoked an insulin-resistant state in our mouse model. We observed a significant increase in the rate of fatty acid oxidation by *trans*10, *cis*12-CLA, but not *cis*9, *trans*11-CLA, as levels of long-chain acyl-CoA thioester hydrolase were significantly increased upon treatment with this isomer. Acyl-CoA thioesterase hydrolyzes CoA esters of various lengths to free fatty acids and CoA-SH, and it is likely to play an important role in maintaining appropriate CoA-SH levels during periods of increased β-oxidation and fatty acid overload (31). The existence of selective acyl-CoA thioesterases could provide important control points in the oxidation of many peroxisomal substrates, and they may regulate intracellular levels of CoA esters and CoA-SH. To date, several thioesterase isoforms have been identified in peroxisomes, cytoplasm, and mitochondria, where they are thought to have distinct functions in lipid metabolism (31). Increased activity and mRNA expression of various mitochondrial and peroxisomal fatty acid oxidation enzymes upon treatment with a CLA mixture have been described before in C57Bl/6J mice (17) and in apolipoprotein E*3-Leiden transgenic mice (13). Moreover, *trans*10, *cis*12-CLA, and to a lesser extent *cis*9, *trans*11-CLA, also increased the rate of gluconeogenesis, as evidenced by elevated levels of phosphoenolpyruvate carboxykinase, α enolase, and fructose 1,6-bisphosphatase, all representing key enzymes in the gluconeogenesis pathway (Fig. 1). In addition, *trans*10, *cis*12-CLA also increased the rate of gluconeogenesis from glycerol, as evidenced by elevated levels of glycerol-3-phosphate dehydrogenase and glycerol kinase (Fig. 1). Principal component analysis revealed that changes in these gluconeogenesis enzymes made a significant contribution to the treatment effect of *trans*10, *cis*12-CLA but not so much to the treatment effect of the *cis*9, *trans*11-CLA isomer (Table 2). Also, both CLA isomers significantly increased levels of pyruvate dehydrogenase and HMG-CoA synthase and decreased levels of isocitrate dehydrogenase (Fig. 1), indicating an increased flux of acetyl CoA toward the formation of ketone bodies at the cost of acetyl CoA entering the citric acid cycle. In our study, *trans*10, *cis*12-CLA increased levels of HMG-CoA synthase with ~1100%, whereas the *cis*9, *trans*11-CLA isomer increased levels of this enzyme with ~500%.

Hyperinsulinaemia in mice fed *trans*10, *cis*12-CLA has occurred in two of our previous studies using ob/ob mice and the apolipoprotein E*3-Leiden transgenic mouse (13, 15) as well as in three other studies with AKR/J, C57Bl/6J, and ICR mice (14, 16, 17). The hyperinsulinaemic effect of *trans*10, *cis*12-CLA is, however, not restricted to mouse models, as in recent literature, concern has been raised about the potential of *trans*10, *cis*12-CLA to increase insulin resistance in humans. This specific isomer increased insulin resistance in obese men with a metabolic syndrome (32) and also increased plasma triglycerides and glucose concentrations in healthy men, although the latter observation might not be clinically relevant since this effect was insufficient to modify the degree of insulin resistance or insulin sensitivity (33). Also, a mixture of CLA significantly increased fasting glucose concentrations and decreased insulin sensitivity in type 2 diabetic patients after 8 wk of treatment (22). However, a similar amount of CLA mixture did not affect insulin resistance in obese men with a metabolic syndrome after 12 wk of treatment (32). The possible health consequences of prolonged treatment periods with CLA are at present unknown, as are the molecular mechanisms behind the hyperinsulinaemic effects of the *trans*10, *cis*12-CLA isomer. Therefore, more studies are needed to assess its long-term effects and safety in humans.

