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RESEARCH ARTICLES

Divergent effects of a CLA-enriched beef diet on metabolic health in ApoE $^{-/-}$ and *ob*/*ob* mice☆,☆☆

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Abstract

Conjugated linoleic acid (CLA) is found naturally in meat and dairy products, and represents a potential therapeutic functional nutrient. However, given the discrepancies in isomer composition and concentration, controversy surrounds its proposed antidiabetic, antiobesity effects. This study focused on the effects of CLA-enriched beef (composed predominantly of c9, t11-CLA) in two separate models of metabolic disease: proatherosclerotic ApoE$^{-/-}$ mice and diabetic, leptin-deficient *ob*/*ob* mice. Animals were fed CLA-enriched beef for 28 days, and markers of the metabolic syndrome and atherosclerosis were assessed. Comprehensive hepatic transcriptomic analysis was completed to understand divergent metabolic effects of CLA. CLA-enriched beef significantly reduced plasma glucose, insulin, nonesterified fatty acid and triacylglycerol and increased adiponectin levels in *ob*/*ob* mice. In contrast, plasma lipid profiles and glucose homeostasis deteriorated and promoted atherosclerosis following the CLA-enriched beef diet in ApoE$^{-/-}$ mice. Hepatic transcriptomic profiling revealed divergent effects of CLA-enriched beef on insulin signaling and lipogenic pathways, which were adversely affected in ApoE$^{-/-}$ mice. This study demonstrated clear divergence in the effects of CLA. CLA-enriched beef improved metabolic flexibility in *ob*/*ob* mice, resulting in enhanced insulin sensitivity. However, CLA-enriched diet increased expression of lipogenic genes, resulting in inefficient fatty acid storage which increases lipotoxicity in peripheral organs, and led to profound metabolic dysfunction in ApoE$^{-/-}$ mice. While CLA may have potential health effects, in some circumstances, caution must be exercised in presenting this bioactive lipid as a potential functional food for the treatment of metabolic disease.

Keywords: Conjugated linoleic acid; ApoE; *ob*/*ob*; Atherosclerosis; Metabolic syndrome

1. Introduction

Conjugated linoleic acid (CLA) represents the positional and geometric isomers of linoleic acid. Found in meat and dairy produce of ruminant animals, up to 28 isomers have been identified [1]. The two most abundant isomers, *cis*9, *trans*11-CLA (c9,t11-CLA) and *trans*10, *cis*12 (t10,c12)-CLA, have focused attention on the role of CLA in human health. C9,t11-CLA accounts for up to 90% of total CLA and is reported to have anti-inflammatory properties demonstrating potential for treatment of proinflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease and insulin resistance [2-4]. T10,c12-CLA is less abundant, representing 1%–10% of total CLA, and has received considerable attention as an antiobesity agent [5]. However, use in human subjects remains a controversial issue, as its prolonged use has been associated with dysfunctional glucose homeostasis and increased risk of developing type 2 diabetes [6–8].

The anti-inflammatory, antiobesity effects of CLA make it a prime candidate for the treatment of metabolic syndrome. This syndrome includes an array of metabolic complications, such as dyslipidemia, hypertension, low-grade inflammation and insulin resistance, which predispose to type 2 diabetes and atherosclerosis [9–11]. Reports indicate divergent metabolic mechanisms for both c9,t11-CLA and t10,c12-CLA in animal feeding studies [12]. C9,t11-CLA has potent anti-inflammatory effects leading to a reduction in adipose tissue macrophage infiltration and to improved insulin and lipid profiles, thus ameliorating the progression of insulin resistance and atherosclerosis [4,13,14]. T10,c12-CLA is associated with a reduction in fat mass; however, subsequent increases in plasma lipids increase the risk of lipotoxic effects in peripheral insulin-sensitive organs [15]. It is important to note that these effects are observed in studies using high concentrations of synthetic CLA isomers rather than naturally occurring sources.

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CLA is a natural by-product of the biohydrogenation of linoleic acid to stearic acid in ruminants [16,17]. Indeed, increasing the pasture to grain ratio of cattle feed dramatically enhances CLA concentrations in beef and dairy produce [18]. Furthermore *trans*-vaccenic acid, the most abundant *trans*-fat in ruminant produce, can be processed to c9, t11-CLA by stearoyl-CoA-desaturase (SCD), further enhancing CLA levels endogenously [19]. Previous research by this group indicated that the most dramatic changes in gene expression in response to CLA-enriched beef occurred in the liver (1270 genes) rather than the adipose tissue (16) or skeletal muscle (601) [20]. Also, given the central role of the liver in key metabolic pathways common to both metabolic disease and atherosclerosis, this study specifically focused on differential liver transcriptomic profiles. The objective of this study was to determine the effect of feeding CLA-enriched beef in two mouse models of metabolic dysfunction: apolipoprotein E knockout (ApoE$^{-/-}$) and leptin-deficient *ob/ob* mice. Feeding CLA-enriched beef had beneficial effects on plasma lipid biomarkers in *ob/ob* but not ApoE$^{-/-}$ mice. Hepatic transcriptomic profiling highlighted divergent molecular mechanisms which identified important variable effects of beef enriched with CLA on insulin signaling and lipogenic pathways.

2. Methods

2.1. Animals and diets

Homozygous ApoE-deficient mice on a C57BL6 background (C57BL/6J-Apoe^{tm1Unc}, 10th generation backcrossed from 129/B6 F1 heterozygous to C57BL6) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). *Ob/ob* (B6.V-Lep^{ob}/ola Hsd) mice were purchased from Harlan, UK. Animals were housed in a facility with a 12-h light cycle and had free access to food and water. Mice were fed an irradiated diet from 6 weeks of age. The mice were randomly assigned to either a low-CLA beef or a high-CLA beef diet group. Food consumption and weight were monitored. All diets were stored at 4°C and were provided fresh to the animals every second day.

2.1.1. Generation of beef-derived feeds

Charolais-cross steers were fed a high-concentrate/straw ration to generate the low-CLA beef diet. For the high-CLA beef diet, charolais-cross heifers were offered grazed perennial ryegrass supplemented with sunflower oil and fish oil. After 150 days, psoas muscle, longissimus muscle and subcutaneous adipose tissue were collected and pooled within ration type to yield a 35% fat beef product. This was then freeze dried and incorporated into mouse feed.

2.1.2. Fatty acid composition of dietary feeds

The composition of the diets, in particular, the fatty acid composition of the high- and low-CLA beef-enriched diets, is presented in Supplemental Table 1. Briefly, lipid was extracted according to Folch et al., dried lipid samples were methylated, and acidic *trans*-esterification was carried out as outlined by Kramer and Zhou [21].

2.1.3. Disease models

ApoE$^{-/-}$ mice were randomised at 40 days to receive standard chow (0.5% total fat and 0.022% cholesterol; n=8; Diet A) for 13 weeks or 1% cholesterol (standard chow with 15.8% fat and 1% cholesterol; Special Diet Services, Essex, UK; n=32) for 8 weeks. After 8 weeks, the mice were further randomised into three groups — 1% cholesterol diet (n=8; Diet B), diet supplemented with low-CLA beef (n=8; Diet C) and diet supplemented with high-CLA beef (n=8; Diet D) — for a further 5 weeks. The *ob/ob* mice received either diet supplemented with low-CLA beef (n=8) or diet supplemented with high-CLA beef (n=8) for 4 weeks. At the time of sacrifice, whole blood was taken by cardiac puncture. Aorta samples were harvested, and samples for histology analysis were placed in 10% formal saline. White adipose tissue, liver and aorta samples for protein and mRNA analysis were frozen in liquid nitrogen.

