Isomer specific effects of Conjugated Linoleic Acid on macrophage ABCG1 expression

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1 Introduction

1.1 Conjugated linoleic acid (CLA)

Conjugated linoleic acid (CLA) is a collective term for a group of positional and geometrical isomers of linoleic acid with a conjugated double bond system. It is formed in the digestive tracts of ruminant animals such as cows, sheep and goats by fermentative bacteria. *Butyrivibrio fibrisolvens* is a fermentative anaerobic bacterium that isomerizes cis-9,trans-12-octadecenoic acid (linoleic acid) to cis-9,trans-11-octadecenoic acid (cis-9,trans-11-CLA), followed by the hydrogenation of the cis-double bond of the conjugated diene to yield trans-11-octadecenoic acid (trans-vaccenic acid) (figure 1). Trans-vaccenic acid is further converted to cis-9,trans-11-CLA within mammalian cells through stearoyl-CoA desaturase (SCD), a δ -9 desaturase [1]. In addition to rumenal bacteria, enteric bacteria such as *Bifidobacterium* species and *Lactobacillus* species can produce various CLA isomers from linoleic acid [2]. The most common sources of CLA are beef, dairy products and partly hydrogenated vegetable oils [3]. The daily intake of CLA has been calculated for various countries and estimated at several hundred mg/day in a typical diet [4]. The cis-9,trans-11 (c9,t11)- and trans-10,cis-12 (t10,c12)-CLA isomers are the major dietary forms of CLA, but lower levels of other isomers such as trans-9,trans-11 (t9,t11)-CLA are also present in CLA food sources (figure 2) [5].

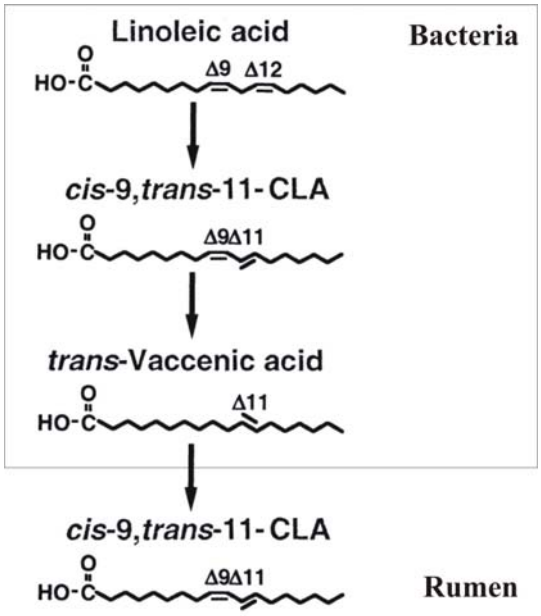

Figure 1: Formation of c9,t11-CLA in the digestive tracts of ruminant animals by *Butyrivibrio fibrisolvens*.

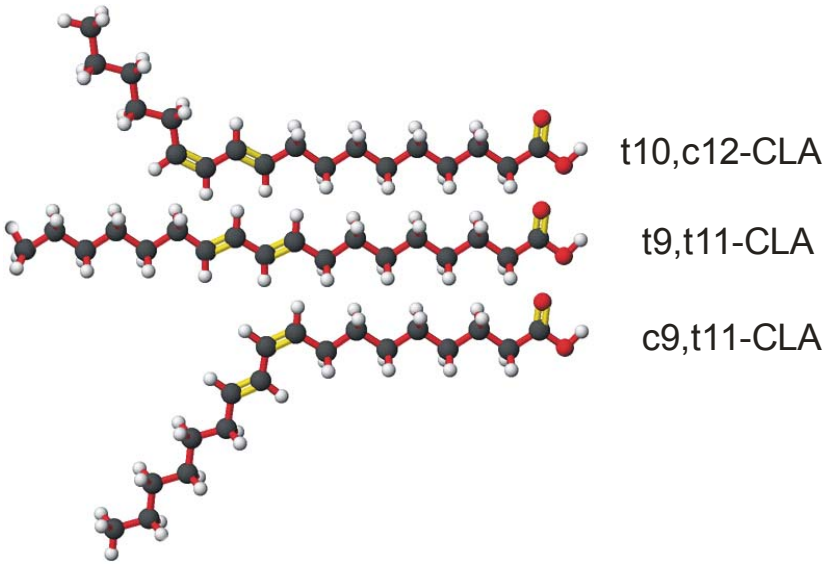

Figure 2: CLA isomers of major and minor abundance.

Major interest in CLA had emerged about 20 years ago, because CLA obtained from lipids extracted from barbecued beef displayed anti-carcinogenic

properties [6]. These anti-carcinogenic activities of CLA have been confirmed in a chemically induced rat mammary tumor model with an effective range of 0.1-1.0% CLA in the diet [7]. The growth inhibitory effects of CLA have also been studied in various human cancer cells including hepatoma, colorectal, breast and lung cancer. C9,t11-CLA, t10,c12-CLA and a CLA mixture inhibited proliferation of these cancer cells [8]. These reported effects from animal studies and *in vitro* studies are supported by several findings in humans such as an inverse relationship between milk consumption and breast cancer risk in woman [9]. Further, dietary CLA intake and serum CLA levels of Finnish woman were significantly lower in cancer patients than in controls [10].

These very promising findings led to various studies investigating beneficial health effects of CLA. Most studies use animal models and a mixture of the two major CLA isomers. It has been shown that feeding mice a mixture of c9,t11- and t10,c12-CLA mediates protection from chemically induced inflammatory bowel disease (IBD). The CLA mixture activates nuclear peroxisome proliferator activated receptors (PPARs) γ and δ and represses expression of inflammatory genes such as tumor necrosis factor (TNF) α and nuclear factor-kappa B (NFκB), while inducing the immuno-regulatory cytokine transforming growth factor (TGF) β 1 [11]. Studies on CLA supplemented pigs support these findings. Thus, the onset of IBD is delayed and colitis less severe. Moreover the growth suppression diminished in pigs fed CLA correlating with the induction of colonic PPAR*γ* and downregulation of TNF*α* [12].

Several studies have shown that CLA inhibits the development and progression of atherosclerosis. A mixture of the major occurring isomers c9,t11-

and t10,c12-CLA induced a profound resolution of pre-established atherosclerosis in mice. CLA negatively regulated the expression of pro-inflammatory genes and induced apoptosis in atherosclerotic lesions primary via activation of PPARα and γ [13]. Interestingly, another study describes a selective isomer-dependent effect of conjugated linoleic acid on atherosclerotic lesion development. Supplementation of apolipoprotein E deficient mice with c9,t11-CLA diminished, whereas t10,c12-CLA promoted atherosclerosis [14]. However to date, no study has determined the effect of CLA on atherosclerosis in humans. Several studies in humans found a lipid lowering and therefore anti-atherosclerotic effect, but the results of different studies are still inconsistent [15].

The underlying molecular mechanisms that lead to most physiological effects of CLA are a result PPARα, γ and δ activation. PPARs are well described ligand activated nuclear transcription factors that play important roles in cellular differentiation, cancer, insulin sensitization, atherosclerosis and several metabolic diseases [16]. CLA isomers are high affinity ligands and activators of peroxisome proliferator-activated receptors [17].

CLA seems to have various health benefits, but there is a marked variation between findings of different studies. This variation reflects the isomer specific effect of the individual CLA isomers. The composition of CLA mixtures strongly influences its biological and physiological effects.

1.2 ATP-binding cassette transporter G1 (ABCG1)

The ATP-binding cassette (ABC) transporters are a large super-family of trans-membrane proteins that facilitate the transport of a wide variety of substrates across cellular membranes. The human superfamily containing 48 ABC-transporters is divided into seven subfamilies (A-G). All ABC transporters use ATP to generate the energy required for the transport of metabolites across membranes. Structurally, they fall into two groups, full size transporters having two similar structural units joined covalently and half-size transporters of single structural units that form active heterodimers or homodimers. ABCG1 belongs to the family of half size transporters, it contains one trans-membrane domain consisting of six trans-membrane spanning α-helices, and an ATP-binding cassette (figure 3). ABCG1 mRNA and protein are broadly expressed with high levels in macrophages [18; 19].

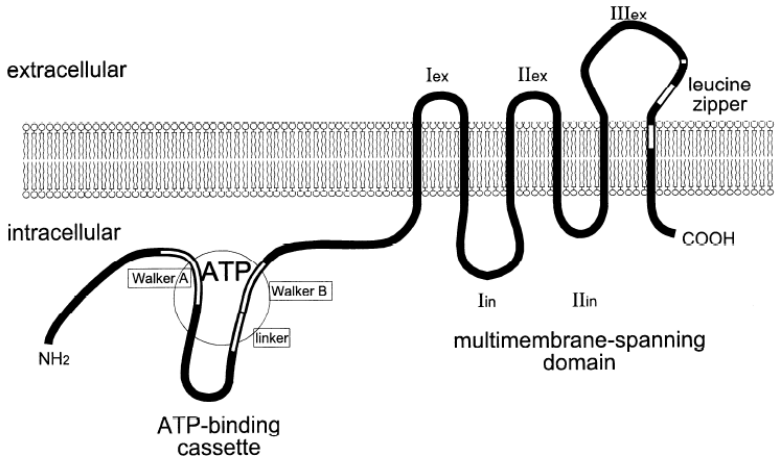

Figure 3: Domain organization of ABCG1 from [20].

ABCG1 is a mediator of macrophage cholesterol efflux to mature high density lipoproteins (HDLs). Macrophages lacking ABCG1 expression have

impaired cholesterol efflux to HDL but not to lipid-free apolipoprotein AI (ApoAI) *in vitro* [21-23]. Cholesterol efflux from macrophages to ApoAI as acceptor particle is mediated by ABCA1 [24]. In contrast to ABCA1, which transports also phospholipids and other lipophilic compounds, ABCG1 is mainly a cholesterol transporter with only minor phospholipid transport capacity [25]. ABCA1 and ABCG1 have been shown to function cooperatively to remove cholesterol from cells *in vitro*, both transporters promote macrophage reverse cholesterol transport *in vivo* and are additive in their effects [26; 27]. ABCA1 converts lipid-poor ApoAI to partially lipidated nascent lipoproteins, which are then effective acceptors for cholesterol exported by ABCG1 (figure 4).

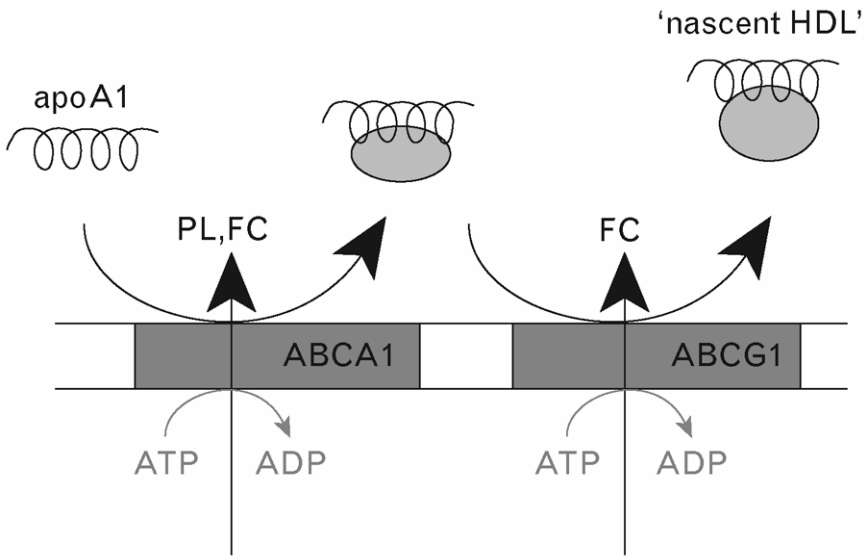

Figure 4: Cholesterol efflux mediated by ABCA1 and ABCG1. PL,phospholipids; FC, free cholesterol; adapted from [28].

Since ABCG1 mediates cholesterol export in macrophages, it is most likely that this transporter is cardioprotective and loss of function is atherogenic.

Surprisingly, three studies showed that transplantation of bone marrow from ABCG1 deficient mice into atherogenic mice caused only a moderate increase or even decreased atherosclerotic lesions [29-31]. Decreased atherosclerosis was associated with increased macrophage apoptosis and enhanced expression of ApoE and ABCA1. Therefore, it was suggested that potential harmful effects of impaired ABCG1 may be compensated by beneficial effects on clearing apoptotic cells and increasing other compensatory efflux pathways.

Various transcripts of ABCG1 are produced as a result of the use of alternate exons and alternative promoters. The different transcripts are predicted to encode ABCG1 proteins, that differ only at the N-terminus (figure 5). Current evidence suggests that there is only one major transcript in humans, hABCG1-a, which is generated after activation of the second promoter (figure 6). Its regulatory region contains multiple SP1 binding sites and a consensus sequence for binding of sterol regulatory element binding protein (SREBP) [32]. hABCG1-a expression and function is repressed by zinc finger protein 202 (ZNF202) [33]. Variant hABCG-1b is produced after induction of its regulatory region with oxysterols and liver X receptor (LXR) ligands, because its promoter is made up of two liver X receptor (LXR) response elements [34]. Transcript hABCG1-c might be synthesized after activation of a putative promoter containing a predicted nuclear factor κB (NFκB) binding site [35].

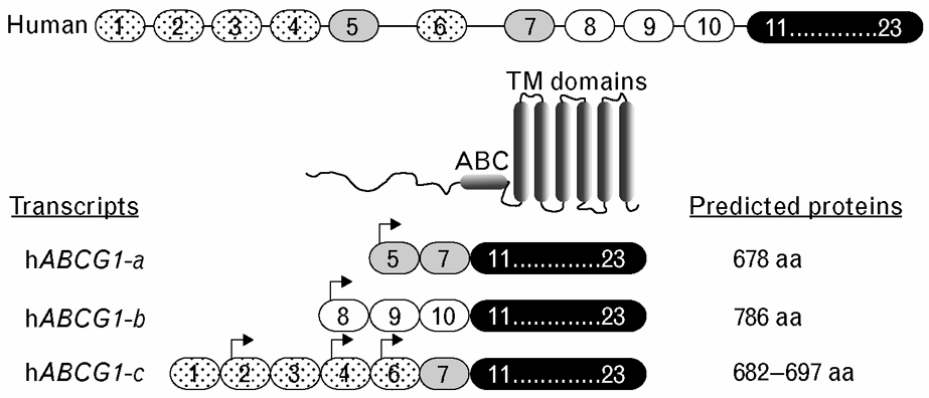

Figure 5: Exon configurations of the human ABCG1 transcripts and predicted proteins. aa, amino acid; adapted from [36].