CLA and inflammation

*cis*9, *trans*11-CLA significantly increased levels of five different posttranslationally modified forms of heat shock protein (HSP) 70 kD, whereas levels of this protein were not altered by *trans*10, *cis*12-CLA. HSPs act as molecular chaperones that ensure correct protein folding of other proteins within the cell. Although HSPs were originally identified on the basis of their induction by elevated temperature, these proteins are in fact induced by a wide range of stimuli that are potentially damaging to the cell, or by cytokines interleukin-6 and interleukin-10 in a nonstressful manner (34). Several in vitro studies have demonstrated that exposure to mildly stressful stimuli that can induce HSP 70 kD expression, or overexpression of HSP 70 kD by transfection, can protect cells against exposure to a more severe stress (34). In addition, transgenic mice overexpressing HSP 70 kD are protected against the damaging effects of ischemia (35). High levels of human HSP 70 kD have been associated with a low coronary artery disease risk, independent of traditional risk factors (36). The protective mechanisms responsible for this relationship might include antiinflammatory pathways. HSP 70 kD forms complexes with inhibitory κB levels and attenuates NF-κB activation. Since NF-κB is a key transcription factor modulating the expression of proinflammatory genes, this could be a key antiinflammatory activity of HSP 70 kD (37). The enhanced protein levels of IKBα in our study do indeed indicate that there is at least some inhibition of degradation of IKBα upon treatment with *cis*9, *trans*11-CLA, which could subsequently lead to a decrease in NF-κB activity.

Furthermore, an inverse relationship has been found between intracellular levels of HSP 70 kD and the activity of cyclo-oxygenase (COX-)2, a major proinflammatory enzyme (38). Therefore, the increased levels of the HSP 70 kD protein observed after intervention with specifically the *cis*9, *trans*10-CLA isomer might contribute toward inhibition of enzymatically (mainly via COX)-induced lipid peroxidation. Although one study suggests that the *cis*9, *trans*10-CLA appears to be able to increase the urinary 15-keto-dihydro-PGF2α to some extent, at least in a group of abdominally obese men (20), other studies report that CLA-induced lipid peroxidation is indeed mainly triggered by the *trans*10, *cis*12-CLA isomer. This isomer, compared with a CLA mixture, induced a significant higher increase in the urinary excretion of 15-keto-dihydro-

PGF2α (a main metabolite of PGF2α that is used as an indicator COX-2-induced lipid peroxidation) in obese men, in men with metabolic syndrome, and in healthy humans (18, 19, 39). The apparent dissimilar ability of both CLA isomers to affect the pathway of COX-induced lipid peroxidation might therefore represent a potential important antiinflammatory effect of HSP 70 kD.

CLA and atherogenesis

A mixture of CLA decreased the development of early atherosclerotic lesions in rabbits (1, 2), hamsters (3–5), and transgenic mice (6). In hamsters, both *cis*9, *trans*11-CLA and *trans*10, *cis*12-CLA reduced the number of lesion sections when compared with a control group of linoleic acid (5). A recent paper by Kritchevsky et al. also shows that both individual CLA isomers, as well as a CLA mixture, were able to significantly inhibit atherogenesis and effect significant regression of established lesions (40). However, in our study we observed a clear difference in lesion development between both CLA isomers. The decrease in lesion area upon intervention with *cis*9, *trans*11-CLA is in agreement with previous studies using different animal models. A pro-atherogenic effect of *trans*10, *cis*12-CLA has not been observed before, but the obvious increase in insulin resistance upon intervention with this CLA isomer in our sensitive animal model might explain the increase in lesion area.

An interesting finding in this respect was the down-regulation of macrophage migration inhibitory factor (MIF) mainly by *cis*9, *trans*11-CLA but also by *trans*10, *cis*12-CLA. MIF is a proinflammatory cytokine expressed widely by vascular cells. In vivo evidence supports direct participation of MIF in atherogenesis as deficiency of MIF decreases atherogenesis in LDLr–/– mice (41). In addition, neutralizing MIF bioactivity after experimental angioplasty in atherosclerosis-susceptible mice reduces vascular inflammation, cellular proliferation, and neo-intimal thickening (42). Therefore, the decreased protein levels of MIF could potentially play a role in the decreased development of atheroslerotic lesions observed upon intervention with *cis*9, *trans*11-CLA.

CLA and body composition

Several animal studies have shown that dietary CLA can substantially reduce the proportion of body fat in mice, and to a lesser extent also in pigs, rats, hamsters, and chickens. Effects from human studies are less convincing than those seen in mice, although doses of CLA used in both studies are comparable (43). The effects of CLA on body weight and body fat have been ascribed to reduced adipocyte size (44), inhibition of adipocyte proliferation (45), increased adipocyte lipolysis (46), greater fatty acid oxidation, and energy expenditure (16), and they are believed to be attributable to the *trans*10, *cis*12 isomer (47).