2.1.4. Biochemical analysis

Plasma triacylglycerol (TAG), cholesterol, nonesterified fatty acid (NEFA) and glucose levels were determined by enzymatic assay using commercially available kits (Randox Laboratories, Antrim, UK). Plasma insulin levels were determined by enzyme-linked immunosorbent assay (ELISA) (Mercodia Inc., NC, USA). Fasting plasma insulin and glucose levels were used to calculate insulin resistance using the homeostasis model assessment for insulin resistance (HOMA-IR) [(glucose$_{fasting}$×insulin$_{fasting}$)/22.5], with the knowledge that HOMA-IR is validated for humans and not mice [4]. Plasma adiponectin and CRP were measured by ELISA (R&D Systems, Abingdon, UK, and Alpco Diagnostics, NH, USA).

2.1.5. Hematoxylin and eosin (H&E) staining

Aorta samples were perfused *in situ* with phosphate-buffered saline and formal saline (0.9% NaCl+10% formaldehyde). The aorta was removed from the aortic arch to the iliac bifurcation and fixed in formal saline for 24–48 h. The vessels were paraffin embedded, and serial sections (5 μM thick) were cut transversely from the aortic arch (Leitz 1512 microtome, Weltzar GmBH, Germany). Sections from the aortic arch were stained using standard H&E procedures. Images of section were captured using a (Nikon E80i) microscope and Polaroid digital camera, and the image analysis was carried out using ImageJ software (NIH, MD, USA). The total area of the aorta and the area of the lesion(s), if any, were measured accurately for each section. Atherosclerosis was quantified as percentage lesion area per total surface area of the aorta.

2.1.6. Protein isolation and Western blotting

Total protein was isolated from the aorta and adipose tissue using the nuclear extract kit from Active Motif (Carlsbad, CA, USA). Protein concentrations were determined by Bradford assay (BioRad, CA, USA). Fifteen micrograms of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis for Western immunoblotting. Antibodies used for analysis were PPARγ, CD68, MMP-9 and actin (Santa Cruz, CA, USA).

2.1.7. mRNA analysis

Tissues were harvested in TRIreagent (Molecular Research Center Inc., OH, USA), and total RNA was extracted according to the manufacturer's instructions. Single-stranded cDNA was prepared using the High Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). mRNA expression was quantified by real-time polymerase chain reaction(PCR) on an ABI 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Warrington, UK). TaqMan real-time PCR was performed for FASN, PPARγ and F4/80 using predeveloped assay reagent kits. mRNA levels were normalized to 18S for each sample by subtracting the C_t for 18S from the C_t for the gene of interest, producing a ΔC_t value. The ΔC_t for each treatment sample was compared to the mean ΔC_t for control samples using the relative quantitation $2^{-(\Delta\Delta Ct)}$ method to determine fold-change.

2.1.8. Analysis of microarray data

Microarray data were processed and analysed using Bioconductor software [22]. Basic QC was performed using the affyQCReport library and hierarchical clustering. Following background correction and normalization using the gcrma approach, differential gene expression was identified by the limma library [23]. Genes exhibiting an false discovery rate-adjusted P value [24] of less than .05 were deemed to be differentially expressed.

2.2. Canonical correlation analysis (CCA) of metabolic markers and gene expression in ApoE mice

Regularized CCA using the Bioconductor library mixOmics [25] was used to examine correlations between differentially expressed genes (P value<.01) and levels of metabolic markers. Data were standardized over the different diets in order to identify correlation independent of diet group. Genes showing little variation over all conditions (measured by an Interquartile range <0.5) were removed. CCA was run with M-fold cross-validation using the default number of folds.

2.3. Pathway analysis of differentially expression

Pathway analysis was performed using the Gene set enrichment analysis (GSEAlm) library [26]. KEGG pathways with more than five genes present on the microarray chip were used as gene-sets. In calculating gene-set level t-statistics, both the original and absolute values of the per-gene t-statistics were examined, allowing analysis of significant unidirectional and bidirectional changes in gene sets [20]. Pathways with P values less than .01 were considered significantly altered.

2.3.1. Statistics

Two-way analysis of variance was used to determine significant differences between dietary conditions. Data are presented as means±S.E.M. When this indicated significance (P<.05), post hoc Bonferroni test analysis was used to determine which conditions were significantly different from each other. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Divergent effects of low- and high-CLA beef diets on plasma markers of metabolic dysfunction in ApoE$^{-/-}$ and ob/ob mice

CLA-enriched beef diets exhibited contrasting effects on plasma markers of metabolic dysfunction in ApoE$^{-/-}$ and *ob/ob* mice. ApoE$^{-/-}$ mice were adversely affected by both low- and high-CLA-enriched beef diets. Plasma glucose, insulin, NEFA, TAG and cholesterol were all significantly elevated (P<.001) following consumption of beef-enriched diets when compared to standard or high-cholesterol diets (Fig. 1A–G), while the insulin-sensitising