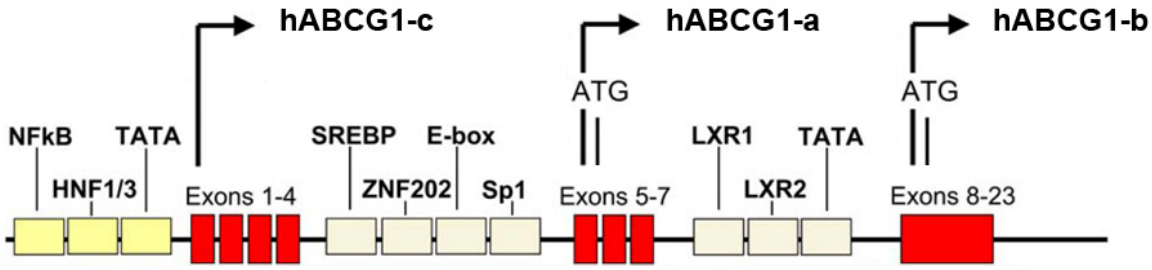

Figure 6: ABCG1 regulatory region, adapted from Langmann T., Habilitationsarbeit 2003.

1.3 Sterol regulatory element binding protein (SREBP)

Lipid homestasis in vertebrate cells is regulated by a family of membrane-bound transcription factors described as sterol regulatory element binding proteins (SREBPs). SREBPs are basic helix loop helix leucine zipper (bHLH-LZ) transcription factors synthesized as 1150 amino acid inactive precursors bound to the membranes of the endoplasmatic reticulum (ER) [37]. SREBP precursors are organized into three domains, a N-terminal transactivation domain for DNA binding and dimerization, two hydrophobic transmembrane

spanning segments and a C-terminal regulatory domain. Upon activation the ER anchored premature SREBP (pSREBP) undergoes a sequential two-step cleavage process to release its N-terminal activation domain, designated the nuclear SREBP form (nSREBP). Three members of the SREBP transcription factors have been described in mammalian species (figure 7). SREBP-1a and SREBP-1c are produced from the single gene SREBF-1 through the use of alternate promoters and they are identical except the N-terminal trans-activation domains. SREBP-1c is expressed in most mouse and human tissues, with high levels in macrophages, liver, white adipose tissue, adrenal gland and brain, whereas SREBP-1a is mainly expressed in cell lines and in tissues with a high capacity of cell proliferation such as spleen and intestine [38]. SREBP-2 is derived from the separate gene SREBF-2 and shows a 50% homology with the SREBP-1 amino acid sequence. [39].

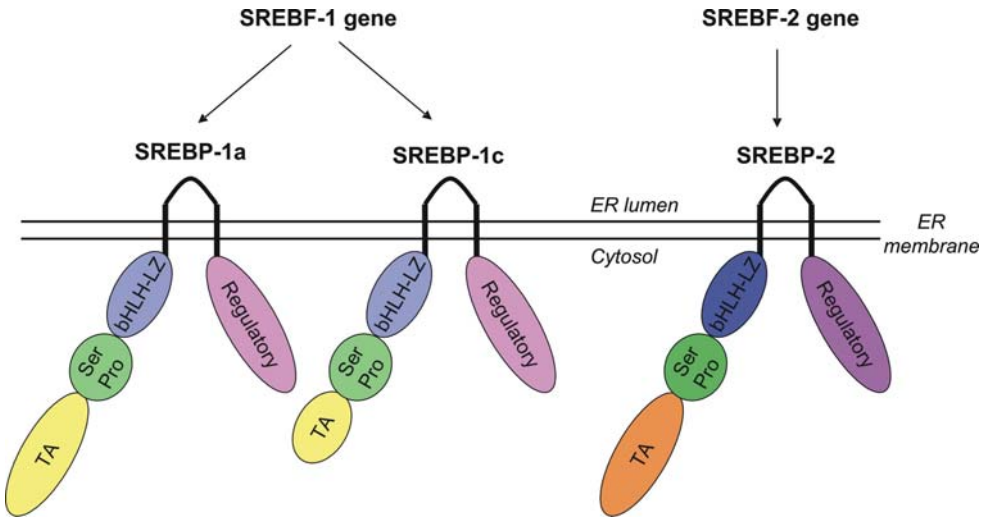

Figure 7: SREBP genes and isoforms. bHLH-LZ, basic helix loop helix leucine zipper, Pro, proline; Ser, serine; TA, trans activation.

As indicated in figure 8, SREBP-1c responsive targets in mammalian cells include genes of fatty acid metabolism such as fatty acid synthase (FASN) or stearoyl-CoA desaturase (SCD), which generate saturated and monounsaturated fatty acids, and genes that control triacylglyceride and phospholipid biosynthesis such as glycerol 3-phosphat acyltransferase (GPAT). SREBP-2 responsive genes include cholesterol biosynthesis enzymes such as HMG-CoA synthase (HMGCS) and HMG-CoA reductase (HMGCR) [40].

In contrast to mammalian cells, Drosophila and C. elegans express genes that encode only a single SREBP, its processing is regulated by phospholipids and SREBP targets are enzymes required for saturated fatty acid biosynthesis [41; 42].

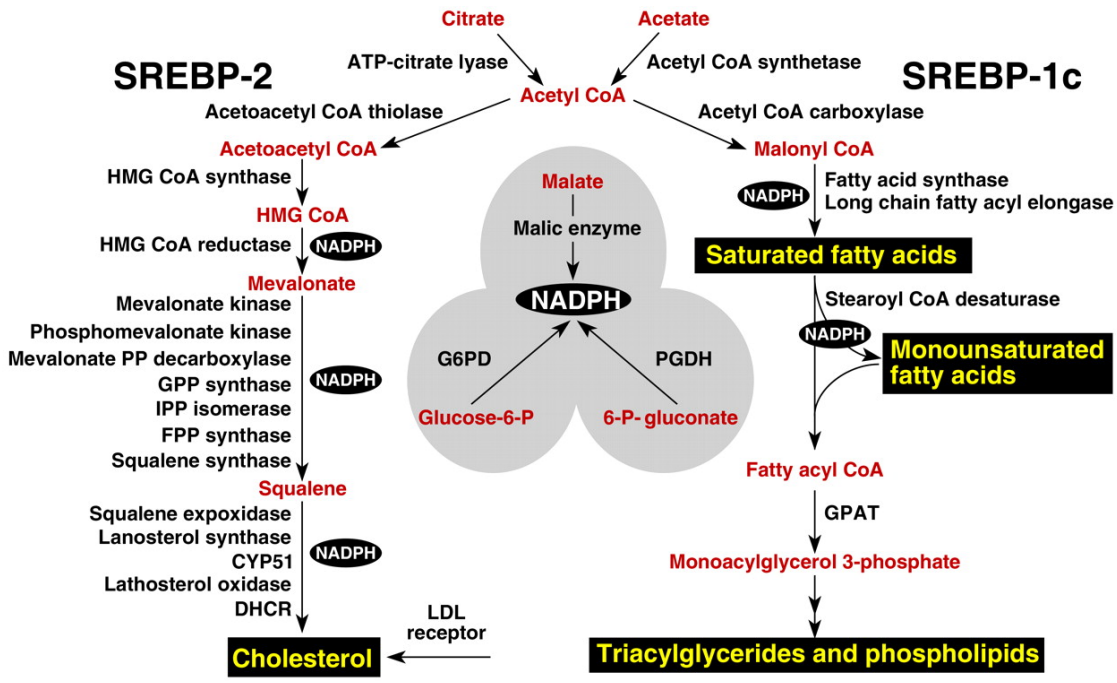

Figure 8: SREBP-2 and SREBP-1c controlled genes and metabolic pathways, from [43].

SREBP-1a and SREBP-2 activation is mainly controlled by proteolytic cleavage of SREBP precursors, whereas SREBP-1c activation is mainly regulated on the transcriptional level. SREBP-1a and SREBP-2 are inserted into the membranes of the endoplasmatic reticulum (ER). Its C-terminal regulatory domain binds to the C-terminal domain of the sterol sensitive SREBP cleavage-activating protein (SCAP) (figure 9). When cells are depleted of sterols, SCAP transports SREBPs from the ER to the Golgi apparatus where Site-1 protease (S1P) and Site-2 protease (S2P) act to release the N-terminal domain of SREBP, the nuclear SREBP (nSREBP), from the membrane. Nuclear SREBP enters the nucleus, binds to sterol regulatory elements (SREs) in the promoters of target genes and activates transcription. When the cellular cholesterol content rises, the SCAP/SREBP complex is no longer incorporated in ER transport vesicles, SREBPs do not reach the Golgi apparatus, nSREBP cannot be released from the membrane and transcription of target genes is shut off [40; 43; 44].

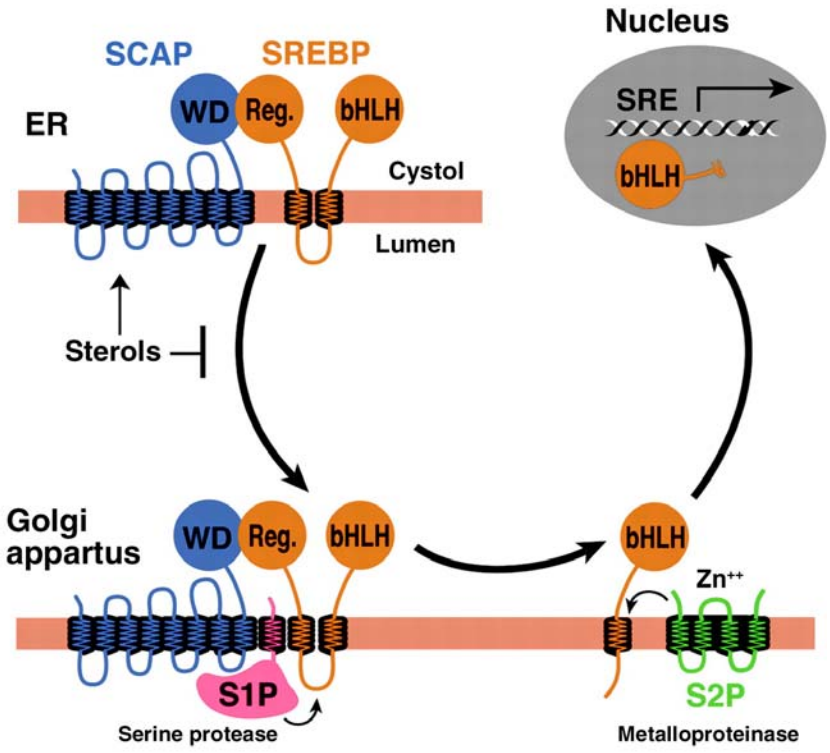

Figure 9: Regulation of SREBP-1a and SREBP-2 activation by proteolytic cleavage, from [43].

In contrast to SREBP-1a and -2, nuclear abundance of SREBP-1c is not regulated by cholesterol levels [45]. However, SREBP-1c expression is induced by oxysterols and the nuclear transcription factor liver X receptor (LXR). LXR can directly promote SREBP-1c transcription through two LXR binding elements (LXR-Es) in the SREBP-1c promoter (figure 10) [46]. LXR agonists up-regulate SREBP-1c expression *in vivo* in rodents and *in vitro* in various cell models, including human cells [47; 48].

SREBP-1c expression is depressed in fasting animals but increases when animals are re-fed with a high carbohydrate diet, because it can be induced by insulin [49; 50]. The effects of insulin on SREBP-1c expression are mediated by

a PI(3)-kinase dependent pathway, but the downstream effectors are unclear [51].

Remarkably, the SREBP-1c promoter can be directly activated by nuclear SREBPs in an auto-regulatory loop, because SREs are present in its regulatory region [52].

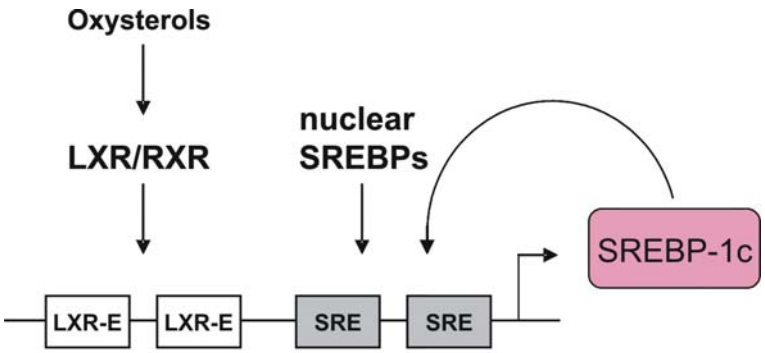

Figure 10: Promoter of SREBP-1c and its regulation. LXR-E, LXR binding element; SRE, SREBP response element; RXR, retinoid X receptor.

SREBPs affect the development of several human metabolic diseases including atherosclerosis, type 2 diabetes, obesity and lipodystrophy. In adipose tissue of obese and type 2 diabetic patients, SREBP-1c mRNA expression is decreased in comparison to controls [53]. Consistent with this finding weight loss in obese patients is associated with an improved insulin sensitivity and leads to an increase of SREBP-1c expression in adipose tissue.

In contrast to the adipose tissue, where SREBP-1c levels are diminished, SREBP-1c levels are elevated in the fatty livers of obese and insulin resistant mice [54]. Despite the profound insulin resistance of the liver, insulin continues

to activate SREBP-1c transcription and protein expression. Elevated SREBP-1c increases lipogenic gene expression, enhances fatty acid synthesis, and accelerates triacylglyceride accumulation. Thus many individuals with obesity and insulin resistance also have fatty livers [43].

1.4 Liver X receptor (LXR)

Liver X receptors (LXRs) are ligand dependent transcription factors belonging to the nuclear receptor superfamily of proteins. These proteins typically contain a N-terminal transcriptional activation domain, a core zinc finger DNA binding domain, a hinge region for receptor dimerization and a C-terminal ligand-binding domain [16]. LXRs form heterodimers with retinoid X receptor (RXR) and then bind to LXR-responsive elements (LXR-Es) in the promoters of target genes, which mainly consist of direct repeats of the core sequence AGGTCA separated by four nucleotides (DR-4 elements) (figure 11) [16]. Within the nucleus, LXR/RXR heterodimers are bound to LXR-Es in the promoter of target genes in a complex with co-repressors such as silencing mediator of retinoic acid, thyroid hormone receptor and nuclear receptor co-repressor [55; 56]. In response to binding of ligands, the co-repressor compexes are exchanged for co-activator complexes and target gene expression is de-repressed and induced.