As expected, dietary intervention with *trans*10, *cis*12-CLA significantly decreased body weight gain as well as the weight of white and brown adipose tissue in our animals, confirming the findings in our study in ob/ob mice, which were fed exactly the same diets (15). However, we also observed a significant, albeit smaller, reduction in body weight and fat depots upon intervention with *cis*9, *trans*11-CLA. This finding is puzzling and has not been observed before. This effect might be specific to the apolipoprotein E knockout animal model, which may be more

sensitive to the anti-adipogenic effects of trace levels of *trans*10, *cis*12-CLA in the *cis*9, *trans*11-CLA-rich diet.

Comparative analysis of the two CLA isomer treatments

The distinctive color patterns in the two correlation plots of the individual CLA isomers (Fig. 4) highlight the divergent effects that *cis*9, *trans*11-CLA and *trans*10, *cis*12-CLA had on physiological parameters as well as hepatic protein levels, indicating that separate pathways were affected. Across treatments, this correlation plot revealed expected and less obvious clusters of associated variables. Most noticeable were two clusters that were related to glucose metabolism. The first cluster (A) contained all the plasma-derived parameters relating to glucose metabolism and insulin resistance (plasma triglycerides, plasma NEFA, plasma glucose, plasma insulin), as well as three derived plasma indices of insulin resistance: HOMA, QUICKI, and the revised QUICKI. This cluster was plausibly linked to three posttranslational forms of phosphoenolpyruvate carboxykinase, a rate-limiting enzyme in the gluconeogenesis pathway. However, this link was unexpectedly represented by the enzyme glutathione-S-transferase zeta (also known as maleylacetoacetate isomerase), catalyzing the fifth step in phenylalanine catabolism. Currently, we cannot explain how this enzyme could physiologically link several indices of insulin resistance. The second cluster (B) contained enzymes involved in glucose metabolism, for example, glucose phosphomutase, fructose 1,6-bisphosphatase, and glycerol-3-phosphate dehydrogenase, as well as serotransferrin, an iron binding transport protein. Indeed, both glucose and iron metabolism were affected upon consumption of *trans*10, *cis*12-CLA, as we observed a simultaneous up-regulation of these gluconeogenic enzymes as well as significantly higher levels of hepatic serotransferrin and serotransferrin precursor, indicative of increased levels of hepatic iron. Insulin resistance-associated hepatic iron overload combined with liver steatosis has been observed before (48), although mechanisms underlying this association have not yet been identified. Hepatic iron overload with hepatic steatosis may down-regulate insulin receptors and hence insulin action. Alternatively, insulin resistance may lead to the redistribution of transferrin receptors to the cell surface, where they could mediate the uptake of extracellular iron. Another explanation involves inflammation: since the insulin-resistant syndrome is an atherogenic state, widespread activation of inflammatory cytokines in the subendothelial space increases transcription of ferritin mRNA in macrophages, and these cells may subsequently transfer ferritin to hepatocytes (49).

Conclusion

This study was undertaken to identify mechanisms by which *cis*9, *trans*11-CLA and *trans*10, *cis*12-CLA differently affect pathways that underlie the change in physiological markers for the development of atherosclerosis in the apolipoprotein E knockout mice. Proteomic analysis of liver proteins provided insight into both novel and recognized mechanisms related to lipid degradation, gluconeogenesis, and ketone body formation. Altered expression of acyl CoA thioester hydrolase, α enolase, pyruvate dehydrogenase, glycerol-3-phosphate dehydrogenase, fructose 1,6-bisphophatase, phosphoenolpyruvate carboxykinase, and hydroxymethylglutaryl CoA synthase proteins explained most of the antidiabetic treatment effects of *cis*9, *trans*11-CLA and the prodiabetic effects of *trans*10, *cis*12-CLA in the apolipoprotein E knockout mice. In addition, for the first time, we showed that the *cis*9, *trans*11 isomer of CLA specifically induces an increased expression of the antiinflammatory HSP 70 kD protein, as well as a decreased

expression of the proinflammatory macrophage migration inhibitory factor. Both effects could potentially contribute to a less severe inflammatory response, or protection against the development of atherosclerosis upon consumption of *cis*9, *trans*11-CLA.