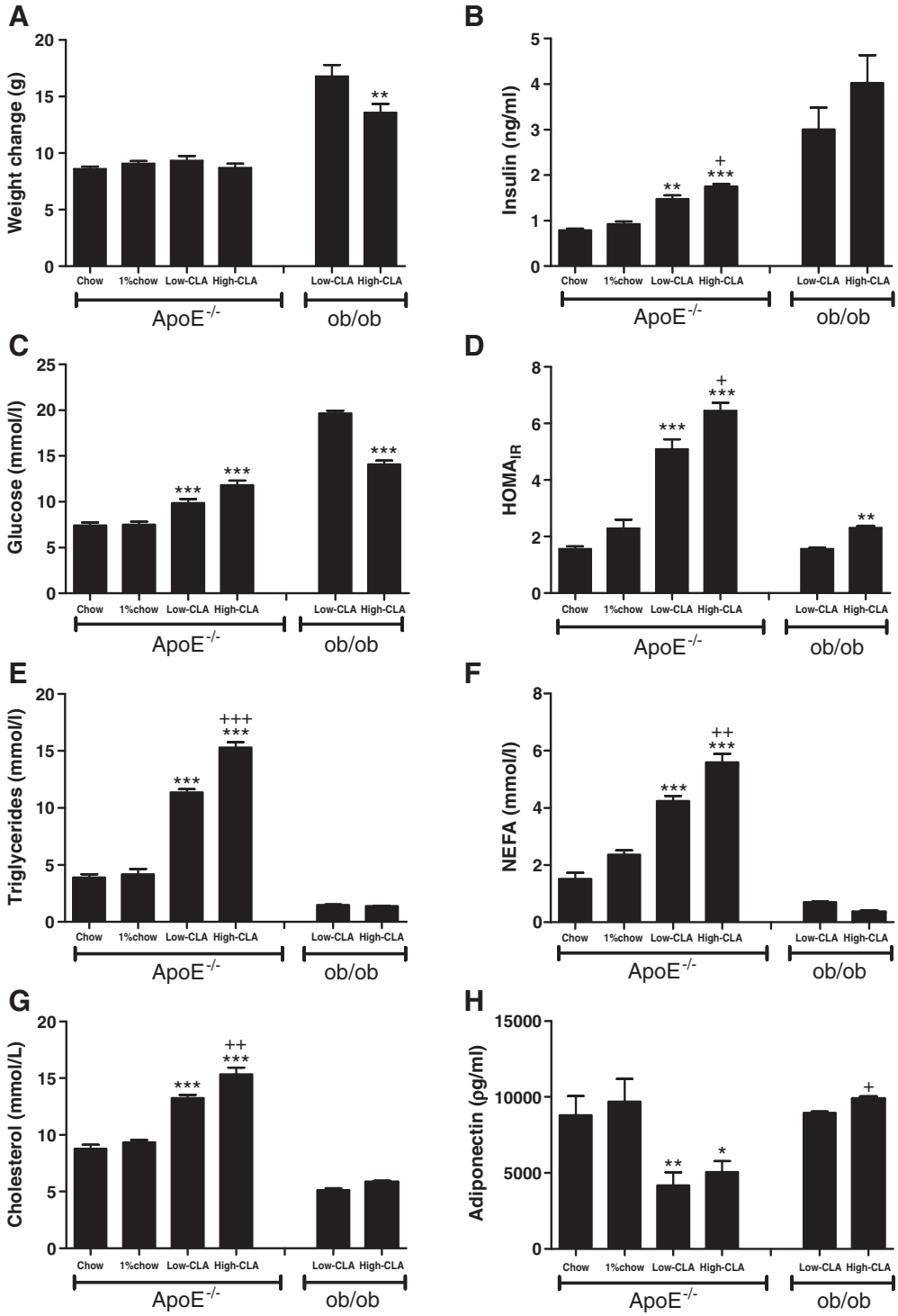

Fig. 1. Changes in plasma markers of insulin resistance in ApoE$^{-/-}$ and *ob/ob* mice in response to CLA-enriched beef diets. (A) Weight change of animals over course of metabolic challenge (**P<.01 w.r.t. chow, n=8). (B & C) Basal plasma insulin and glucose concentrations were measured using commercially available kits (Mercodia) (**P<.01, ***P<.001 w.r.t. chow; +P<.05 w.r.t low-CLA, n=8). (D) HOMA$_{IR}$ was calculated in the following way [(glucose$_0$*insulin$_0$)/22.5] (***P<.001 w.r.t chow; +P<.05 w.r.t low-CLA). (E–G) Triglycerides, NEFA and cholesterol were determined using commercially available kits (Randox) (***P<.001 w.r.t chow; ++P<.01, +++P<.001 w.r.t low-CLA). (H) Adiponectin was determined using DuoSet ELISA (R&D) (*P<.05, **P<.01 w.r.t chow; +P<.05 w.r.t low-CLA).

adipokine adiponectin was significantly reduced (Fig. 1H). Furthermore, beef-enriched diets promoted insulin resistance (HOMA-IR), an effect which was further augmented by the high-CLA-enriched beef diet (Fig. 1D). Interestingly, this group exhibited a more severe effect on NEFA, TAG and cholesterol concentrations (P<.01). In contrast, obese, insulin-resistant *ob/ob* mice fed the high-CLA-enriched beef diet had significantly lower plasma glucose concentrations and increased adiponectin levels compared to the low-CLA beef diet group. This did not translate into improved insulin sensitivity (HOMA-IR). Plasma lipid profiles remained unchanged

in the low- compared to high-CLA-enriched beef diets (Fig. 1A–H). There was no difference in food intake across genotypes or diet groups (data not shown).

3.2. CLA-enriched beef diets promote atherosclerosis progression in ApoE$^{-/-}$ but improve insulin signaling in adipose tissue of ob/ob mice

Representative aorta cross sections from ApoE$^{-/-}$ mice demonstrated early atherosclerotic lesion development in chow-fed mice. Mice fed high-cholesterol chow developed atherosclerotic lesions covering approximately 31% of the total vessel area (31.48%±1.9%) compared to the chow-fed controls (P<.001) (Fig. 2). The low-CLA-enriched beef diet group displayed equivalent effects. Interestingly, the high-CLA beef group exhibited advanced atherosclerotic lesion development (39.03%±4.33%), which was significantly greater than that in the high-cholesterol chow group (P<.05) (Fig. 2).

Adipose tissue biology is fundamental to obesity-induced insulin resistance characteristic of type 2 diabetes; therefore, markers of insulin sensitivity were assessed in the white adipose tissue of the *ob/ob* mice. The high-, but not the low-, CLA-enriched beef diet enhanced GLUT4, IRS-1 and adiponectin mRNA expression, key targets that regulate insulin sensitivity, compared to the chow diet (P<.05, P<.01) (Fig. 3A–D). Also, Western blot analysis demonstrated enhanced GLUT4 and pIRS1Ser307 expression in the high-CLA- compared to the low-CLA-enriched beef diet group, thus promoting insulin sensitivity despite the obese phenotype. Overall, these results confirm the diverging effects of a high-CLA-enriched beef diet in different mouse models of metabolic dysfunction.

3.3. CLA-enriched beef exerts a strong effect on differential gene expression in ApoE$^{-/-}$ mice

CLA-enriched beef fed ApoE$^{-/-}$ mice evoked substantial differential gene expression (4900+) within the hepatic transcriptomic signature compared to control and low-CLA beef diets (Table 1). In contrast, the chow- and cholesterol-enriched diets were not significantly different. Furthermore, beef-fed versus non-beef-fed control mice elicited the largest change in gene expression (8613 genes), demonstrating that CLA-enriched beef diets exerted strong effects on gene expression in the ApoE$^{-/-}$ mice. Differentially expressed gene numbers are presented in Table 1. GSEAlm analysis was performed individually on the *ob/ob* and ApoE$^{-/-}$ data, and resulting pathways from both analyses were compared to understand the basis of the different metabolic phenotype. Compared to their corresponding controls, ApoE$^{-/-}$ mice fed a CLA-enriched beef diet had more altered pathways (183) than *ob/ob* mice fed CLA-enriched beef (131). Fifty-two of the 183 pathways altered in ApoE$^{-/-}$ mice were not altered in *ob/ob* mice, while all pathways altered in *ob/ob* mice were also altered in ApoE$^{-/-}$ mice. Sixteen pathways were identified as significantly up-regulated in ApoE$^{-/-}$ mice, a number of which were involved in metabolism (Fig. 4).

3.4. Correspondence analysis identifies a predominant beef signal in the gene expression data

Correspondence analysis was performed using the Bioconductor library made4 [27]. Correspondence analysis on normalized gene