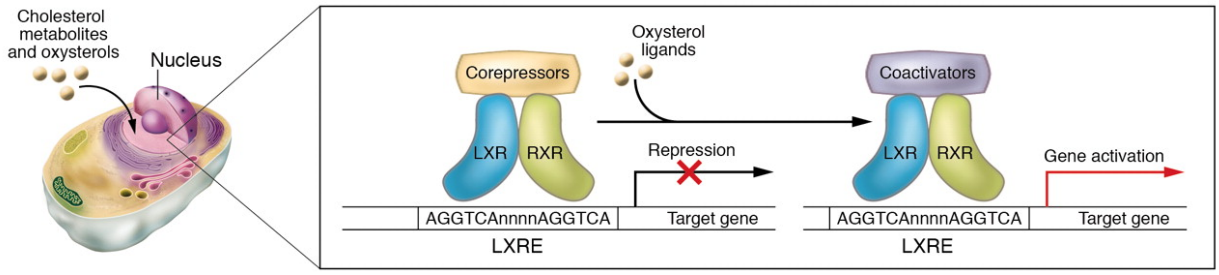

Figure 11: LXR mediated activation of target genes, from [57].

The liver X receptor (LXR) subfamily consists of two members, LXRα and LXRβ. The two LXRs share considerable amino acid sequence homology (77%), but their tissue distribution differs. LXRα is highly expressed in liver, adipose tissue and macrophages, whereas LXRβ is universally expressed [58].

LXRs control various genes of reverse cholesterol transport (figure 12). The first identified direct target gene for LXRs in mice was cholesterol 7-α-hydroxylase (Cyp7a1), which encodes the rate limiting a enzyme in hepatic bile acid synthesis. Cyp7a1 is up-regulated in response to cholesterol rich diet, whereas LXRα deficient mice show no induction of Cyp7a1 expression in response to high cholesterol diet [59]. Several ABC transporters, such as ABCA1 and ABCG1, which mediate cholesterol efflux of macrophages, and ABCG5 and G8, which promote cholesterol excretion into the bile, are controlled by LXR [18; 60]. Interestingly, Apolipoprotein E (ApoE), was the first gene shown to be regulated in a tissue specific manner by LXR [61]. Its expression is induced by LXR in adipose tissue and macrophages, but not in liver.

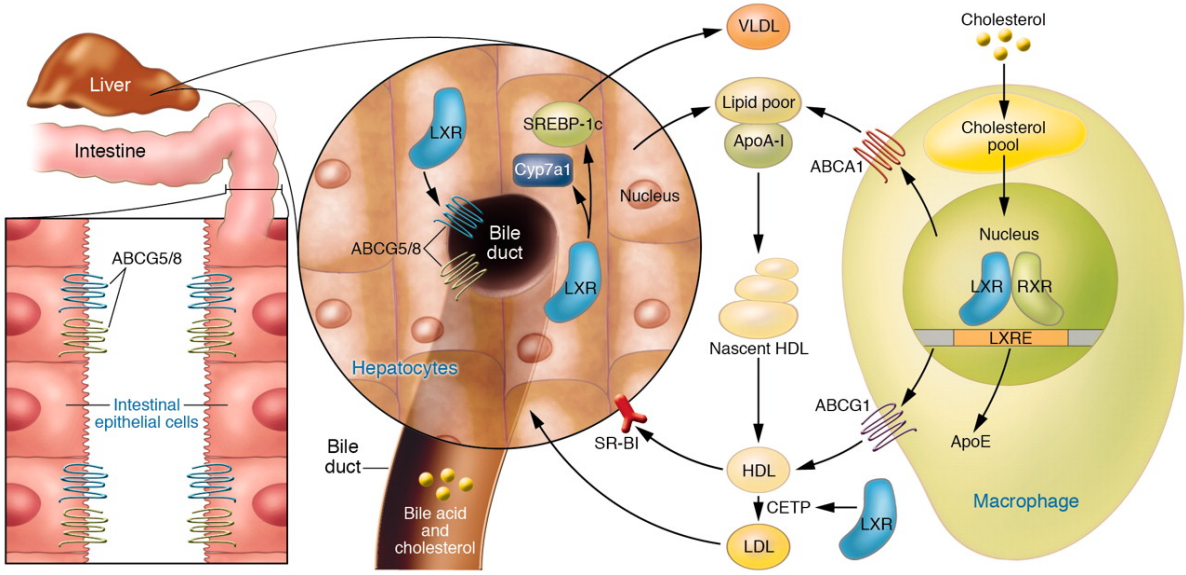

Figure 12: LXR and its target genes in reverse cholesterol transport, from [57].

Additionaly LXRs modulate the expression of several lipoprotein-remodeling enzymes, including lipoprotein lipase (LPL), cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP) [62-64]. LPL catalyzes the hydrolysis of lipoprotein tryglycerides. CETP mediates the transfer of HDL cholesterol esters to apoB containing particles for return to the liver. PLTP remodels HDL particles into large α-HDL and small pre-β-HDL particle fractions, which are efficient acceptors of cholesterol from peripheral cells [65]. Besides cholesterol and lipoprotein metabolism, LXRs are also implicated in the control of fatty acid metabolism via SREBP-1c as described above.

Consistent with their physiological roles, endogenous activators of LXRs are oxidized cholesterol derivates (oxysterols). The most potent natural activators inducing LXR at physiological levels are 22-(R)-, 20-(S)-, 24-(S)-hydroxycholesterol, 24-(S),25-epoxycholesterol and 27-hydroxycholesterol [66;

67]. Whereas most LXR ligands activate both LXRα and β, 24-(S),25-di-epoxycholesterol and 6α-hydroxy bile acids are selective agonists for LXRα [68]. In addition to natural ligands, a number of synthetic LXR ligands have been developed. The compounds T0901317 and GW3965 are activators of both LXR isoforms [69].

In contrast to oxidized cholesterol derivatives, that enhance transcriptional activity of LXRs, geranyl,geranyl-pyrophosphate, an intermediate of cholesterol biosynthesis and unsaturated fatty acids antagonize LXR activation [70].

The regulation of LXRα in the liver, adipose tissue, muscle and macrophages is mainly controlled by PPARs. PPARα and γ agonists induce LXRα in mouse and human macrophages [71]. Additionally, an auto-regulatory loop, which is limited to human cells, controls the expression of LXRα [72].

Recent data indicate multiple roles of LXRs in anti-inflammatory pathways that are involved in pathogenisis of cardiovascular and metabolic diseases. A recent study addressed the importance of macrophage LXR signaling using bone marrow transplantation studies in ApoE and LDLR deficient mice [73]. This approach allowed an analysis of LXR null macrophages in the setting of normal LXR function in liver and intestine, which led to a significant increase in atherosclerosic lesion formation in these mice. These studies provide strong evidence that LXR activity in macrophages is an important determinant in the development and progression of atherosclerosis. Evidence for the potential utility of LXR activation in atherosclerosis has come from intervention studies in mouse models. The LXR agonist GW3965 was shown to decrease lesion area

approximately 50% in both ApoE and LDLR knockout mice and to increase ABCA1 and ABCG1 expression in the atherosclerotic aortas of these mice [74]. In addition to enhancing cholesterol efflux through activation of ABCA1 and ABCG1, LXR agonist treatment led to an increase of cholesterol-accepting lipoproteins to be used as acceptors cholesterol efflux.

2 Aims

Since most studies analyzed the effects of CLA mixtures of different composition and purity, the aims of this work were to investigate whether the single isomers c9,t11- and t10,c12- and t9,t11-CLA have distinct effects on gene expression in human macrophages. Gene expression of t9,t11-CLA treated *in vitro* differentiated human macrophages from three healthy donors was examined with Affymetrix U133 Plus 2.0 DNA-microarrays. After validation of candidate genes with TaqMan^{TM} RT-PCR mRNA expression was compared to c9,t11-CLA and t10,c12-CLA treated macrophages. We found a t9,t11-CLA specific induction of ABCG1. To characterize this activation in detail, gene reporter assays with deletion plasmids of the ABCG1 regulatory region were carried out. To investigate the effects of the CLA isomer on ABCG1 function, cholesterol efflux was determined in t9,t11-CLA treated human macrophages.

3 Materials and Methods

3.1 Materials

3.1.1 Technical equipment

2100 Bioanalyzer,	Agilent, Palo Alto, CA, USA
Autoclave Steam Sterilizer Type 24	Melag, Berlin, Germany
Biofuge 15R	Heraeus, Hanau, Germany
Cell culture Incubator 6000	Heraeus, Hanau, Germany
ELISA-reader	Tecan, Stuttgart, Germany
Horizontal Shaker GFL-3016	GFL, Großburgwedel, Germany
Incubator B 6120	Heraeus, Hanau, Germany
Instant Camera MP4	Polaroid, Offenbach, Germany
Kodak X-Omat 2000 processor	Kodak, Rochester, NY, USA
LaminAir Hood	Heraeus, Hanau, Germany
Liquid Scintillation Counter Wallac 1410	Berthold, München, Germany
Lumi Imager F1	Boehringer, Mannheim, Germany
LUMAT LB9501	Berthold, München, Germany
Megafuge 1.0 R	Heraeus, Hanau, Germany
Microscope (Visible) Leitz Laborlux S	Leitz GmbH, Wetzlar, Germany
Milli-Q UF Plus System	Millipore, Bradford, VT, USA
MiniSpin Plus Centrifuge	Eppendorf, Hamburg, Germany
Mini Transblot Cell	BioRad, München, Germany
Nano Drop	PeqLab, Erlangen, Gernany
Precision Balance Sartorius MD BA 200	Sartorius, Göttingen, Germany
Power Supply PAC 300	BioRad, München, Germany
Princeton MicroMax CCD-1317-K/1	Roper Scientific, Trenton, NJ, USA

Shaking Incubator GFL-3032	GFL, Großburgwedel, Germany
Shaking Water Bath Julabo SW-20C	Julabo, Seelbach, Germany
Stirrer with Heating Surface IKAMAG	Labor Center, Nürnberg, Germany
SpeedVaq Alpha RVC	Christ, Osterode, Germany
Sysmex Micro-Cell Counter F-300	Digitana AG, Hamburg, Germany
Thermocycler Gene Amp PCR System 9600	Perkin Elmer, Uberlingen, Germany
Thermomixer Comfort	Eppendorf, Hamburg, Germany
Ultrasonic Disintegrator Soniprep 150	MSE, Watford Herts, United Kingdom
Ultracentrifuge (fixed angle) J2-21 M/E	Beckman, München, Germany
Ultracentrifuge L-70	Beckman, München, Germany
Ultracentrifuge Optima TLX	Beckman, München, Germany
Vortex-Mixer REAX 2000	Heidolph, Kelheim, Germany
Zeiss Axiovert S-100 Spectral Microscope	Carl Zeiss, Goettingen, Germany

3.1.2 Consumables

Cell culture flascs	Nunc, USA
Cell scraper	Sarstedt, Nümbrecht, Germany
Cups (0,5, 1,5, 2,0ml)	Eppendorf, Hamburg, Germany
Falcon Tubes (15, 50ml)	Sarstedt, Nümbrecht, Germany
Filter tips (2, 10, 20, 200, 1000µl)	Eppendorf, Hamburg, Germany
Latex gloves	Hartmann, Heidenheim, Germany
Optical adhesive cover	Applied Biosystems, USA
Sterile Filter	PALL, USA
384-Well Thermo-Fast plate	ABgene, UK

3.1.3 Reagents

Trans-9,trans-11-CLA (98% pure)	Cayman Chemicals (IBL), Hamburg, Germany

Cis-9,trans-11- CLA (96% pure)	Cayman Chemicals (IBL), Hamburg, Germany
Trans-10,cis-12-CLA (98% pure)	Cayman Chemicals (IBL), Hamburg, Germany

3.1.4 Gene Expression Assays

Gene	Applied Biosystems-ID
Acetoacetyl-CoA synthetase	Hs00225090_m1
ATP-binding cassette transporter A1	Hs00194045_m1
ATP-binding cassette transporter G1	Hs00245254_m1
7-Dehydrocholesterol reductase	Hs00154732_m1
Fatty acid synthase	Hs00188012_m1
Farnesyl-diphosphate farnesyltransferase1	Hs00189506_m1
Farnesyl diphosphate synthase	Hs00266635_m1
Insulin induced gene 1	Hs01650979_m1
3-Hydroxy-3-methylglutaryl-CoA reductase	Hs00168352_m1
3-Hydroxy-3-methylglutaryl-CoA synthase 1	Hs00266810_m1
Low density lipoprotein receptor	Hs00181192_m1
Liver X receptor	Hs00172885_m1
MID interacting protein 1	Hs00221999_m1
Phospholipid transfer protein	Hs00272126_m1
Stearoyl-CoA desaturase	Hs00748952_s1
Sterol regulatory element binding protein 1c	Hs00231674_m1
Vascular endothelial growth factor	Hs00173626_m1

3.1.5 Enzymes, inhibitors and kits for molecular biology

BCA Protein Assay Kit	Pierce, Rockford, IL, USA
BigDye Terminator Cycle Sequencing Kit v.1.1	Applied Biosystems, Darmstadt, Germany
ECL+ Western Blotting Analysis System	Amersham Pharmacia Biotech, Freiburg, Germany
Galactosidase enzyme assay	Promega, Madison, AL, USA
MagNa Pure LC DNA isolation Kit	Roche, Mannheim, Germany

Nucleasefree water	Promega, USA
PCR Purification Kit	Qiagen, Hilden
QIAamp Blood DNA midi Kit	Qiagen, Hilden, Germany
QIAprep Spin Miniprep Kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QIAshredder	Qiagen, Hilden, Germany
Reverse Transcription System	Promega, USA
RNAse Inhibitor RNase ZAP	Ambion, USA
RNeasy mini Kit	Qiagen, Hilden, Germany
RNeasy midi Kit	Qiagen, Hilden, Germany
TaqMan Universal PCR Master Mix	Applied Biosystems, USA

3.1.6 Cells

THP-1	ATCC (LGC Promochem) , Wesel, Germany
RAW 264.7	ATCC (LGC Promochem) , Wesel, Germany

3.1.7 Plasmids

pCMV-nSREBP-1a plasmid, a cytomegalovirus driven expression vector encoding amino acids 1-460 of human nuclear SREBP-1a, and pCMV-nSREBP-1c plasmid, encoding amino acids 1-436 of human nuclear SREBP-1c were gifts from Dr. Timothy F. Osborne (Department of Molecular Biology & Biochemistry, University of California).