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Table 1

Fasting plasma triglycerides, nonesterified fatty acids (NEFA), glucose and insulin concentrations, HOMA, and revised QUICKI in apolipoprotein E knockout mice fed a high-fat, high-cholesterol diet supplemented with 1% (w/w) linoleic acid (control), 1% (w/w) *cis*9, *trans*11-CLA, or 1% (w/w) *trans*10, *cis*12-CLA for 12 wk

	control (*n*=10)	*cis*9, *trans*11-CLA (*n*=9)	*trans*10, *cis*12-CLA (*n*=9)
Triglycerides (mmol/l)	2.2 ± 0.5	1.5 ± 0.6[a]	6.5 ± 4.0[a]
NEFA (mg/dl)	3.2 ± 1.0	2.1 ± 1.0	8.6 ± 4.1[a]
Glucose (mmol/l)	17.3 ± 2.8	14.4 ± 2.3[a]	22.6 ± 6.4[a]
Insulin (pmol/l)	295.7 ± 102.7	217.8 ± 23.2[a]	304.5 ± 105.3
HOMA	32.7 ± 12.8	20.3 ± 4.8[a]	44.6 ± 20.3
revised QUICKI	0.21 ± 0.01	0.22 ± 0.01[a]	0.18 ± 0.01[b]

[a]Values represent the mean ± SD. HOMA, homeostasis model for insulin resistance; (revised) QUICKI, quantitative insulin sensitivity check index.
[b]Significantly different from the control group: *$P < 0.05$; **$P < 0.01$.

Table 2

Liver cytosolic proteins representing the highest positive and negative loadings towards the dietary treatment effects in principal component analysis.

Treatment	Proteins representing the highest positive loadings toward treatment	Proteins representing the highest negative loadings toward treatment
Control	3203 Thioether-S-methyltransferase 3208 Thioether-S-methyltransferase 3504 ATP-dependent 26S proteasome regulator unit 3506 Ornithine-oxo-acid transaminase 4602 Aldehyde dehydrogenase family 7, A1 5002 D-dopachorme tautomerase 5003 Pterin-4-alpha-carbinolamine dehydratase 5203 Peroxiredoxin 6 5204 Haao protein	2608 Glycerol kinase 3101 Ferritin light chain 1 5708 Phosphoenolpyruvate carboxykinase
*c*9,*t*11 CLA	1606 Heat shock protein 60 kD 2602 Heat shock protein 60 kD 702 Heat shock protein 70 kD 1704 Heat shock protein 70 kD 1705 Heat shock protein 70 kD 2712 Heat shock protein 70 kD 2708 Stress 70 protein, GRP 75 kD 3706 Heat shock protein 75 kD 1503 Protein disulfide isomerase A6 precursor 3601 Selenium binding protein 2 3623 Selenium binding protein 2 3608 Thioredoxin (GRP) 3801 10-Formyltetrahydrofolate dehydrogenase 4810 Glutathion-S-transferase Z 5707 Phosphoenolpyruvate carboxykinase 6504 Elongation factor 1-gamma 6701 Phosphoenolpyruvate carboxykinase	2104 Proteasome B 7412 Isocitrate dehydrogenase
*t*10,*c*12 CLA	1404 Apolipoprotein A4 1412 Apolipoprotein A4 2710 Albumin 1 2714 Albumin 1 3702 Albumin 1 3505 Alpha enolase 5501 Alpha enolase 6503 Alpha enolase 4408 Pyruvate dehydrogenase 4401 Fructose 1,6-bisphosphatase 5519 Acyl-CoA thioester hydrolase (Bile acid) 5515 Hydroxymethylglutaryl CoA synthase 6506 Hydroxymethylglutaryl CoA synthase 7511 Hydroxymethylglutaryl CoA synthase 4107 Thioredoxin peroxidase 5306 Delta 3,5-delta 2,4-dienoyl-CoA dehydrogenase 7507 Homogentisate 1,2-dioxygenase 6707 Serotransferrin 7702 Serotransferrin precurosr 7703 Serotransferrin precursor	304 Senescence marker 30 2604 Heat shock protein 60 kD 3503 Adenosin kinase 3605 Selenium binding protein 2 4104 Glutathion peroxidase 4205 Thiopurine-S-methyltransferase 4305 Malate dehydrogenase 4501 Ornithine-oxo-acid transaminase precursor 4704 Histidine ammonia lyase 5506 Adenosylhomocysteinase 4608 Aldehyde dehydrogenase family 7, A1 5601 Aldehyde dehydrogenase family 7,A1 6501 Aldehyde dehydrogenase 6203 Sulphotransferase 6402 Ureido propionase beta 6407 Isovaleryl CoA dehydrogenase 6409 Arginase-1 liver