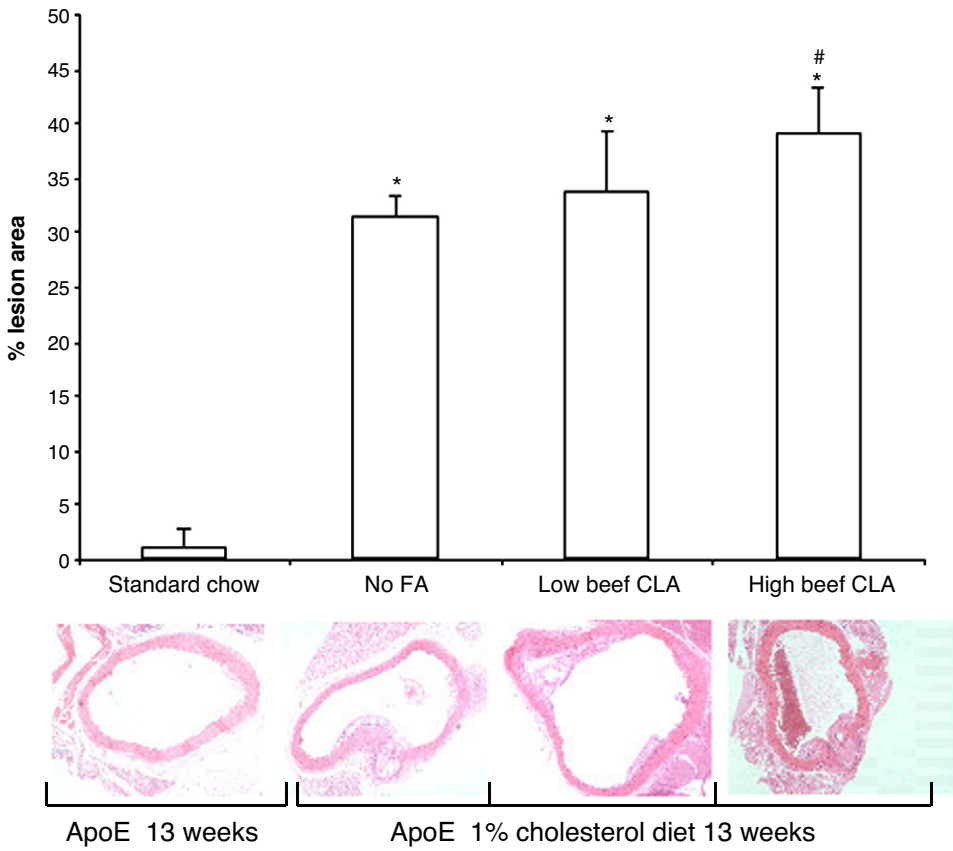

Fig. 2. Effect of CLA-enriched beef diet on progression of atherosclerosis in ApoE$^{-/-}$ mice. Diet A, control diet; Diet B, 1%cholesterol diet; Diet C, low-CLA-enriched beef diet; Diet D, high-CLA-enriched beef diet. Representative cross sections from the aorta (at 2.5× magnification) of ApoE$^{-/-}$ mice from different treatment groups stained with H&E. Percentage lesion area in ApoE$^{-/-}$ animals fed a standard chow diet for 13 weeks (n=8), a 1% cholesterol diet for 8 weeks (n=24), followed by 1% cholesterol chow alone (n=8), 1% cholesterol chow+low-CLA beef (n=8) or 1% cholesterol chow+high-CLA beef (n=8) for a further 5 weeks (*P<.05 w.r.t chow diet; #P<.05 w.r.t ApoE 1% cholesterol chow).

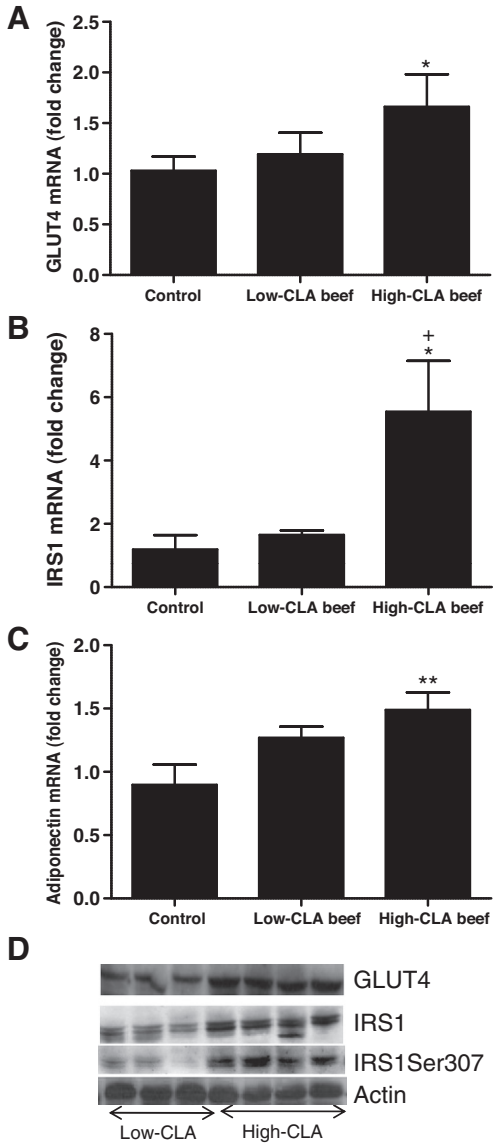

Fig. 3. Effect of low- and high-CLA-enriched beef diets on markers of insulin sensitivity in *ob*/*ob* mice. Diet C, low-CLA-enriched beef diet; Diet D, high-CLA-enriched beef diet. Expression of (A) GLUT4, (B) IRS1 and (C) adiponectin was measured using Taqman reverse transcriptase PCR. mRNA levels were normalised to18S, and the results are expressed as fold induction relevant to Diet A. The results represent the mean±S.E.M. for three independent experiments (*P<.05, w.r.t. Diet A, **P<.01, w.r.t Diet A, +P<.05, w.r.t, Diet C). (D) Protein lysates were prepared from adipose tissue of mice fed low- and high-CLA-enriched beef. Levels of GLUT4, IRS1 and serine (307) phosphorylated IRS1 were assessed by immunoblotting.

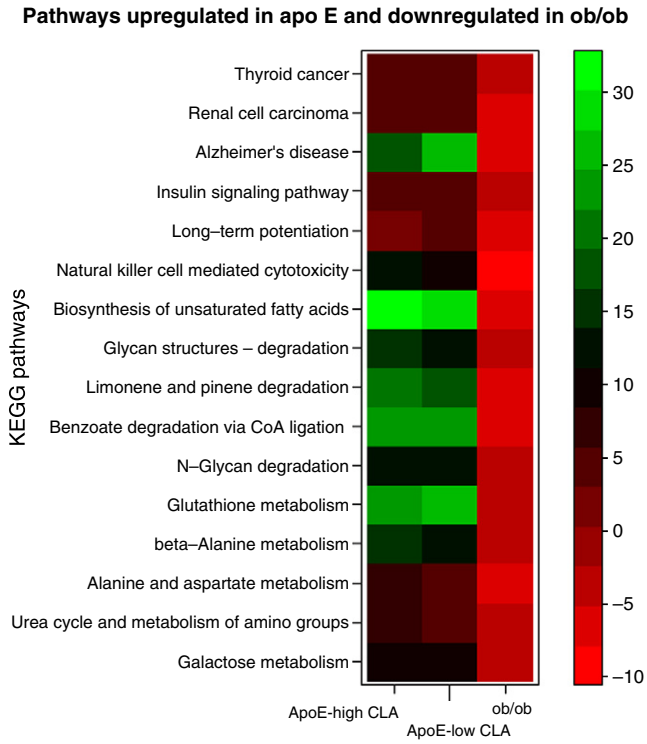

Fig. 4. Pathways up-regulated in CLA-fed ApoE mice with a corresponding down-regulation in CLA-fed *ob*/*ob* mice. All 16 pathways are significantly altered (P value<.01) in all three groups.

expression of the ApoE$^{-/-}$ mice data revealed two groups: mice fed the standard chow (control and cholesterol groups) and mice fed CLA-enriched beef (Fig. 5), suggesting that CLA-enriched beef diets exhibited the main trend in this data set. Correspondence analysis was applied to ApoE$^{-/-}$ liver microarray data normalized with corresponding *ob*/*ob* data to explore the differences between the positive outcome in *ob*/*ob* compared to the negative outcome in ApoE$^{-/-}$. Fig. 5 confirms three clear groups: (a) *ob*/*ob* mice, (b) ApoE$^{-/-}$ mice fed standard chow and (c) ApoE$^{-/-}$ mice fed CLA-enriched beef. The primary signal along the *x*-axis (accounting for 72.95% of the variation) splits beef- and non-beef-fed mice, suggesting that the trends observed in the ApoE$^{-/-}$ data were beef dependent rather than CLA dependent. The secondary signal along the *y*-axis (accounting for 4.17% of the variation) mirrors the overall health of mice at the end of the studies. *Ob*/*ob* had the best outcome, while the ApoE$^{-/-}$ mice fed CLA-enriched beef had the worst metabolic outcome.