Deletion constructs of the ABCG1 promotor (-2912/+50bp, -957/+50bp, -457/+50bp and -234/+50bp) have been described by Langmann et al [32].

SREBP-1c-Luc plasmids and the constructs with mutated LXR binding sites hLXRE-1mut, hLXRE-2mut, hLXRE-1/2mut were provided by Oberkoffler and collegues [75; 76].

The thymidine kinase (TK)-Luc plasmid was generated by ligating the BglII-HindIII fragment derived from the pRL-TK vector (Promega) containing the herpes simplex virus (HSV) minimal TK promoter region into the pGL3-Basic Vector upstream of the luciferase reporter gene. For construction of the 3xSRE-TK-Luc plasmid, three copies of the SREBP-responsive region of ABCG1 (5`-TGCCAGGGGTCACCCCACACCGGTGCTG-3`) were inserted into the TK-Luc plasmid using the NheI and KpnI restriction sites of its multiple cloning site.

3.2 Methods

3.2.1 Working with cells

3.2.1.1 Cell culture and stimulation

THP-1 cells and RAW 264.7 cells were cultured in RPMI 1640 medium or DMEM medium (Sigma) supplemented with 10% fetal calf serum (Gibco BRL), 100 U/ml penicillin, 100 µg/ml of streptomycin and incubated in 10% CO_2 in air at 37 °C. THP-1 monocytes were differentiated over night in the presence of 160 nM PMA prior to treatments and RNA or protein extraction.

Human monocytes were obtained from healthy donors by leukapheresis and counterflow elutriation [20]. The cells were cultured on plastic petri dishes in macrophage SFM medium (Gibco BRL) and allowed to differentiate for 4 days

in the presence of 50 ng/ml recombinant human macrophage colony stimulating factor (MCSF) from R&D Systems. Cells were stimulated with the indicated concentration of t9,t11-, c9,t11- and t10,c12-CLA or ethanol as solvent control.

3.2.1.2 Transfections and reporter gene assays

THP-1 cells were electroporated with Amaxa`s Nucleofector-Kit-V and RAW 264.7 cells were transiently transfected with FuGENE 6 (Roche) according to the manufacturer`s instructions. A promoterless pGL3-basic vector served as negative control, while a pGL3-control vector containing the CMV promoter was used as positive control. A cotransfected β-galactosidase plasmid was used to estimate transfection efficiency. Luciferase assays and β-galactosidase assays were carried out 36h after transfections.

3.2.2 Working with DNA

3.2.2.1 Isolating and purifying DNA

Plasmids were purified from overnight cultures with the QIAprep Spin Miniprep Kit (Qiagen). PCR-fragments were purified with the PCR Purification Kit (Qiagen). DNA fragments were extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen).

3.2.2.2 Analyzing DNA

DNA restriction digest was performed using 10 units of the appropriate restriction endonuclease per one µg of DNA. DNA fragments were separated using 0.8-2% agarose gels containing 0.01mg/ml ethidium bromide at 90V. Agarose gels were scanned with a Lumi-imager (Boehringer).

3.2.2.3 Amplification of DNA

DNA was amplified using polymerase chain reaction (PCR) with Taq-DNA polymerase (Qiagen) or Pfu Polymerase (Fermentas). Thermocycling was performed in a Gene Amp PCR System 9600 (Perkin Elmer). The template was denatured for 2 min at 95°C, followed by 35 cycles of denaturation (30 sec, 95°C), annealing (45sec, 50-60°C) and elongation (30-120 sec, 72°C). After the elongation was completed by incubation for 15 min at 72°C, the reaction was cooled down to 4°C.

3.2.2.4 Cloning of DNA

DNA fragments were ligated using T4-DNA-ligase (Invitrogen) at room temperature for two hours or at 4°C overnight. When the vector and insert had a similar length, a molar ratio of 1:3 (vector : insert) was used. When vector and insert were not similar in length, a molar ratio of 1:1 or 1:2 was used. Transformation of DH5α competent bacterial cells (Invitrogen) was performed according to the manufacturer`s manual.

3.2.2.5 Sequencing of DNA

Cycle sequencing was performed in a Gene Amp PCR System 9600 (Perkin Elmer) using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v.1.1 (Applied Biosystems). After purification of the sequencing reaction using Centrisept Spin Colums (Princeton Separations) 4 µl of the sample were mixed with 21 µl HighDye Formamid (Applied Biosystems) in 96-well plates (Applied Biosystems). Signal detection was carried out on an ABI PRISM 3130xl Genetic

Analyser equipped with Data Collection Software v3.0 (both Applied Biosystems).

3.2.3 Working with RNA

3.2.3.1 Isolation and analysis of RNA

Total RNA was extracted from cultured cells using the RNeasy Protect Midi Kit (Qiagen). Purity and integrity of the RNA was assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip® reagent set (Agilent Technologies) according to the manufacturer`s recommendations. The RNA was quantified on NanoDrop (Peqlab) and then stored at −80 °C.

3.2.3.2 Reverse transcription of RNA

cDNAs were generated by using the Reverse Transcripton System from Promega. Reverse transcription was performed in 40µl reaction volume containing 2µg RNA.

3.2.3.3 Real-time quantitative RT-PCR (TaqMan™) analysis

Real-time quantitative RT-PCR analysis was performed with an ABI7900HT machine (Applied Biosystems). All reagents necessary for running a TaqMan™ RT-PCR assay were purchased from Applied Biosystems and used according to the manufacturer's instructions. TaqMan™ analysis of the transcript for hABCG1-a and hABCG1b has been determined with Assays by Design (Applied Biosystems). All other transcripts have been specified with predesigned and optimized Gene Expression Assays (Applied Biosystems).

In detail 2,5 µL of single-stranded cDNA (10ng/µl) was mixed with 0.5µl Gene Expression Assay or Assay by Design, 5µl of TaqMan Universal PCR Master Mix and 2µl nuclease-free water. After the prepared mixture was loaded into a 384 well-plate (Thermo Fast) the plate was sealed with a optical adhesive cover (Applied Biosystems) and then placed in the 384 Well Block of the ABI7900HT machine. The thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 30 s at 97 °C and 1 min at 59.7 °C. Each sample was analyzed in duplicates.

Relative quantitation was carried out with the Applied Biosystems software SDS 2.2. In detail, gene expression values were calculated based on the comparative threshold cycle (Ct) method, in which RNA samples were designated as calibrators to which the other samples were compared. The Ct data for the analyzed gene and 18S rRNA in each sample were used to create δCt values ($Ct_{gene} - Ct_{18S\ rRNA}$). Thereafter, $\delta\delta$Ct values were calculated by subtracting the δCt of the calibrator from the Ct value of each target. The RQs were calculated with the equation: $RQ = 2^{-\delta\delta Ct}$. For calculating the RQ of the analyzed gene mRNA in CLA treated compared with untreated macrophages, untreated samples were designated as calibrators. The standard deviations (SDs) for δCt and $\delta\delta$Ct values were calculated from the single Ct values with the equation: $SD\delta Ct = \sqrt{(SD_1^2 + SD_2^2)}$. δCt values >25 were selected as the cut-off for absence of expression. All data are expressed as changes in transcript levels relative to the control group.

3.2.3.4 DNA-Microarray analysis

Gene expression profiles were determined using U133 Plus 2.0 GeneChips, which cover more that 47.000 human transcripts. We had three RNAs from three healthy donors per condition and each isolated RNA was hybridized to a single microarray by the Kompetenzzentrum für Fluoreszente Bioanalytik in Regensburg.

In a first step of gene array analysis expression signals for each transcript and comparisons between control and treated macrophages were calculated with the Affymetrix GCOS (gene chip operating software) 1.4 and Microsoft Excel (Microsoft Corp., Redmond, WA). Genes were picked out, which showed similar significant regulation in each replicate per condition. In a second step microarray data were analyzed on single probe level using the Genomatix microarray analysis software ChipInspector. This method uses the significance analysis of microarrays (SAM) algorithm [77; 78]. After total intensity normalization of the microarrays we used the exhaustive comparison mode for analyzing the raw data. Subsequently a SAM algorithm with a false discovery rate of 0% and probe coverage of three was used to identify significantly regulated genes. Finally the results of step one and two were combined and biomedical pathway analysis of the resulting genes was performed. All data are expressed as fold changes in transcript levels relative to the control group.

3.2.4 Working with proteins

3.2.4.1 Isolation and quantification of proteins

For protein isolation cells were washed in 1xPBS and lysed in RIPA buffer (Roche). Protein concentration was determined using the BCA protein assay reagent (Pierce Biotechnology).

3.2.4.2 Western blot analysis

Equal amounts of total cellular protein were denaturated at 70°C for 10 min after addition of NuPage-Sample Buffer (Invitrogen) and were separated on 4-12% NuPage-SDS-gels (Invitrogen). After protein transfer on polyvinylidene fluoride (PVDF) membranes (Roche), membranes were blocked in 3% BSA/PBS for 1h and incubated with a 1:1000 dilution of primary polyclonal anti-SREBP-1 antibody (Santa Cruz) or incubated with a dilution of 1:1000 primary monoclonal anti-ABCG1 antibody (Gene Tex) for 1h. Both antibodies were detected using a 1:20000 dillution of a peroxydase conjugated anti-rabbit immunoglobulin G (Dianova). Proteins were visualized with the ECL Plus™ Western Blotting Detection System (Amershan) on film.

3.2.4.3 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared with NE-PER-Kit (Pierce). *In vitro* synthesized human SREBP-1c was generated using the pCMV-SREBP-1c plasmid described before and the TNT Quick Coupled Transcription and Translation System from Promega. EMSAs were performed with the digoxygenin (DIG) gel shift kit 2nd generation for 3`-end labelling of oligonucleotides (Roche). The used

oligomers 5`-ACTGCCAGGGGTCACCCCACACCGGTGCTGTT-3` and 5`-AACAGCACCGGTGTGGGGTGACCCCTGGCAGT-3`contained the predicted SRE site [32]. These oligos were annealed, labelled and used in gel shift reactions. For electrophoretic separation retardation gels (6%) purchased from Invitrogen were used. Blotting was performed using a Biorad electroblotting system. Chemiluminescence of DIG labelled DNA was detected as described in the manufacturer`s instructions.

3.2.5 Cholesterol efflux assays

Cholesterol efflux assays were performed as described [79]. In detail, 850000 monocytes were seeded in 6-well plates and incubated for 96 h in M-SFM containing 50ng/ml MCSF for differentiation of macrophages. Then, macrophages were incubated for 24h in RPMI containing 0.2% BSA and 15µg/ml D_5-Cholesterol (Larodan) for 24h. Cells were then rinsed with PBS and incubated in RPMI containing 0.2% BSA and 10µM t9,t11-CLA or EtOH as control for another 24 h. Cells were rinsed again with PBS and incubated with or without 10µg/ml ApoAI or 100µg/ml HDL_2 or 100µg/ml HDL_3 and +/-10µM t9,t11-CLA or EtOH as control. Efflux of D_5-cholesterol from cells was measured by the appearance of label in the medium. In detail, the cholesterol efflux was calculated as percent fraction of the D_5-cholesterol in media over the total D_5-cholesterol (D_5-cholesterol in media + D_5-cholesterol in cells). The efflux was calculated in the presence of ApoAI, HDL_2 or HDL_3 with or without t9,t11-CLA minus the efflux in the presence of BSA and in absence of the respective

agonist (ApoAI, HDL_2 or HDL_3). Cholesterol was quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS) in the positive ion mode after acetylation as described [80]. D_5-cholesterol was monitored by a SRM transition of *m/z* 451.4 > 374.3. Samples were quantified using the analytical setup and the data analysis algorithms described by Liebisch et al [81].

3.2.6 Statistical analysis

Statistical analysis was examined with the independent student`s t-test of the MedCalc software for Windows. Significance levels of differences were set to $p<0.05$ (*) and $p<0.01$(**).

4 Results

4.1 Gene expression analysis of t9,t11-CLA treated primary human monocytes derived macrophages with DNA-microarrays

To determine the effects of t9,t11-CLA on global gene expression the expression of more than 47000 known human transcripts was analyzed using Human Genome U133 Plus 2.0 Affymetrix DNA-microarrays. *In vitro* for 4 days in MCSF differentiated monocyte derived macrophages from three healthy donors were treated with 100µM t9,t11–CLA and ethanol as control (figure 13).

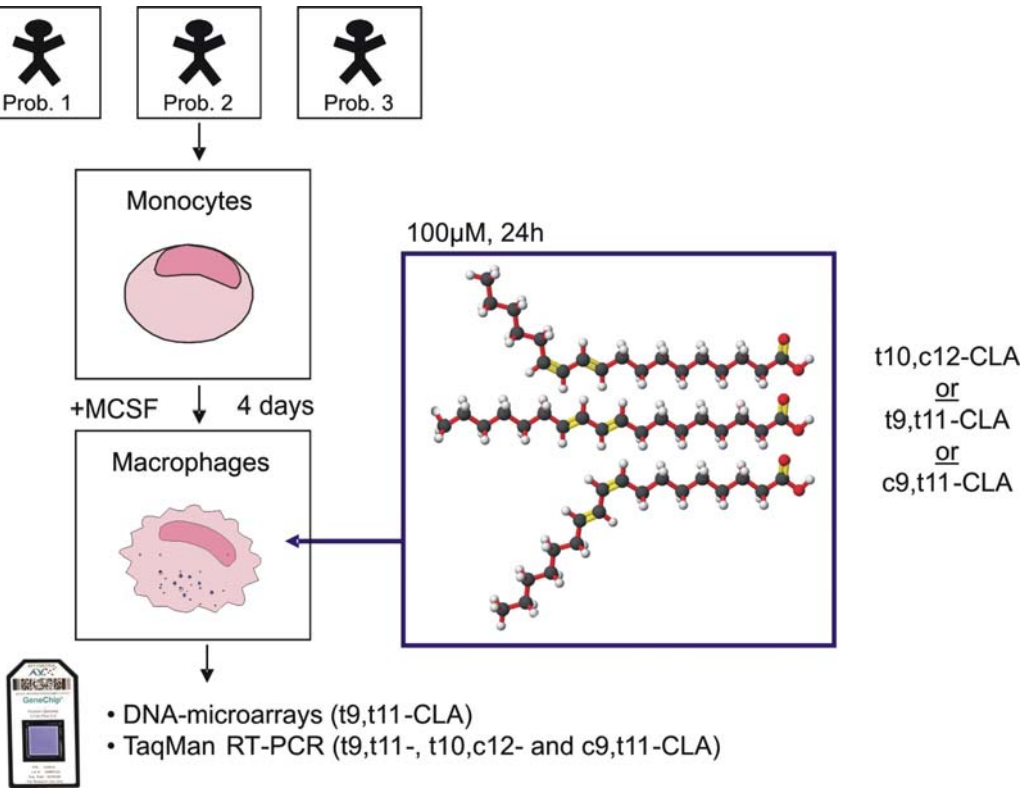

Figure 13: Experimental strategy for mRNA expression analysis of CLA treated primary macrophages from three healthy volunteers. MCSF, macrophage colony stimulating factor.