Fig. 1

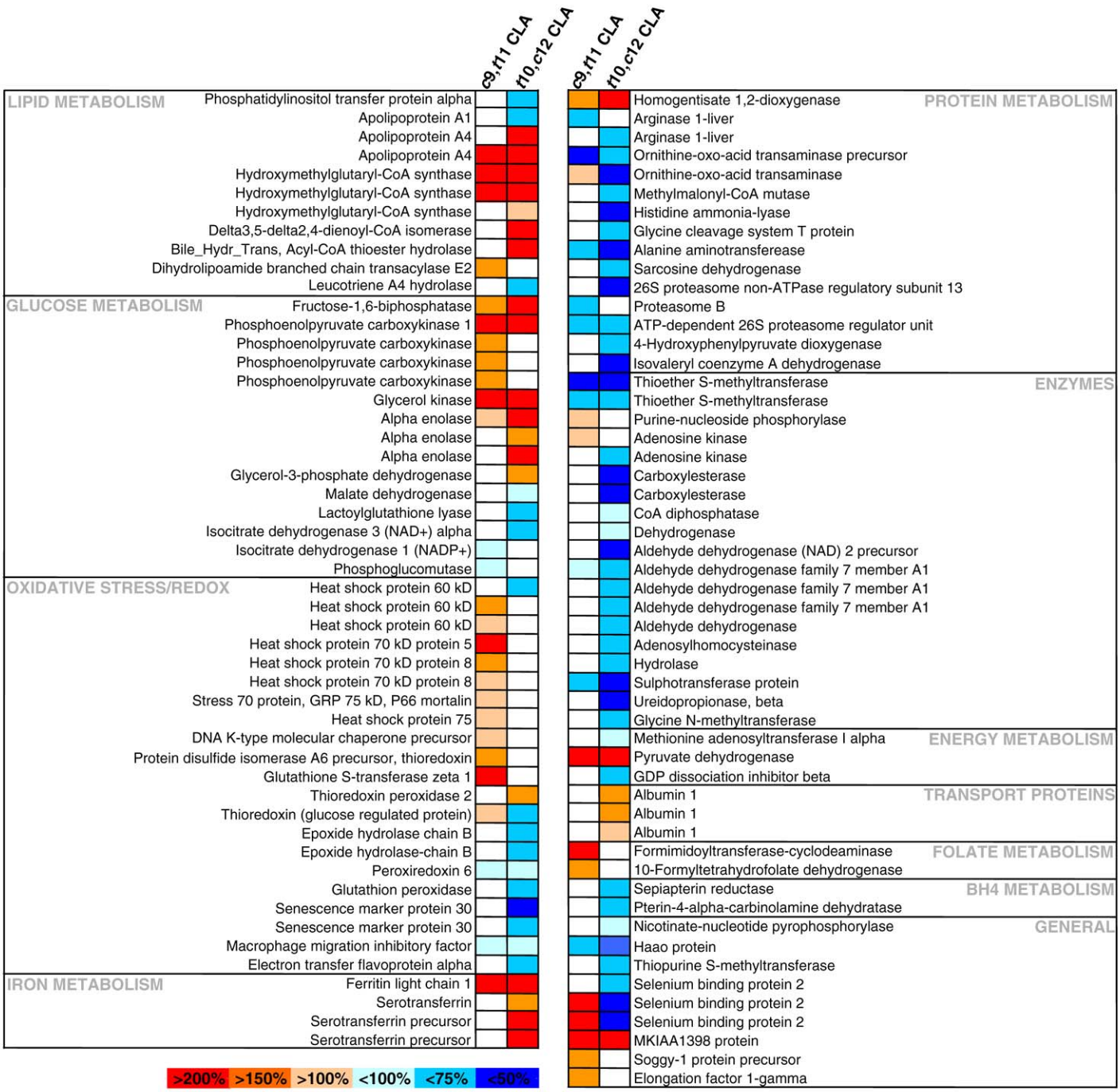

Figure 1. Overview of cytosolic proteins that were significantly increased or decreased in the *cis*9,*trans*11-CLA or the *trans*10,*cis*12-CLA group, as compared with the control group. Relative protein masses were calculated using the PDQuest software as described in Materials and Methods. The colored code indicates the percentage increase or decrease in protein mass in any dietary intervention group as compared with the control group; the white color indicates a nonsignificant change. Proteins were identified by MALDI-TOF mass spectrometry and/or LC-MS/MS as described in Materials and Methods.