Table 1
Numbers of differentially expressed genes (DEGs) in ApoE$^{-/-}$ mice

Contrasts	Number of DEGs (P value<.05)
Cholesterol vs. control	0
Low CLA vs. control	5123
High CLA vs. control	5165
Low CLA vs. cholesterol	4910
High CLA vs. cholesterol	4915
High CLA vs. low CLA	1
Beef vs. no beef	8613

3.5. Changes in insulin signaling in ApoE$^{-/-}$ but not ob/ob mice

Fig. 6 and Table 2 show differential expression in insulin signaling pathways in CLA-enriched beef fed ApoE$^{-/-}$ and *ob*/*ob* mice. Several mediators of PI3K-independant insulin signaling are differentially regulated in ApoE$^{-/-}$ and *ob*/*ob* mice on the CLA-enriched beef diet. *Sorbs1*, a key adaptor protein in this pathway, demonstrated significant up-regulation in ApoE$^{-/-}$ mice (logFC of 1.74). *Sorbs1* increased in *ob*/*ob* mice fed CLA-enriched beef, however, to a lesser extent (logFC of 0.36). Changes in insulin signaling upon exposure to CLA-enriched beef diets was not restricted to the PI3K-independent pathway. *Ship*, a negative regulator of PI3K-dependant insulin signaling, was up-regulated in ApoE$^{-/-}$ mice fed CLA-enriched beef, while this effect was not observed in *ob*/*ob* mice, reflecting the observation from plasma metabolic markers that ApoE$^{-/-}$ mice on the CLA-enriched beef diet are less sensitive to insulin than *ob*/*ob* mice. Fatty acid synthase (*Fasn*) is a key lipogenic protein and catalyzes the conversion of malonyl-CoA to palmitic acid. *Fasn* is

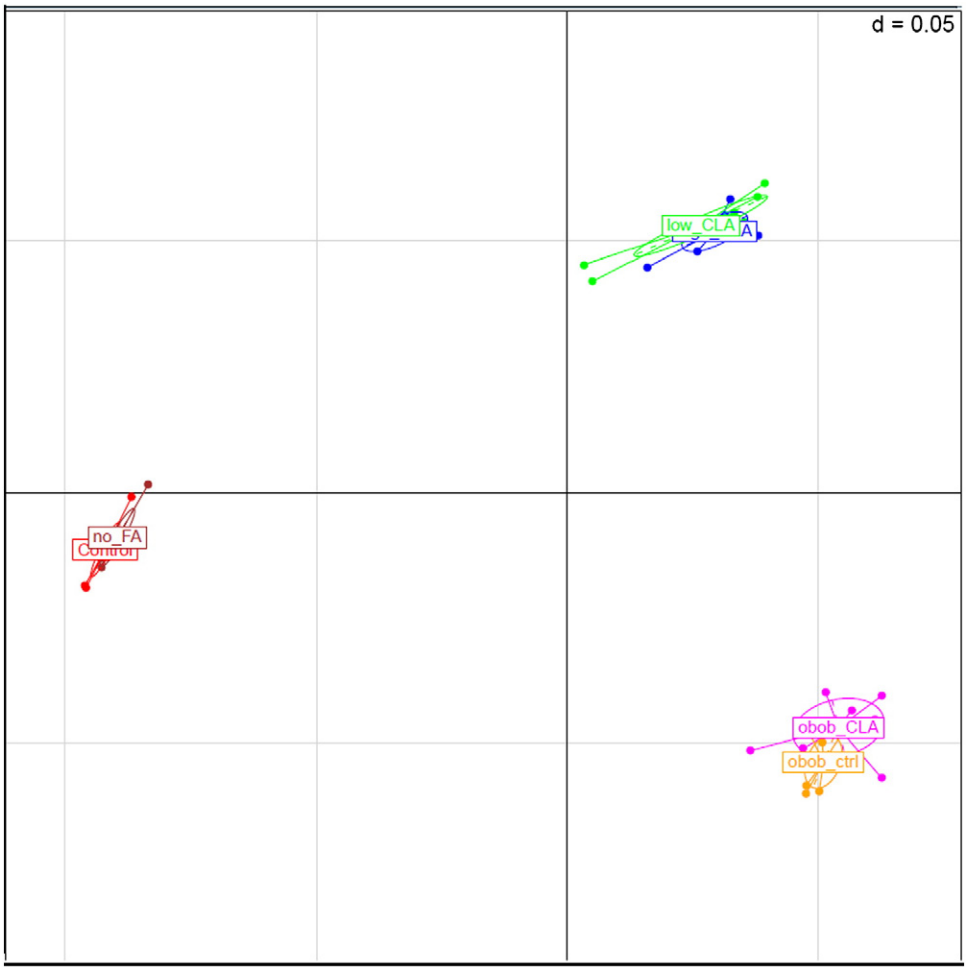

Fig. 5. Correspondence analysis of ApoE and *ob*/*ob* gene expression data. The primary signal of the data (along the *x*-axis) is a beef-based signal. It divides the beef-supplemented diets from the nonbeef chow. The second most dominant signal in the data (along the *y*-axis) mirrors the overall health of mice at the end of the studies; the *ob*/*ob* mice had the best outcome, while the ApoE mice fed CLA-enriched beef had the worst outcome.

down-regulated in *ob*/*ob* but up-regulated in ApoE$^{-/-}$ on the high- compared to the low-CLA beef diet.

Interestingly, microarray gene expression of F4/80 and CD11c, which traditionally indicates presence of resident proinflammatory macrophages (M1 macrophages), is significantly up-regulated in high- compared to the low-CLA-enriched beef fed ApoE$^{-/-}$ mice with a corresponding down-regulation of the anti-inflammatory macrophage (M2 macrophages) marker CD163. These genes were not differentially expressed in the other diet groups. Furthermore, gene expression of proinflammatory cytokines TNFα and IL-1β are up-regulated in the ApoE$^{-/-}$ mice on a high- compared to low-CLA-enriched beef diet.

3.6. Alterations in the biosynthesis of unsaturated fatty acids

Fig. 7 and Table 3 show the differential expression of ApoE$^{-/-}$ and *ob*/*ob* mouse genes involved in the biosynthesis of unsaturated fatty acids. *Scd1*, the rate-limiting enzyme of monounsaturated FA biosynthesis, is up-regulated in ApoE$^{-/-}$ mice but down-regulated in *ob*/*ob* mice on the high- but not low-CLA-enriched beef diet, potentially suggesting an increase in monosaturated fatty acid levels. *Acot 2* and *4*, involved in the endogenous biosynthesis of oleic acid, are up-regulated in ApoE$^{-/-}$ mice on high- compared to the low-CLA-enriched beef diet.