RNA was isolated and six independent RNA samples (3 control RNAs and 3 RNAs from t9,t11-CLA treated macrophages) were separately hybridized on DNA-microarrays by the Kompetenzzentrum für Fluoreszente Bioanalytik in Regensburg. DNA-microarrays were analyzed as described in the methods section. In the first step of our analysis genes where selected, which showed a similar significant regulation in each of the t9,t11-CLA treated replicates. This analysis revealed more than 100 regulated genes. With the second step, the analysis of the microarrays on a single probe level with the Genomatix microarray analysis software ChipInspector, 49 differently regulated transcripts could be identified. Combining of both analyses finally revealed 36 transcripts, which were significantly regulated in control versus t9,t11-CLA treated macrophages. Several genes of fatty acid synthesis, β-oxidation and also target genes of the PPAR family were present. Most importantly, as indicated in table 1 target genes of the nuclear transcription factor SREBP and ABCG1 were significantly induced [40; 44]. SREBP-1c, its target genes such as fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD), as well as SREBP-2 target genes such as 7-dehydrocholesterol reductase, 3-hydroxy-3-methylglutaryl-CoA reductase and synthase 1 were significantly induced. Remarkably, the CLA isomer had strongest effects on the expression of the ATP-binding cassette transporter G1, which was induced 4.4 fold (3.2-6.5 fold). These results indicate that t9,t11-CLA has a strong effect on gene expression related to lipid metabolism in primary human monocyte derived macrophages.

Changes of gene expression (Affymetrix DNA-microarrays)

Gene	Symbol	Mean expression Control	Mean FC t9,t11-CLA
SREBP-1c target genes			
Acetoacetyl-CoA synthetase	AACS	152 (132-168)	1.5 (1.4-1.5)
Fatty acid synthase	FASN	177 (157-196)	3.1 (2.1-4.0)
Stearoyl-CoA desaturase	SCD	4356 (3553-4771)	2.0 (1.6 -2.0)
Sterol regulatory element binding protein 1c	SREBP-1c	207 (140-247)	2.0 (1.7-2.5)
SREBP-2 target genes			
7-Dehydrocholesterol reductase	DHCR7	308 (261-352)	2.1 (1.7-2.6)
Farnesyl-diphosphate farnesyltransferase 1	FDFT1	1208 (1006-1335)	1.4 (1.1-1.6)
Farnesyl diphosphate synthase	FDPS	865 (822-898)	1.5 (1.4-1.5)
3-Hydroxy-3-methylglutaryl-CoA reductase	HMGCR	508 (428-637)	1.6 (1.4-1.7)
3-Hydroxy-3-methylglutaryl-CoA synthase 1	HMGCS	146 (128-165)	1.9 (1.6-2.3)
Insulin induced gene 1	INSIG1	1880 (1601-2246)	1.7 (1.4-2.0)
Low density lipoprotein receptor	LDLR	557 (467-628)	1.8 (1.5-2.1
MID1 interacting protein 1	MIDIP1	227 (218-233)	1.8 (1.5-2.1)
ABC-Transporter			
ATP-binding cassette transporter G1	ABCG1	204 (91-358)	4.4 (3.2-6.5)

Table 1: Genes, whose transcripts were increased in 3 probands after stimulation with 100µM t9,t11-CLA for 24h. The expression signals are given for the untreated samples. The fold change (FC) of gene expression between samples and controls is indicated.

4.2 Verification and further analysis of the candidate genes in primary human monocyte derived macrophages and the myeloid cell line THP-1

To validate the results obtained with DNA-microarrays with an independent and more sensitive technique, TaqMan™ RT-PCR was performed for the candidate genes. All genes could be verified in t9,t11-CLA treated primary human monocyte derived macrophages (table 2). Fatty acid synthase, stearoyl-CoA desaturase, 7-dehydrocholesterol reductase, HMG-CoA reductase and HMG-CoA synthase 1 were strongly induced (2.4-3.5 fold), whereas farnesyl-diphosphate farnesyltransferase 1 was not clearly activated (1.3 fold). SREBP-

1c and ABCG1 were found increased in each replicate 1.2-2.0 fold and 3.1-7.1 fold.

For further confirmation of the microarray results stimulation experiments with t9,t11-CLA were repeated in PMA differentiated THP-1 cells and mRNA expression was analyzed with TaqMan™ RT-PCR. All candidate genes showed a clear up-regulation except MID1 interacting protein 1, which was induced only about 1.3 fold. Notably SREBP-1c and ABCG1 were found induced 2.0 fold and 4.8 fold, respectively.

To compare the effects of single CLA-isomers on gene expression among each other, we incubated MCSF differentiated monocyte derived macrophages from the three healthy donors (figure 13) and the THP-1 cells with c9,t11- and t10,c12-CLA and analyzed the mRNA expression. In contrast to t9,t11-CLA treated cells, gene expression of candidate genes in c9,t11-CLA and t10,c12-CLA stimulated primary human monocyte derived macrophages was mainly unchanged, except for 3-hydroxy-3-methylglutaryl-CoA reductase and synthase (table 2). In c9,t11-CLA and t10,c12-CLA treated THP-1 cells most of the genes were decreased or unchanged, clearly suggesting and isomer specific effect of t9,t11-CLA on gene expression in human macrophages.

Changes of gene expression (TaqMan RT-PCR)

Gene	Symbol	FC					
		t9,t11-CLA		c9,t11-CLA		t10,c12-CLA	
		THP1	Proband 1-3	THP1	Proband 1-3	THP1	Proband 1-3
SREBP-1c target genes							
Acetoacetyl-CoA synthetase	AACS	1.6	2.0 (1.5-2.7)	1.0	1.7 (1.3-2.2)	0.9	1.1 (0.5-1.8)
Fatty acid synthase	FASN	1.9	3.1 (2.1-4.0)	0.7	1.3 (1.2-1.5)	0.6	0.9 (0.7-1.0)
Stearoyl-CoA desaturase	SCD	4.9	3.4 (3.0-3.8)	1.0	0.9 (0.5-1.4)	0.8	0.7 (0.3-0.9)
SRE binding protein 1c	SREBP-1c	2.0	1.7 (1.2-2.0)	0.8	0.9 (0.7-1.0)	0.7	0.7 (0.6-0.8)
SREBP-2 target genes							
7-Dehydrochol. reductase	DHCR7	5.5	3.5 (1.7-5.1)	1.0	1.5 (0.5-2.4)	0.9	1.1 (0.5-2.2)
FDF-transferase 1	FDFT1	2.5	1.3 (1.2-1.4)	1.2	1.1 (0.9-1.2)	0.9	1.0 (0.7-1.4)
FDF-synthase	FDPS	1.7	2.1 (1.3-3.5)	0.8	1.5 (1.0-2.5)	0.8	1.1 (0.4-2.1)
HMG-CoA reductase	HMGCR	1.6	3.5 (2.3-4.6)	1.0	2.5 (1.7-3.2)	0.9	2.1 (1.5-2.7)
HMG-CoA synthase 1	HMGCS	3.7	2.4 (2.1-2.7)	0.9	2.0 (1.2-3.0)	0.9	1.6 (0.6-2.7)
Insulin induced gene 1	INSIG1	3.0	1.9 (1.7-2.3)	1.1	1.2 (0.7-1.6)	1.0	1.1 (0.9-1.6)
LDL receptor	LDLR	3.0	1.8 (1.7-1.9)	1.1	1.3 (1.1-1.4)	1.2	1.1 (0.8-1.4)
MID1 interacting protein 1	MIDIP1	1.3	1.8 (1.1-2.7)	1.1	1.3 (0.8-2.0)	1.0	1.1 (1.0-1.2)
ABC-Transporter							
ABC transporter G1	ABCG1	4.8	4.5 (3.1-7.1)	1.3	1.2 (0.9-1.6)	0.9	1.0 (0.8-1.3)

Table 2: T9,t11-, c9,t11-, t10,c12-CLA induced mRNA expression in THP-1 cells and primary human macrophages from three healthy donors. Cells were treated 100µM for 24h with the appropriate substance and EtOH as control. Gene expression was monitored using TaqMan™ RT-PCR standardized to 18S rRNA as reference. The fold change (FC) in THP-1 cells and the mean FC in primary human macrophages of gene expression between samples and controls is indicated.

4.3 Analysis of SREBP-1c and ABCG1 protein expression in t9,t11-CLA treated human macrophages

T9,t11-CLA induced SREBP-1c transcription 2.0 fold and 1.7 fold and ABCG1 transcription 4.8 fold and 4.5 fold in the THP-1 cell line and primary macrophages. To verify these results on the protein level, THP-1 cells were differentiated to macrophages with PMA and stimulated with 100µM t9t11-CLA or ethanol as control for 24h. Isolated proteins were used for western blot analysis.

As indicated figure 14, expression of the premature form pSREBP-1 was increased as well as expression of its nuclear and transcriptional active form SREBP-1c. Protein levels of ABCG1 were also elevated after treatment of cells with t9,t11-CLA. These findings are in good agreement with the identified transcriptional changes.

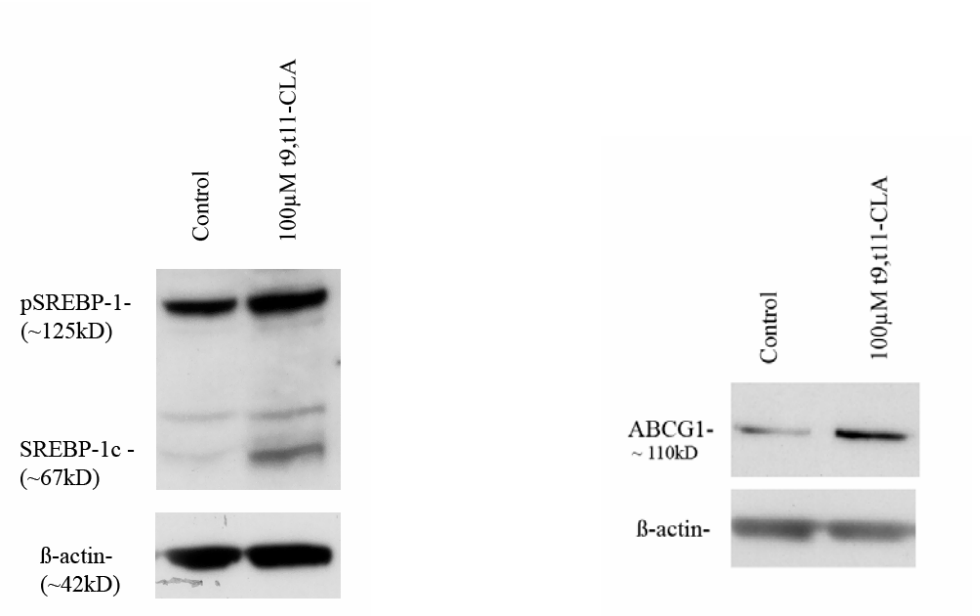

Figure 14: T9,t11-CLA induced protein expression of SREBP-1c and ABCG1. THP-1 macrophages were treated with 100µM t9,t11-CLA or EtOH as control for 24h.

4.4 Analyis of t9,t11-CLA mediated activation of ABCG1

The interesting finding that t9,t11-CLA, but not c9,t11- and t10,c12-CLA induce transcription of ABCG1 and SREBP-1c suggests that ABCG1 is induced via SREBP-1c. To investigate this hypothesis, mRNA expression of the isoform hABCG1-a of ABCG1 was determined with TaqMan™ RT-PCR in human macrophages. T9,t11-CLA significantly induced the transcription of hABCG1-a in the THP-1 cell line and in primary human monocyte derived macrophages

from 2.3 to 5.8 fold (figure 15). In macrophages stimulated with c9,t11-CLA transcription was decreased (0.9-0.5 fold) apart from proband 1, where mRNA expression was increased 1.8 fold. Treatment with t10,c12-CLA lead to changes between 0.6 and 0.7 fold. These results show that transcription of hABCG1-a is strongly increased in t9,t11-CLA, but not in c9,t11- and t10,c12-CLA stimulated cells.

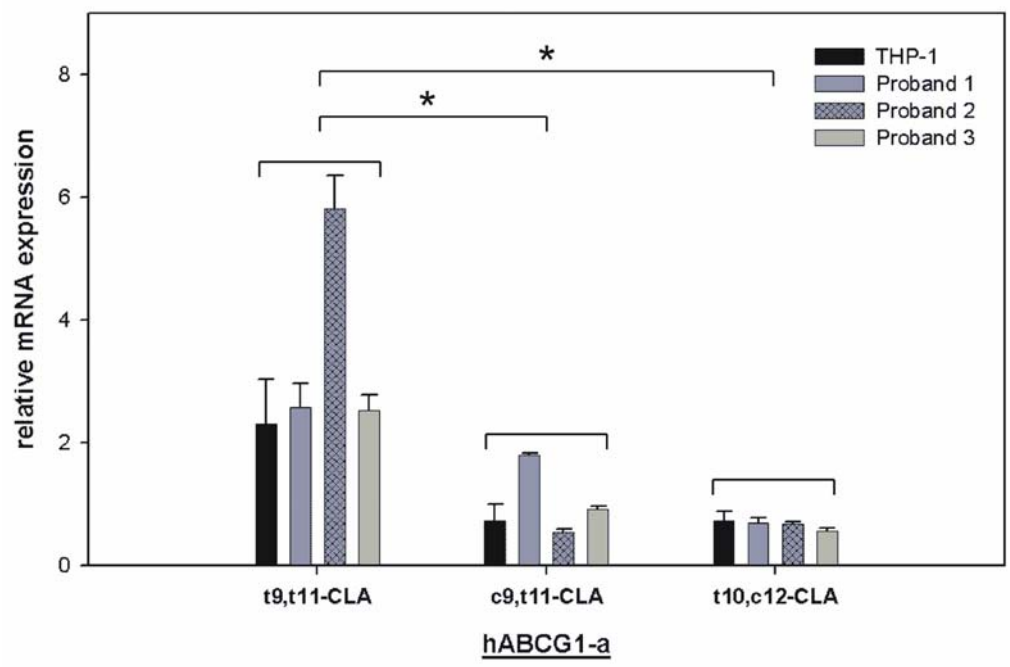

Figure 15: T9,t11-, c9,t11, t10,c12-CLA induced mRNA expression of hABCG1-a in THP-1 cells and primary human monocyte derived macrophages from three probands. Cells were treated 100µM for 24h with the appropriate substance and ethanol as control. Gene expression was monitored using TaqMan™ RT-PCR standardized to 18S rRNA as reference. $p<0.05$(*).