Fig. 2

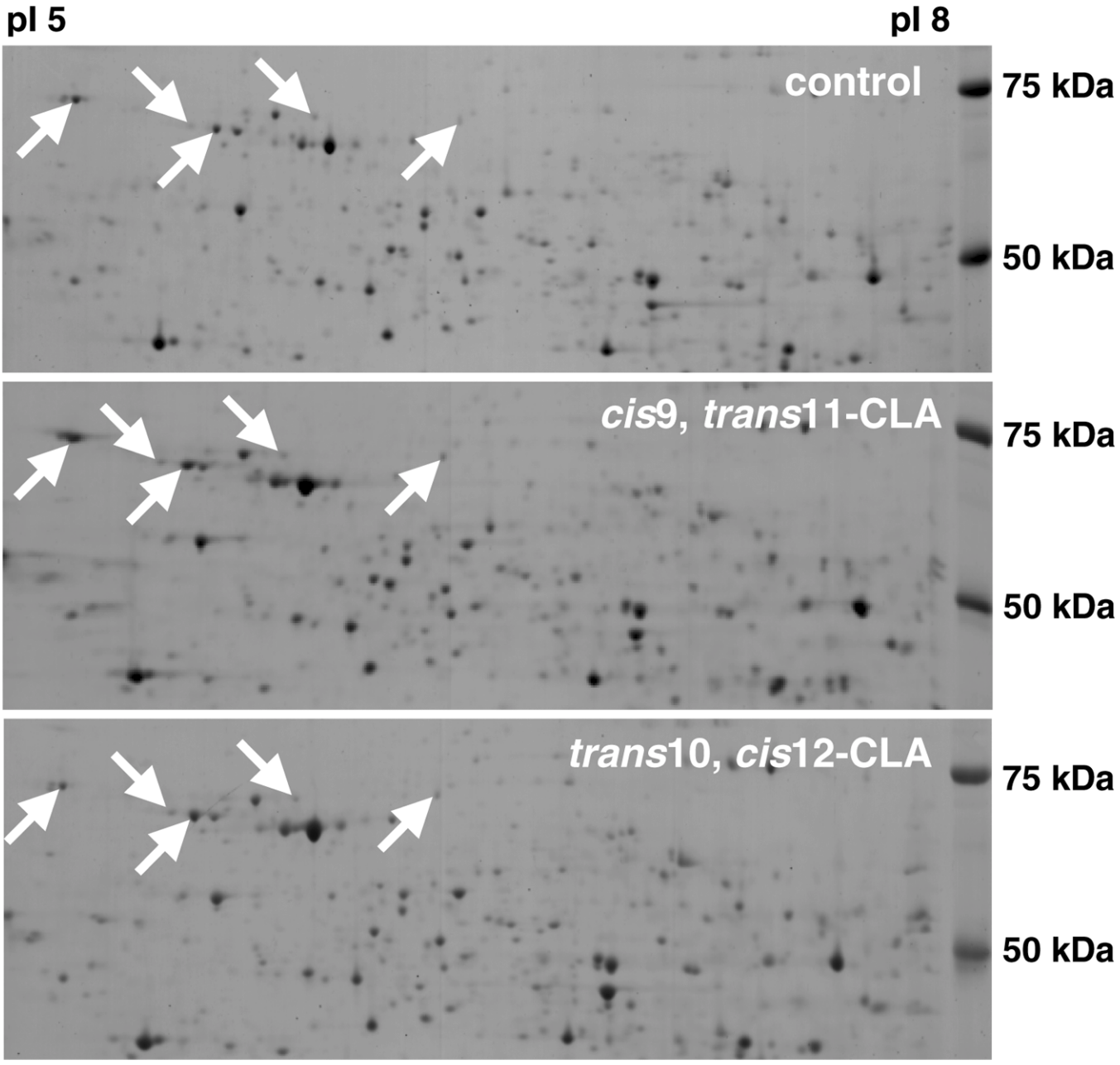

Figure 2. Representative two-dimensional gel electrophoresis gels of the three intervention groups indicating the differential protein expression of heat shock protein (HSP) 70 kD, as identified by MALDI-TOF mass spectrometry and LC-MS/MS and described in Materials and Methods.