Fatty acid elongation is controlled by the family of Elovl elongases, a number of which are altered in ApoE$^{-/-}$ mice following CLA-enriched beef diet. *Elovl2*, involved in elongation of LC-PUFA, is down-regulated in ApoE$^{-/-}$ but not *ob*/*ob* mice fed high- but not low-CLA-enriched beef. This group also demonstrates significant enhancement of *Elovl6*, involved in the elongation of saturated fatty acids. This is accompanied by up-regulation of *Srebf1* (also referred to as *Srebp1*) in ApoE$^{-/-}$ but not *ob*/*ob* mice on the high- compared to the low-CLA-enriched beef diet and may explain the up-regulation of *Elovl 6* in ApoE$^{-/-}$ and the unchanged levels in *ob*/*ob*.

4. Discussion

Adipose tissue inflammation and associated metabolic perturbations are mediating factors which play a central role in the development of type 2 diabetes and atherosclerosis [28]. Potential anti-inflammatory and antiobesogenic effects make CLA a prime candidate for dietary treatment of insulin resistance and atherosclerosis. To date, most studies have focused on synthetic mixtures of the biopotent CLA isomers c9,t11-CLA and t10,c12-CLA. However, mixed CLA formulations between studies have led to variable effects on obesity and diabetes. In this study, we examined the effect of c9,t11-CLA-enriched beef, a natural source of CLA, on two mouse models of metabolic dysfunction: *ob*/*ob* mice which display an obese, diabetic phenotype and the atherosclerotic ApoE$^{-/-}$ mouse model. These

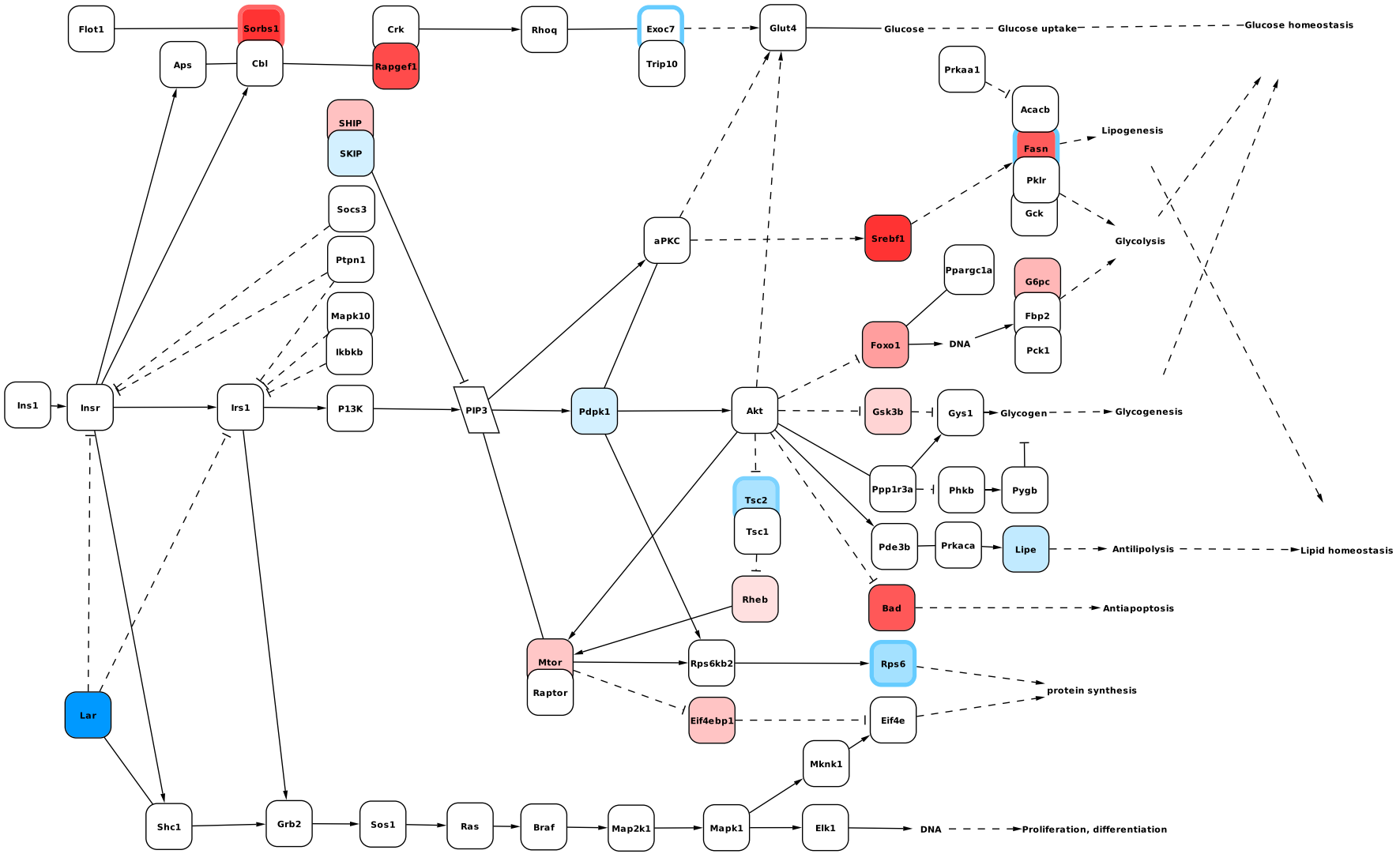

Fig. 6. Effect of CLA-enriched beef diets on the insulin signaling pathway. Node colour reflects the logFC ($\log^2$ fold change) of the gene in ApoE$^{-/-}$ on the high-CLA beef diet. The colour of a node border reflects the logFC of the gene in *ob*/*ob* mice fed CLA-enriched beef. Blue reflects a down-regulation or negative logFC for a gene, while red reflects an up-regulation or a positive change in logFC.

Table 2
Differentially expressed genes involved in insulin signaling

Entrez ID	Symbol	ApoE				*Ob/ob_*	
		High CLA_ logFC	Regulation	Low CLA logFC	Regulation	CLA logFC	Regulation
64930	Tsc1	−0.80	Down	−0.87	Down	–	–
19062	Inpp5k (SKIP)	−0.28	Down	−0.28	Down	–	–
16331	Inpp5d (SHIP)	0.30	Up	–	–	–	–
19268	Ptprf	−2.67	Down	−2.63	Down	–	–
14104	Fasn	0.80	Up	0.97	Up	−0.56	Down
16890	Lipe	−0.40	Down	–	–	–	–
13685	Eif4ebp1	0.29	Up	0.23	Up	–	–
20104	Rps6	−0.59	Down	−0.51	Down	−0.59	Down
56458	Foxo1	0.47	Up	–	–	–	–
12015	Bad	0.82	Up	0.88	Up	–	–
22084	Tsc2	−0.54	Down	−0.56	Down	−0.31	Down
14377	G6pc	0.35	Up	0.28	Up	–	–
56717	Mtor	0.27	Up	0.18	Up	–	–
20411	Sorbs1	1.74	Up	1.71	Up	0.36	Up
19744	Rheb	0.15	Up	0.17	Up	–	–
56637	Gsk3b	0.21	Up	–	–	–	–
20787	Srebf1	1.60	Up	1.53	Up	–	–
107746	Rapgef1	0.88	Up	0.71	Up	–	–
18607	Pdpk1	−0.28	Down	−0.27	Down	–	–
53413	Exoc7	–	–	–	–	−0.38	Down

models of metabolic dysfunction are particularly pertinent as single nucleotide polymorphisms which are associated with atherosclerosis and IR have been identified in humans [29,30]. C9,t11-CLA-enriched beef was attained by feeding cattle pasture, and levels of the t10,c12-CLA isomer were undetectable in the CLA-enriched feeds. Interestingly, these models show divergent effects in terms of plasma lipid profiles and ultimate disease outcome.