The promoter of hABCG1-a contains a consensus sequence for a putative SREBP response element (SRE) at position -660/-648bp with the sequence 5`-

GTCACCCCAC-3 [32]. This putative SRE element is very similar to other described SRE promoter sequences of SREBP target genes (table 3).

Gene	Symbol	SRE promoter sequence (5`-3`)	Reference
ATP-binding cassette transporter G1	ABCG1	G T C A C C C C A C	[32]
Fatty acid synthase	FAS	G C C A C G C C A C	[82]
(2 binding sites)		G T C A G C C C A T	
Glycerol-3-phosphate acyl transferase	GPAT	C T C A G C C T A G	[83]
(3 binding sites)		C T C A C C C C A G	
		G A C A C C C C A G	
3-Hydroxy-3-methylglutaryl-CoA synthase 1	HMGCS	C T C A C C C C A C	[84]
(2 binding sites)		G C C A C C C T A C	
Low density lipoprotein receptor	LDLR	A T C A C C C C A C	[85]
Sterol regulatory element binding protein 1c	SREBP-1c	C T C A C C C C A G	[52]

Table 3: SRE binding sites of selected SREBP responsive genes and putative SRE sequence of ABCG1.

To characterize the activation of the hABCG1-a promoter, two deletion constructs were assayed in reporter gene assays. RAW 264.7 macrophages and plasmids containing the 2912/+50bp or -957/+50bp region of the hABCG1-a promoter driving a luciferase gene were used. Both constructs contain the consensus sequence of the putative SRE (figure 16). T9,t11-CLA stimulation of transfected macrophages resulted in a concentration dependent activation of both promoter constructs.

For further analysis of the regulatory region of the hABCG1-a promoter, the activity of two shorter deletion constructs with -457/+50bp and -234/+50bp length was determined, which do not contain the SRE (figure 2). Treatment with 100µM t9, t11-CLA did not affect the activity of these two promoter constructs lacking the SRE.

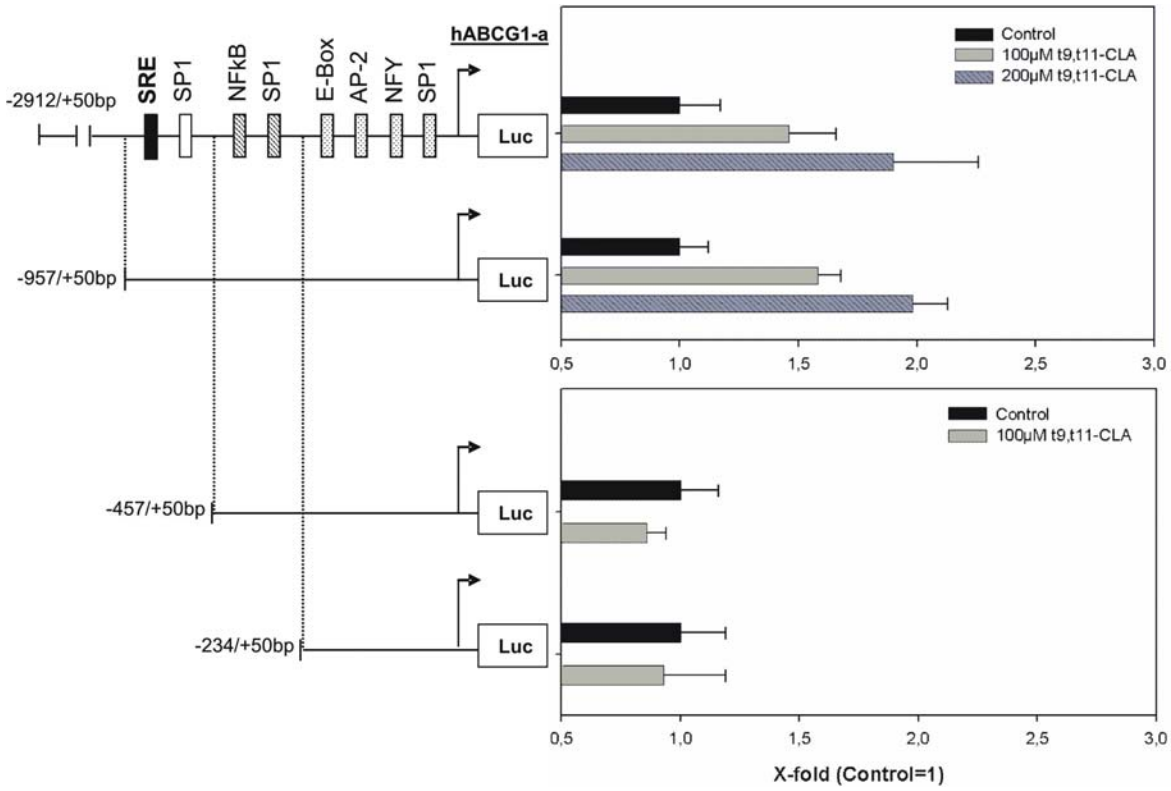

Figure 16: Correlated luciferase activity (X-fold; EtOH-Control=1) of RAW 264.7 cells transiently transfected with the indicated gene reporter reporter plasmids. Cells were treated with the appropriate concentration of t9,t11-CLA or EtOH as control for 36h.

To confirm that ABCG1 can be activated by SREBP-1 and to determine the isoform inducing the SRE, the -957/+50bp ABCG1 promoter construct was cotransfected with expression constructs of the two nuclear forms SREBP-1a and SREBP-1c. Cotransfection with pCMV-nSREBP-1c significantly increased luciferase activity (2.5 fold), whereas cotransfection with pCMV-nSREBP-1a showed no change in luciferase activity, indicating that ABCG1 can be specifically activated by SREBP-1c in macrophages (figure 17).

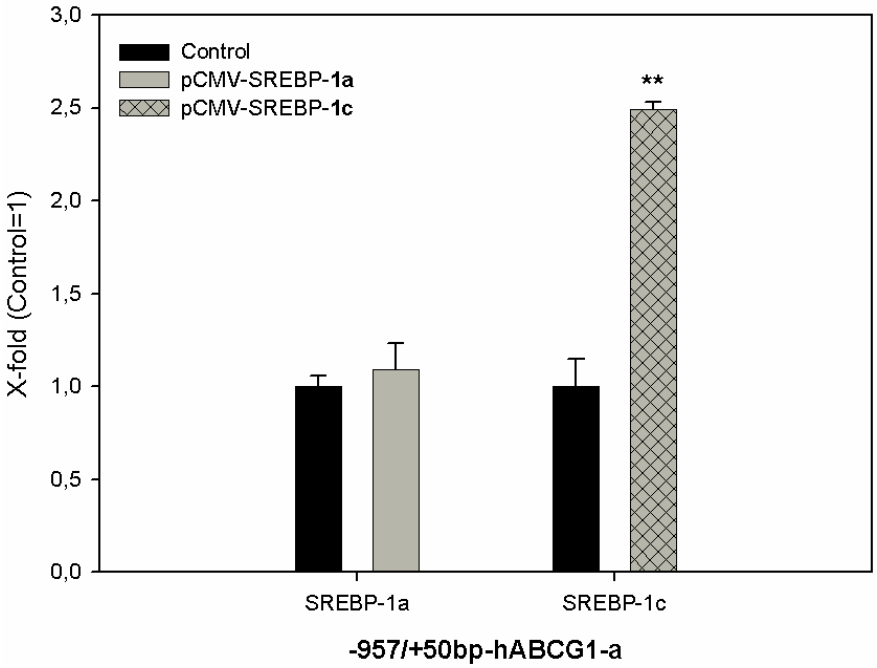

Figure 17: Correlated luciferase activity of RAW 264.7 macrophages transiently transfected with a -957/+50bp-hABCG1-a luciferase reporter plasmid and expression plasmids for SREBP-1a or SREBP-1c. Cells were cotransfected for 36h. $p<0.01$(**).

To analyze the functionality of the putative SRE motif in the hABCG1-a promoter as independent transcriptional regulator, the gene construct pTK3xSRE was developed (figure 18). It contains three copies of the specific SREBP responsive element from hABCG1-a in front of the minimal thymidine kinase (TK) promoter driving the luciferase gene. Cotransfection of RAW 264.7 cells with pCMV-nSREBP-1c, significantly increased reporter gene activity. Notably, even cotransfection of 1ng SREBP-1c led to a significant 2 fold increase in luciferase activity. Cotransfection with increasing amounts of the transcription factor led to 5-20 fold enhanced activities of the gene reporter,

demonstrating that the SRE motif markedly contributes to SREBP-1c mediated induction of the ABCG1 promoter.

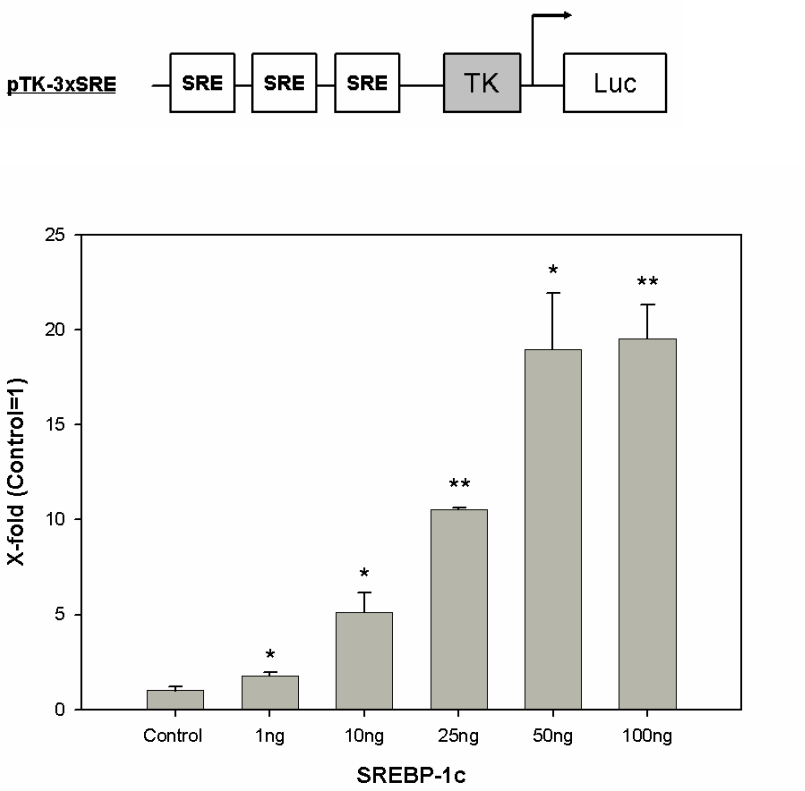

Figure 18: Correlated luciferase activity (X-fold; Control=1) of RAW 264.7 macrophages transiently transfected with pTK-3xSRE and cotransfected with the indicated amount of pCMV-SREBP-1c. $p<0.05$(*); $p<0.01$(**).

To determine whether SREBP-1c binds to the ABCG1 SREBP responsive element, oligonucleotide probes containing the putative SRE site from the hABCG1-a promoter were used to perform electrophoretic mobility shift assays. The DNA-protein complex could be detected as shifted band in the presence of a nuclear extracts from THP-1 macrophages (figure 19). Competition with a 50-fold excess of the unlabeled probe revealed sequence specificity of the DNA protein binding reaction, because the shifted band was markedly reduced. The

nuclear extract incubated with an antibody did not produce a supershift band, but nearly abolished the shifted band. This result most likely indicates that the antibody disrupted the DNA-protein interaction, resulting in reduction in the amount of the characteristic gel shift but no supershift. In the presence of *in vitro* synthesized SREBP-1c a DNA protein complex could be detected approximately at the same height as in the presence of the nuclear extract confirming that SREBP-1c binds to the SRE element located in the hABCG1-a promoter.

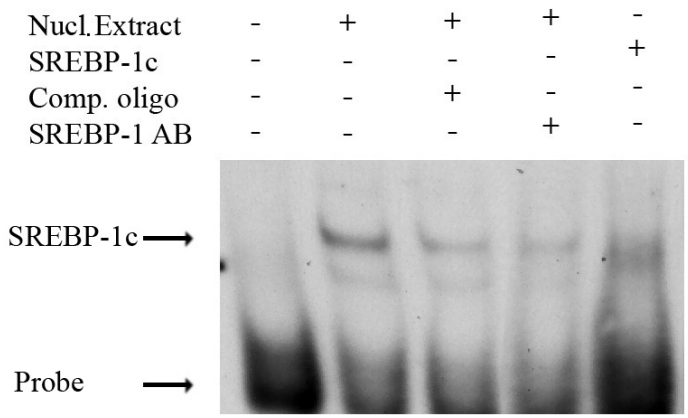

Figure 19: Electrophoretic mobility shift assay using a consensus ABCG1 SRE oligonucleotide, a nuclear extract from THP-1 cells, a unlabeled competition oligo in 50fold excess, a SREBP-1 antibody and in-vitro synthesized SREBP-1c as indicated.

4.5 Analyis of t9,t11-CLA mediated activation of SREBP-1c

LXR is a major activator of SREBP-1c [86], hence we tested the hypothesis that t9,t11-CLA is activator of LXR. Therefore mRNA expression of LXRα and its target genes in primary macrophages and cell line macrophages treated for 24h with 100µM t9,t11-CLA was determined with TaqMan^{TM} RT-PCR. Since

several LXR target genes can also be induced by SREBP-1c, we also analyzed transcription of genes that are exclusively activated by LXR.