Fig. 3

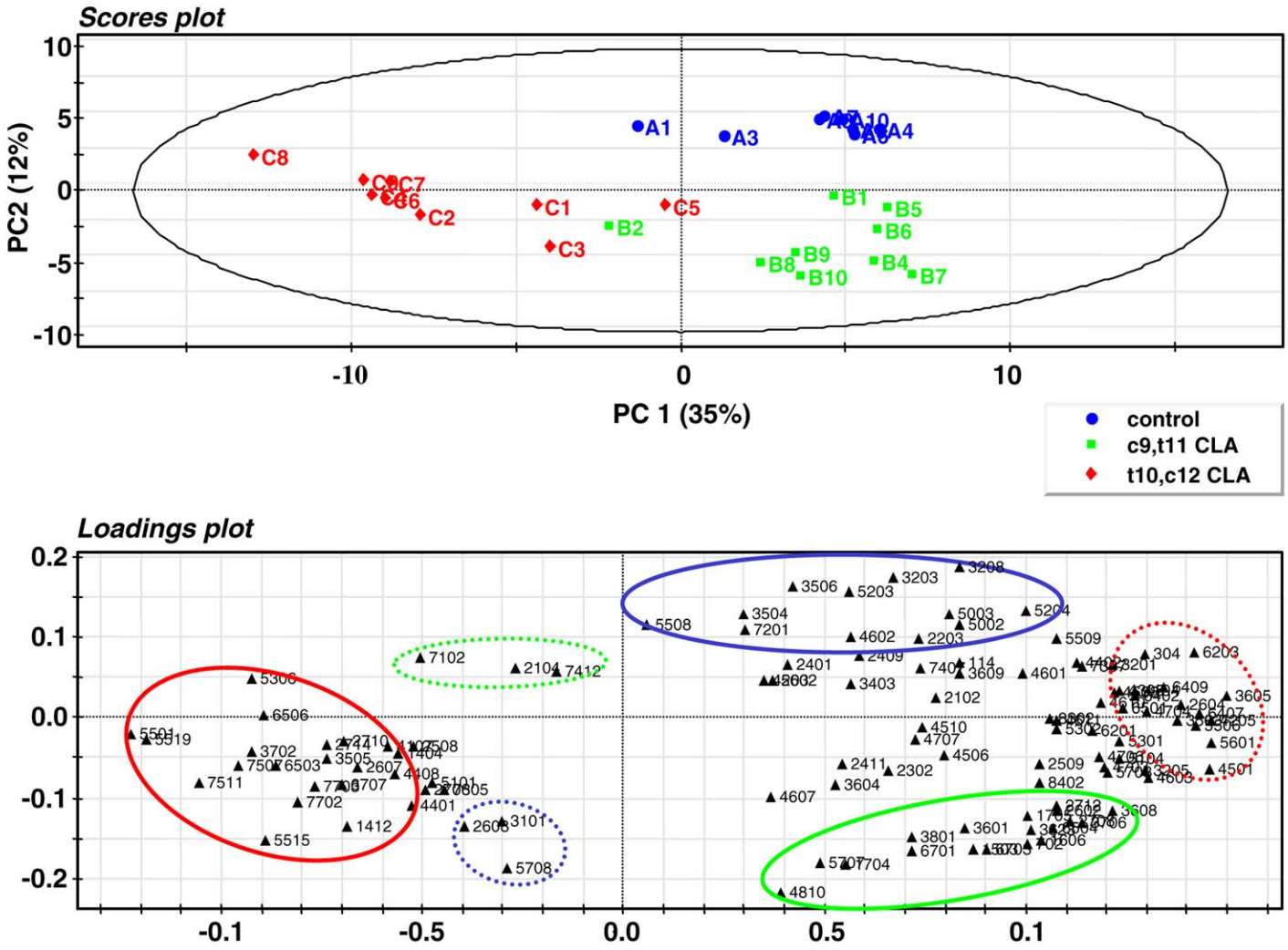

Figure 3. Scores plot and loadings plot of a principal component analysis of hepatic cytosolic proteins that were significantly up-or down regulated by either *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA, as compared to the control group.