Previous studies demonstrated that feeding synthetic forms of CLA improved lipid profiles, impeded the progression and promoted the regression of atherosclerosis in rabbits [31], hamsters [32] and mice [14]. Contrary to our hypothesis, c9,t11-CLA-enriched beef did not protect against the development of atherosclerosis. Supplementation of a 1% cholesterol diet with CLA-enriched beef had a profound proatherogenic effect in the ApoE$^{-/-}$ mouse model of atherosclerosis. The beef-enriched diets, and in particular the high-CLA beef diet, induced a proatherogenic lipid profile with elevated plasma TAG, NEFA and total cholesterol concentrations. This is in contrast to previous studies showing a reduction in total cholesterol and TAG concentrations in CLA-fed C57BL6 mice [33]. The beef-enriched diets also elevated plasma glucose and insulin concentrations, irrespective of the level of CLA in the diets. HOMA-IR, a surrogate marker of insulin resistance was further augmented by the high-CLA beef diet. It is possible that the high saturated fatty acid composition of the beef feed profoundly overwhelmed the ApoE$^{-/-}$ which, with poor capacity to clear circulating lipids, negated any potential positive effects of CLA alone. Indeed, hepatic transcriptomic analysis demonstrated a distinct split between beef- and non-beef-fed mice, suggesting that promotion of atherosclerosis was beef, rather than CLA, dependant.

In agreement with previous studies demonstrating that synthetic CLA isomers resolve insulin resistance in *ob*/*ob* mice [4,34], mice fed CLA-enriched beef were more insulin sensitive with an improved lipid profile. This improved metabolic profile may be partly ascribed to the up-regulated GLUT4, IRS-1 and IRS-1p307 expression in adipose tissue.

In order to ascertain the molecular mechanisms which account for the divergent effects, transcriptomic analysis was carried out in the liver given its central role in lipid and glucose metabolism. GSEA1m analysis on ApoE$^{-/-}$ and *ob*/*ob* data sets was compared to determine pathways which were differentially regulated between mouse models. Interestingly, a strong theme of metabolism was reflected in the pathways identified. Correspondence analysis demonstrated that CLA-enriched beef diets rather than the genotype of the animals accounted for most differentially expressed transcripts. This provided the justification for comparing the effects of CLA between the two different disease models. The main pathways affected by the CLA-enriched beef diet were insulin signaling and biosynthesis of unsaturated fatty acids, both of which are fundamental to insulin resistance and atherosclerosis.

The actions of insulin are regulated through the insulin receptor and a series of tyrosine kinase receptors [35]. Dysregulation of this complex signaling network has major metabolic consequences promoting diabetes and subsequent atherosclerosis. Hepatic transcriptomic analysis showed that several mediators of the PI3K-independent insulin signaling pathway were affected in both genotypes by CLA-enriched beef. SH2 containing 5′ inositol phosphatase 2 (*Ship2*), a lipid phosphatase, is an important negative regulator of insulin signaling and acts by hydrolyzing PI3K [36]. Deletion of *Ship2* ameliorates insulin resistance in mice [37] and in 3T3-L1 adipocytes [38]. *Ship2* was up-regulated in ApoE$^{-/-}$ but not in *ob*/*ob* mice fed CLA-enriched beef, which in turn may have promoted the development of insulin resistance. Sorbin and SH3 containing domain 1 (*Sorbs1*) is an important PPARγ-regulated adaptor protein in the insulin signaling pathway; upon binding of insulin to the insulin receptor, it partially dissociates and binds to c-Abl [39,40]. Human polymorphisms of SORBS1 are positively associated with obesity-induced diabetes [41]. *Sorbs1* was up-regulated in both CLA-enriched beef groups, particularly the ApoE$^{-/-}$ group, which is expected given that c9,t11-CLA is an agonist of PPARγ. It is also interesting to note that *Sorbs1* is linked to macrophage-induced insulin resistance [42] and may account for the detrimental plasma profiles observed in the ApoE$^{-/-}$ mice.

Previous work suggested that c9,t11-CLA was an anti-inflammatory nutrient [43]. Our group showed decreased F4/80^{+} macrophage in adipose following a high-CLA diet [4]. However, hepatic microarray data demonstrated increased F4/80 and CD11c expression, associated with greater TNFα and IL-1β expression, following the CLA-enriched beef in ApoE$^{-/-}$. We speculate that the lipotoxic stress in ApoE$^{-/-}$ mice promoted macrophage infiltration coincident with insulin resistance.

Lipogenic pathways were also markedly affected by the high-CLA beef diet. Fatty acid synthase (*Fasn*) is a key enzyme role in fatty acid biosynthesis and influences energy expenditure rates and fat mass.