As shown in table 4, a 2.3 fold and 1.7 fold up-regulation of Liver X receptor α in primary macrophages and cell line macrophages could be detected. The LXR target gene ABCA1 was significantly activated 2.4 and 1.9 fold, whereas phospholipid transfer protein was induced only in primary macrophages, but not in THP-1 cells. VEGF was activated 7.8 fold in primary macrophages. Interestingly, hABCG1-b, the LXR-controlled form of ABCG1, showed a lower response to the CLA isomer compared to the SREBP-1c regulated isoform hABCG1-a (4.4 fold versus 6.6 fold in primary macrophages and 1.3 fold versus 3.3 fold in THP-1 cells, respectively).

Changes in gene expression (TaqMan RT-PCR)

Gene	Symbol	FC t9,t11-CLA	
		THP1	Proband 1-3
Liver X receptor α	LXRα	1.7	2.3 (2.2-2.5)
Liver X receptor target genes			
ATP binding cassette transporter A1	ABCA1	1.9	2.4 (2.2-2.5)
ATP binding cassette transporter G1 isoform a	hABCG1-b	1.3	4.4 (4.2-4.6)
Phospholipid transfer protein	PLTP	1.0	2.0 (1.9-2.2)
Vascular endothelial growth factor	VEGF	1.5	7.8 (7.4-8.3)
SREBP-1c target gene			
ATP binding cassette transporter G1 isoform b	hABCG1-a	3.3	6.6 (6.4-6.8)

Table 4: T9,t11-CLA induced mRNA expression in human macrophages. Cells were treated 100µM t9,t11-CLA and ethanol as control for 24h. Gene expression was monitored using TaqMan™ RT-PCR standardized to 18S rRNA as reference. FC, fold change.

To analyze the potential LXR dependent induction of SREBP-1c, the activity of its promoter region was investigated with gene reporter assays. Therefore, THP-1 cells were transfected with plasmids containing the promoter region from -422bp to -186bp of SREBP-1-c. This region harbours two LXR binding sites at

positions -349/-334bp and -298/-283bp. Treatment of transfected cells with 100µM t9,t11-CLA resulted in a significant activation of the reporter gene, whereas CLA treatment of cells transfected with constructs containing mutations in the first, second, or both LXR binding sites, did not significantly affect promoter activity (figure 20). These results confirm that t9,t11-CLA activates SREBP-1c via LXR in human macrophages.

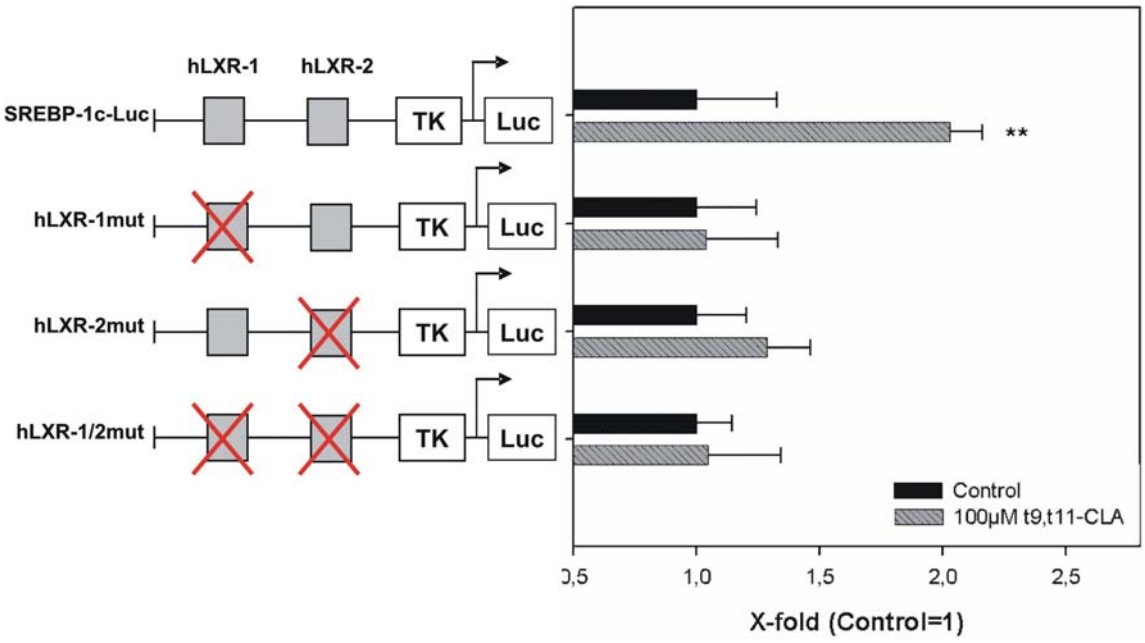

Figure 20: Correlated luciferase activity (X-fold; EtOH-Control=1) of THP-1 cells transiently transfected with the indicated gene reporter reporter plasmids. Cells were treated with 100µM t9,t11-CLA or EtOH as control for 36h. $p<0.01$(**).

4.6 Concentration range of t9,t11-CLA

To determine the effective concentration range by that the CLA isomer induces gene expression, primary macrophages were treated with doses from 5µM to 100µM of t9,t11-CLA for 24h and mRNA expression of LXRα, SREBP-1c, ABCG1 and ABCA1 was analyzed with TaqMan^{TM} RT-PCR. For determination

of ABCG1 transcription a gene expression assay detecting both, hABCG1-a and hABCG1-b was used.

As indicated in figure 21 the CLA-isomer activated transcription of LXRα, SREBP-1c and ABCG1 in a dose dependent manner (figure 21). ABCA1 transcription was activated 2.4-and 2.6-fold with the lower doses, whereas treatment of cells with higher doses induced ABCA1 mRNA expression only 2.0-fold. Notably, a dose of 10µM led to a 2.0-fold induction of SREBP-1c and ABCG1, and to a 2.6-fold induction of ABCA1 indicating that especially the physiological levels of CLA significantly influence gene expression in human macrophages [87].

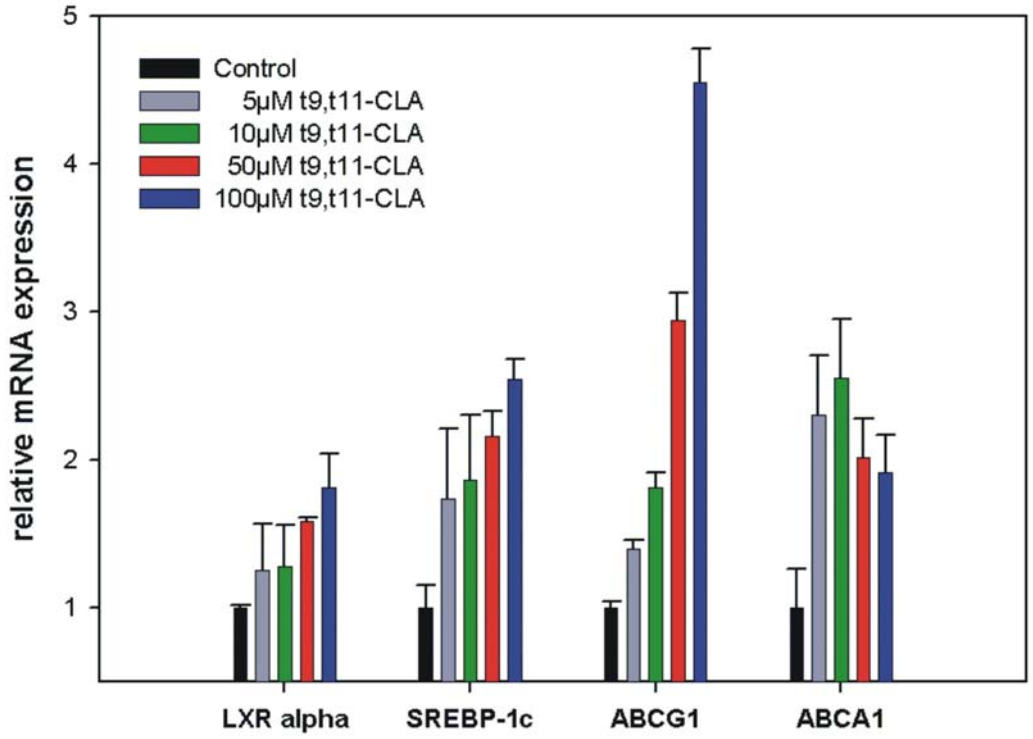

Figure 21: T9,t11-CLA induced mRNA expression in primary macrophages. Cells were treated with the indicated CLA dose and ethanol as control for 24h. Gene expression was monitored using TaqMan^{TM} RT-PCR standardized to 18S rRNA as reference.

4.7 Effects of t9,t11-CLA on ABCA1 and ABCG1 mediated cholesterol efflux

Since ABCG1s and ABCA1s function is export of cholesterol, we examined ApoAI and HDL specific cholesterol efflux of human macrophages after treatment with t9,t11-CLA. Therefore, primary macrophages were loaded with labeld cholesterol and treated with 10µM t9,t11-CLA. Cholesterol efflux was determined with electrospray ionization tandem mass spectrometry (ESI-MS/MS). This dose of CLA was selected, because using this concentration resulted in a significant induction of ABCA1 and ABCG1 mRNA expression. In addition, the dose of 10µM is within the physiological range of CLA in humans [87]. T9,t11-CLA significantly induced the efflux of cholesterol to ApoAI as acceptor particle, mediated by ABCA1, as well as to HDL_2 and HDL_3 as acceptor particles, mediated by ABCG1 (figure 22). ApoAI specific cholesterol export increased from 0,5% to 2,5%. HDL_2 specific efflux was augmented from 39% to 63% and HDL_3 specific efflux from 30% to 40% after treatment of the cells with 10µM t9,t11-CLA. Interestingly, the ABCA1 mediated cholesterol efflux was more effected by the CLA isomer than the ABCG1 mediated efflux.

These results indicate that the increase in mRNA levels of ABCA1 and ABCG1 induced by t9,t11-CLA parallels with the functional response of cholesterol efflux in human macrophages.

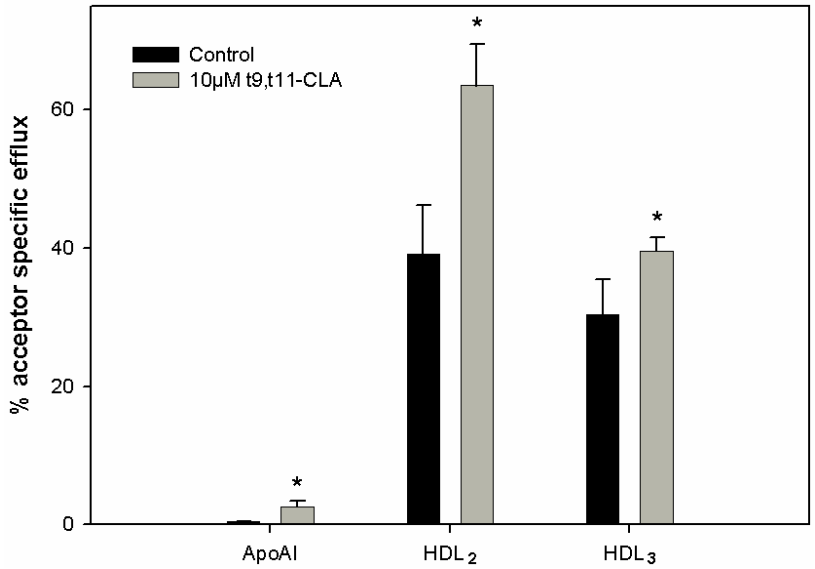

Figure 22: Percent of ApoAI, HDL_2 or HDL_3 specific cholesterol efflux in the presence of 10µM t9,t11-CLA or EtOH as control.

4.8 Summary of the results

In summary, t9,t11-CLA treatment of human macrophages leads to activation of the following regulatory cascade (figure 23). The CLA isomer activates LXRα, which directly induces ABCG1, ABCA1 and SREBP-1c. SREBP1-c activates ABCG1 via binding in its regulatory region. The transcriptional activation of the ABC transporters A1 and G1 parallels with the functional response of cholesterol efflux in human macrophages.

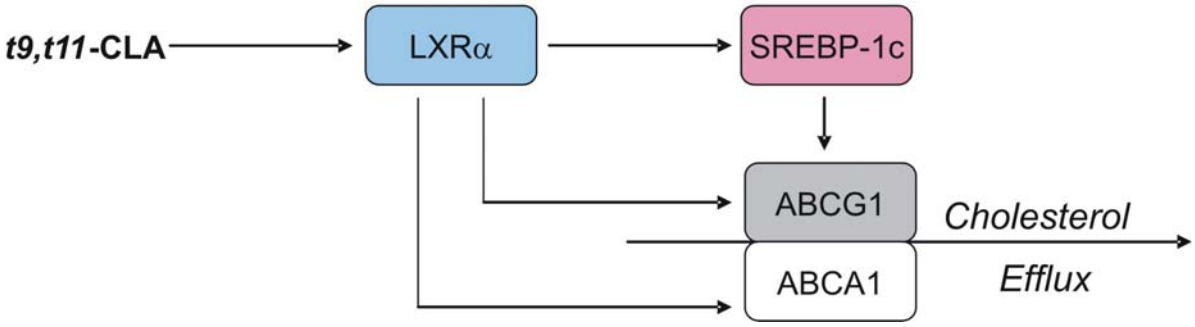

Figure 23: T9,t11-CLA induced transcriptional and functional cascade.

5 Discussion

5.1 Isomer specific effects of CLA on macrophage gene transcription

This study shows that the single isomers t9,t11-CLA, c9,t11-CLA and t10,c12-CLA have individual effects on gene expression in human macrophages. Large scale transcription profiling revealed an up-regulated gene cluster of SREBP target genes in t9,t11-CLA treated *in vitro* MCSF differentiated monocyte derived macrophages from three healthy donors. SREBP-1c, its target genes and SREBP-2 targets were induced, whereas SREBP-2 was not differently regulated in t9,t11-CLA treated macrophages.

Almost all genes identified with DNA-microarrays could be verified with TaqMan™ RT-PCR. Although farnesyl-diphosphate farnesyltransferase 1 showed only a small increase in several samples, the overall tendency in all replicates was upregulation. Further analysis of t9,t11-CLA stimulated THP-1 cells confirmed the results of the microarrays from primary human monocyte derived macrophages. Comparison of mRNA expression to c9,t11-CLA and t10,c12-CLA treated cells with TaqMan™ RT-PCR showed that the effect of t9,t11-CLA on gene expression of candidate genes is clearly isomer dependent.