Fig. 4

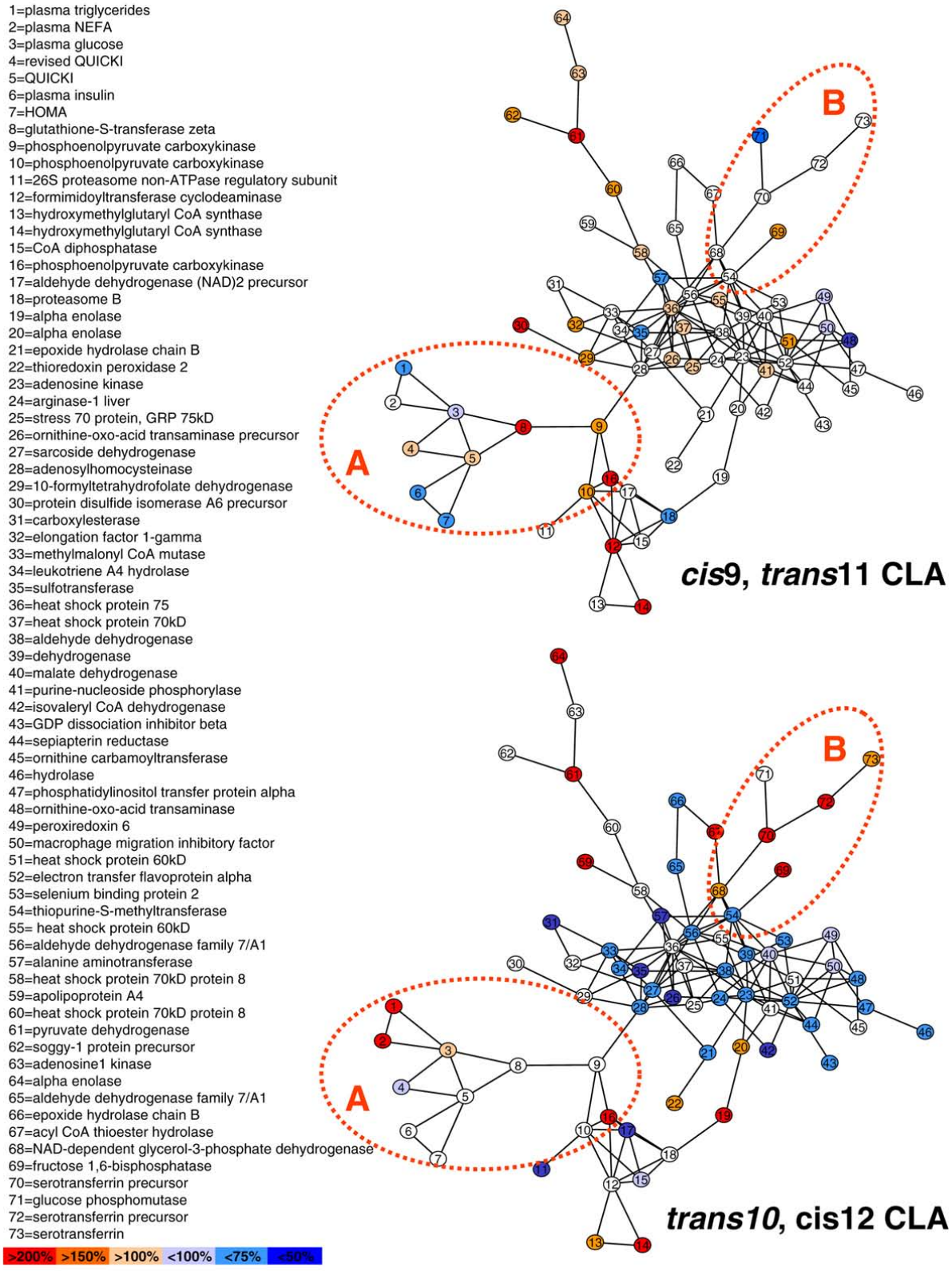

Figure 4. Correlation plot indicating all pair-wise correlations between plasma lipid and liver protein levels that had a Pearson correlation higher than 0.66 and a *q* value lower than 0.005, using the software tool Cytoscape as described in Materials and Methods. The color code indicates the percentage increase or decrease in plasma levels or hepatic protein mass in any dietary intervention group as compared to the control group.