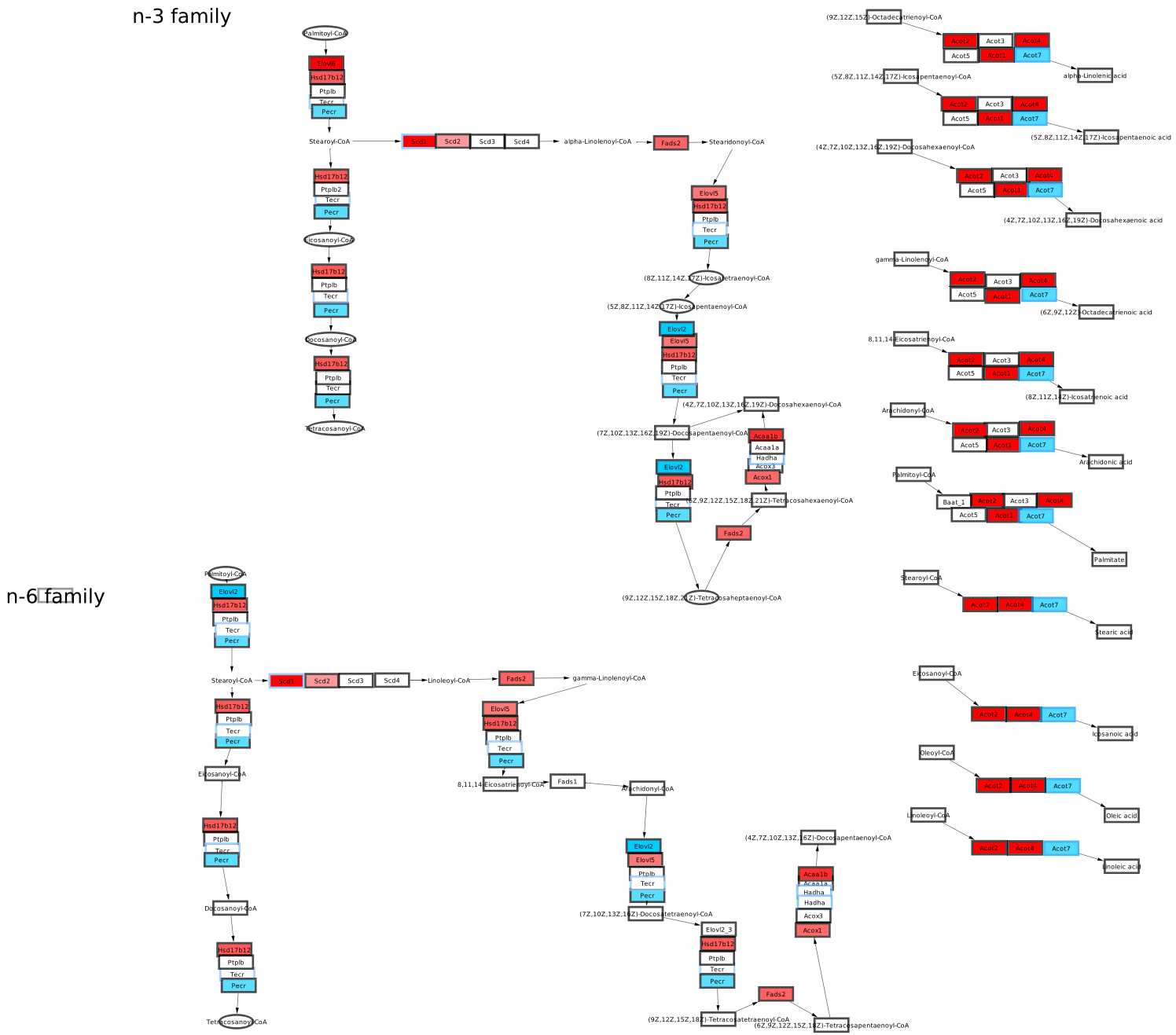

Fig. 7. Effect of CLA-enriched beef diets on biosynthesis of unsaturated fatty acid pathway. Node colour reflects the logFC ($\log^2$ fold change) of the gene in ApoE$^{-/-}$ on the high-CLA beef diet. The colour of a node border reflects the logFC of the gene in *ob*/*ob* mice fed CLA-enriched beef. Blue reflects a down-regulation or negative logFC for a gene, while red reflects an up-regulation or a positive change in logFC.

Table 3
Differentially expressed genes involved in the biosynthesis of unsaturated fatty acids

Entrez ID	Symbol	ApoE				*Ob*/*ob*_	
		High CLA_		Low CLA		CLA	
		logFC	Regulation	logFC	Regulation	logFC	Regulation
54326	Elovl2	−0.68	Down	−0.71	Down	–	–
56348	Hsd17b12	0.63	Up	0.55	Up	–	–
111175	Pecr	−0.32	Down	−0.31	Down	–	–
235674	Acaa1b	1.05	Up	1.05	Up	–	–
11430	Acox1	0.50	Up	0.41	Up	–	–
56360	Acot9	3.61	Up	3.51	Up	–	–
171210	Acot2	2.25	Up	2.14	Up	–	–
171282	Acot4	2.65	Up	2.51	Up	–	–
26897	Acot1	1.82	Up	1.49	Up	–	–
70025	Acot7	−0.34	Down	−0.36	Down	−0.85	Down
170439	Elovl6	2.23	Up	2.35	Up	–	–
20249	Scd1	2.66	Up	2.50	Up	−0.33	Down
20250	Scd2	0.34	Up	0.45	Up	–	–
56473	Fads2	0.55	Up	0.55	Up	–	–
68801	Elovl5	0.45	Up	0.55	Up	–	–
106529	Tecr	–	–	–	–	−0.26	Down
97212	Hadha	–	–	–	–	−0.36	Down

Increased expression is associated with development of diabetes and cardiovascular disease [44]. Transcriptomic results demonstrate that *Fasn* was up-regulated in the CLA-enriched beef ApoE$^{-/-}$ group, but down-regulated in the CLA-enriched beef *ob*/*ob* group. It is possible that increased *Fasn* activity in ApoE$^{-/-}$ mice and saturated-fatty-acid-rich feed also work together to negate the positive effects of CLA. The biosynthesis of unsaturated fatty acid pathway was also enhanced exclusively in ApoE$^{-/-}$ mice, which would promote dyslipidemia and progression of diabetes and atherosclerosis. Stearoyl-CoA desaturase (*Scd*) is the key rate-limiting enzyme in monounsaturated fatty acid biosynthesis; this was significantly enhanced in ApoE$^{-/-}$ mice and reduced in *ob*/*ob* mice receiving the CLA-enriched beef diet. Deletion of *Scd* reduced adiposity and increased insulin sensitivity [45,46]. Furthermore, studies have indicated that *Scd* regulates whole body glucose homeostasis [47], and human polymorphisms in the *Scd* gene resulted in decreased body mass index and enhanced insulin sensitivity [48]. Acyl-CoA thioesterases (*Acot*) are involved in the hydrolysis of acyl-CoAs to free fatty acids. *Acot2* (mitochondrial thioesterase) promotes fatty acid oxidation in conjunction with uncoupling protein 3 [49]. Increased *Acot2* expression is associated with cardiovascular disease risk and lipid dysfunction upon exposure to high-fat diets [50,51]. *Acot4* is similar in structure and function to *Acot2*. Both *Acot2* and *Acot4* (peroxisomal) are up-regulated in ApoE$^{-/-}$ mice on the CLA-enriched beef diet. It is possible that increased activity of these enzymes promoted the release of free fatty acids leading to peripheral lipotoxicity and insulin resistance, promoting atherosclerosis. Fatty acid elongation is another critical point in the biosynthesis of fatty acids, mediated by a group of elongases including elongation of very long-chain fatty acid (*Elovl*) elongases. *Elovl2* elongates arachidonic and eicosapentaenoic acid, promoting the synthesis of peroxisome proliferator-activated receptor (PPARα) ligands [52]. Overexpression of *Elovl2* in adipocytes enhances TAG synthesis and lipid droplet formation [53]. Interestingly, *Elovl2* was down-regulated in ApoE$^{-/-}$ but not *ob*/*ob* mice receiving the CLA-enriched beef diet; this may reflect the inability of ApoE$^{-/-}$ to efficiently process and store fatty acids derived from the beef diet. *Elovl6* is involved in the elongation of saturated fatty acids with 12–16 carbons to palmitic acid. Deletion of *Elovl6* results in resistance to diet-induced insulin resistance [54]. Expression of *Elovl6* was unchanged in *ob*/*ob* mice but significantly enhanced in ApoE$^{-/-}$ receiving the CLA-enriched beef diet, again demonstrating the enhancement of lipogenic pathways upon exposure to CLA.

5. Conclusion

This study clearly illustrates divergent effects of feeding naturally derived c9,t11-CLA to two mouse models of metabolic dysfunction. The work provides greater mechanistic insight which may explain the variable health benefits of CLA in terms of metabolic dysfunction. *Ob*/*ob* mice benefit from a CLA-enriched beef diet, with amelioration of insulin resistance and positive effects on markers related to insulin sensitivity with relatively undisturbed lipogenesis. Contrary to this and, indeed, in previous studies using synthetic CLA isomers, ApoE$^{-/-}$ mice demonstrated adverse effects on plasma lipid profiles, progression of atherosclerosis, insulin signaling and lipogenic pathways. We speculated that the high saturated fatty acid content of the CLA-enriched beef feed overwhelms mice lacking ApoE, negating any potential benefit provided by the CLA. This is highlighted by enhanced expression of lipogenic proteins resulting in inefficient processing and storage of fatty acids and increased accumulation in peripheral tissues and plasma, ultimately leading to profound metabolic dysfunction. Overall, the role of CLA as a potential functional food remains uncertain; its role in atherosclerosis independent of ApoE needs further clarification. Thus, while it may have some benefit, caution must be exercised.

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