The finding that t10,c12-CLA slightly decreases the transcription of SREBP-1c is also supported by microarray studies from Larosa et al [88]. They found significant reductions of SREBP-1c mRNA expression in white adipose tissue of t10,c12-CLA fed mice. Takahashi et al. showed that mice fed a CLA mixture

had an increased mRNA expression of δ-5 and δ-6 desaturases, and SREBP-1c in liver [89]. The used mixture was mainly made of the two major CLA isomers and a smaller amount of minor isomers, among them t9,t11-CLA. Our results show a clear induction of stearoyl-CoA desaturase, a δ-6 desaturase and SREBP-1c in t9,t-11-CLA stimulated macrophages, but treatment with the two major isomers did not affect or slightly decreased the expression of these genes. Shaomei et al. reported that a mixture consisting mostly of the two major isomers up-regulates the LDL receptor gene (LDLR) in the heptoma cell line HepG2 [90]. Ringseis and colleges expanded these findings and reported that LDLR transcription is selectively induced by t10,c12-CLA but not by c9,t11-CLA in HepG2 cells [91]. However, our results clearly demonstrate that the single isomers c9,t11-CLA and t10,c12-CLA are not potent inducers of LDLR expression, at least in macrophages.

Although t9,t11-CLA is a CLA isomer of minor abundance, it seems to have stronger effects on specific cellular processes than its major dietary occurring isomers. This assumption is also supported by studies on CLA isomer specific treated human colon cancer cells. T9,t11-CLA has an much higher inhibitory effect on the growth of Caco-2 cells than c9,t11- or t10,c12-CLA [92]. Furthermore, t9,t11-CLA has as stronger anti-proliferative activity in SW480 and HT-29 cancer cells compared to c9,t11-CLA [93].

5.2 T9,t11-CLA mediated activation of ABCG1

A surprising and interesting finding was that ABCG1 and SREBP-1c were significantly up-regulated in t9,t11- but not in c9,t11- and t10,c12-CLA treated macrophages. From these data we hypothesized that ABCG1 is activated by a SREBP-1c dependent mechanism. The promoter of ABCG1, which is responsible for transcription of the isoform hABCG1-a, contains a putative SREBP responsive element (SRE) and contributes to macrophage specific expression of ABCG1 [32]. Isoform specific transcription analysis confirmed the assumption that the mRNA expression of hABCG1-a is induced by t9,t11-CLA, but not by c9,t11- and t10,c12-CLA. Statistical analysis provided a significant difference between the expression values of the hABCG1-a isoform for t9,t11-CLA, c9,t11-CLA and t10,c12-CLA stimulated cells.

Reporter gene assays with the deletion constructs -2912/+50bp and -957/+50bp of the hABCG1-a promoter containing the putative SRE in RAW 264.7 macrophages showed a dose dependent increase of luciferase activity after t9,t11-CLA treatement, whereas the -457/+50bp and -234/+50bp promoter constructs lacking the SRE were not activated by t9,t11-CLA. Cotransfections with SREBP-1c expression plasmids caused significant induction of the ABCG1 promoter by SREBP-1c but not by SREBP-1a. This finding is plausible, because SREBP-1c is the major isoform in human tissues and it is highly regulated compared to SREBP-1a. We conclude that SREBP-1a isoform has only a minor role *in vivo*. Additionaly, nSREBP-1a overexpression in the liver of transgenic mice lead to fatty livers with accumulated triglycerides and cholesterol. Inversely, overexpression of nSREBP-1c was found to produce a triglyceride-

enriched fatty liver with no increase in cholesterol [43]. The reason for this might be SREBP-1c dependent activation of ABCG1 mediated cholesterol efflux. However, the finding that SREBP-1c, but not SREBP-1a stimulates the ABCG1 promoter is also surprising. In experiments comparing the transcriptional efficiency of these two isoforms in the liver, the SREBP-1a isoform had higher potency compared to SREBP-1c, despite DNA binding of both isoforms was similar [94]. In contrast to the current work, which uses the macrophage model, these experiments were performed in liver cells. Thus, tissue specific function of transcription factors might explain the controversial findings.

The binding of SREBP-1c in the promoter of hABCG1-a has been investigated with electrophoretic mobility shift assays. Kim et al. reported that SREBPs can bind to E-boxes, like all basic helix loop helix proteins, as well as to SRE sequences [95], which are present in the promoter of ABCG1. Since the deletion constructs lacking the SRE could not be activated in luciferase assays, it was very likely that the SRE in the promoter of ABCG1 is responsible for its activation through SREBP-1c. Another reason supporting this suggestion is that in the vicinity of the SRE a SP1-element exists, which is a cofactor of SREBP in most promoters [40]. Our EMSAs also confirmed that SREBP-1c binds to the ABCG1 SRE. In the presence of *in vitro* synthesized SREBP-1c a DNA-protein complex could be detected nearly at the same height as in the presence of a nuclear extract from THP-1 cells. SREBP-1c protein undergoes significant posttranslational modification including phosphorylation, acetylation, sumoylation and ubiquitination [96]. The nuclear extract was prepared from human cells, whereas the *in vitro* translated SREBP-1c was generated in rabbit

reticulocytes. Thus different posttranslational modifications might explain the minimal differences in the height of the shifted bands.

5.3 T9,t11-CLA mediated activation of SREBP-1c

The assumed activation of liver X receptor by t9,t11-CLA has been investigated with RT-PCR analysis of LXRα and its target genes in t9,t11-CLA treated macrophages. The initially analyzed genes FASN, LDLR and SCD are direct target genes of the transcription factors SREBP-1c and LXR and thus genes were analyzed that are induced by LXR but not by SREBP-1c [97-99]. Transcriptional analysis of candidate genes in t9,t11-CLA treated macrophages clearly demonstrated that the CLA isomer induces LXRα and its target genes.

The suggested LXR dependent activation of SREBP-1c has been investigated with luciferase assays. A reporter gene plasmid containing the LXR regulated promoter region of SREBP-1c could be activated by t9,t11-CLA isomer, whereas its alternative forms with mutations in the first LXR binding site could not be activated. hLXR-2mut, a alternative form with a mutation in the second LXR element, could be slightly induced by t9,t11-CLA. This finding supports the findings from Oberkoffler and collegues, that the second LXR binding element has a minor role in SREBP-1c promoter activity compared to its first LXR binding site [75].

As indicated in figure 24 two mechanisms could be estimated explaining the t9,t11-CLA mediated activation of LXRα. The CLA isomer could either be a ligand for LXR and thereby directly induce it or t9,t11-CLA could activate LXRα by a PPARγ dependent mechanism.

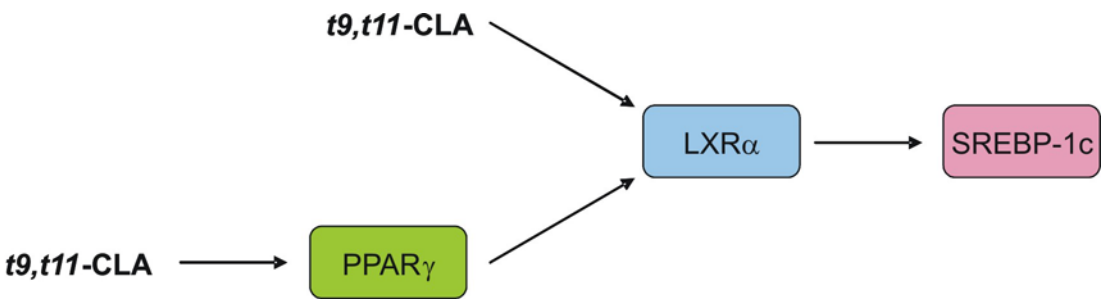

Figure 24: Two possible mechanisms for LXRα activation by t9,t11-CLA.

It is very likely that the CLA isomer activates LXR directly, since the initial isomer specific analysis of candidate genes with RT-PCR showed that t9,t11- but not c9,t11- and t10,c12-CLA induces SREBP-1c and its target genes. If the CLA isomer would activate LXRα via PPARγ, SREBP-1c and its target genes should also be induced by c9,t11-CLA and t10,c12-CLA, which are PPAR ligands. In fact the two major isomers should induce SREBP-1c and its target genes a lot stronger than t9,t11-CLA, because it has been demonstrated that these CLA isomers are ligands for PPARα with a rank order of c9,t11-CLA>t10,c12-CLA>t9,t11-CLA in rat heptoma cells. C9,t11-CLA has even been described to be one of the most avid fatty acids ever described as a PPARα ligand [100]. The hypothesis that the CLA isomer activates LXRα directly is also supported by several studies showing that PPAR induction leads to SREBP-1c and SREBP-2 inhibition. Overexpression of PPARα and γ in human embryonic kidney 293 kidney cells inhibited LXR induced SREBP-1c promoter activity through a reduction of LXR/RXR complex formation [101]. PPARα activation in the rat heptoma cell line Fao led to an inhibited processing of the premature

SREBP-2 protein and thus to reduced levels of nuclear SREBP-2 [102]. Incubation of HepG2 and Caco-2 cells with the PPARγ agonist Troglitazone diminished nuclear SREBP-2 amounts and mRNA expression of SREBP-2 target genes [103]. Since in our experiments t9,t11-CLA treatment led to an increase of SREBP-1c in promoter activity, transcription and protein levels, and to an upregulation of SREBP-2 target genes it is implausible that t9,t11-CLA activates LXRα via PPARγ.

5.4 Effects of CLA isomers on lipid metabolism

The known biological effects of CLA isomers on lipid metabolism are controversial. Most studies are investigating molecular and physiological effects of the major dietary forms of conjugated linoleic acid, c9,t11-CLA and t10,c12-CLA. Our study compares the effects of these two major CLA isomers and the minor isomer t9,t11-CLA on macrophage gene expression. T9,t11-CLA was found to activate LXRα and SREBP-1c transcription.

Granlund and colleges reported that treatment of adipocytes with t10,c12-CLA results in reduced expression of LXRα and SREBP-1c [104]. Surprisingly feeding obese mice with a t10,c12-CLA enriched diet did not affect LXRα expression, whereas c9,t11-CLA fed mice had a markedly reduced LXRα, SREBP-1c and ABCA1 expression [105].

Interstingly, several studies have shown that c9,t11-CLA inhibits atherosclerosis development and progression. A c9,t11-CLA enriched diet led to significantly reduced plasma cholesterol and free fatty acid concentrations, but increased ApoAI concentrations in ApoE deficient mice [106]. Valeille et al found that

hyperlipidemic hamsters fed with a c9,t11-CLA rich oil have increased LXR dependent ABCA1 expression in the aorta and a reduced atherogenic outcome [107]. Moreover, supplementation with natural concentrations of c9,t11-CLA from milk fat led to higher plasma HDL cholesterol concentrations and increased ABCA1 gene expression resulting in increased reverse cholesterol transport in the hyperlipidemic hamster model [108]. However, neither c9,t11-CLA, nor t10,c12-CLA had effects on ABCA1 transcription and cholesterol efflux to ApoA1 in THP-1 derived foam cells [109]. Although the findings are partly controversal, the overall physiological effect of c9,t11-CLA can be considered as anti-atherosclerotic.

In contrast to c9,t11-CLA, t9,t11-CLA activates the transcription factors LXRα and SREBP-1c and thereby leads to enhanced ABCA1 and ABCG1 mediated cholesterol efflux in human macrophages. To estimate if the net effect of t9,t11-CLA on lipid metabolism is beneficial, several different aspects of the induced network controlling intracellular cholesterol homeostasis must be discussed. These are summarized in figure 25.

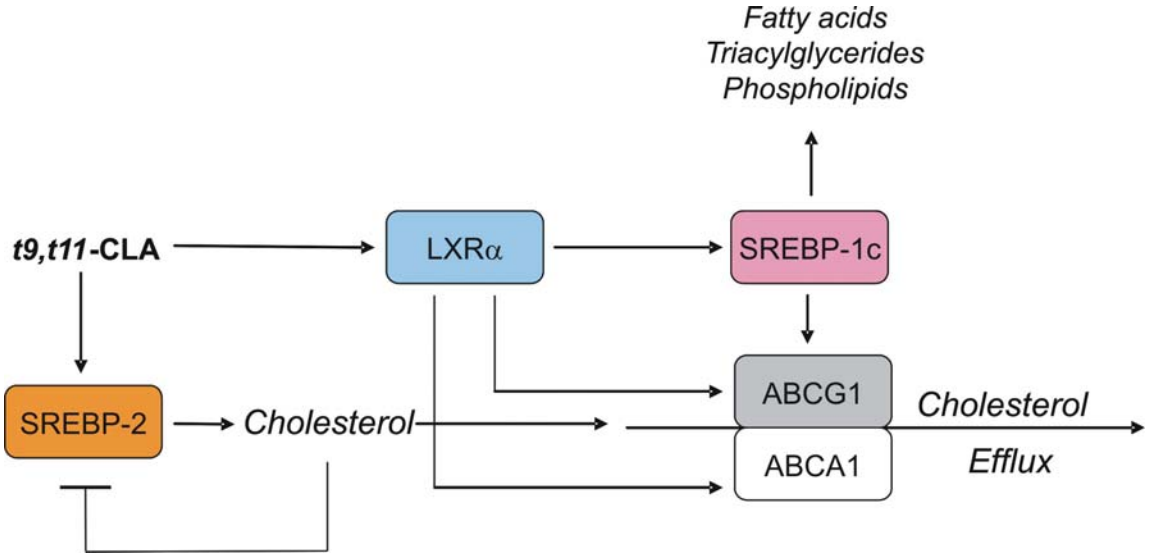

Figure 25: T9,t11-CLA activated cascade and metabolic consequences.

As a response to t9,t11-CLA treatment, transcription of LXR, SREBP-1c, its target genes and SREBP-2 target genes are activated. LXR stimulates ABCA1 directly and ABCG1 directly or via SREBP-1c and thereby promotes cholesterol efflux. SREBP-1c also enhances transcription of fatty biosynthesis genes, such as FASN, SCD and GPAT, which possibly lead to increased synthesis of fatty acids, triacylglycerides and phospholipids. Phospholipids are useful for formation of acceptor particles for ABCG1 mediated cholesterol efflux (HDL). SREBP-2 controlled genes, such as HMGCS and HMGCR, might lead to biosynthesis of cholesterol, which can be effluxed via ABCA1 and ABCG1 and when generated in excess inhibit SREBP-2 processing and thereby its own synthesis.

Interestingly several studies have demonstrated that synthetic LXR agonists, have beneficial health effects and inhibit atherosclerosis development and progression in mice [74; 110; 111]. Despite the undesirable effects of LXR

mediated SREBP-1c induction (raised triacylglyceride levels) the net effect of LXR activation was found to be anti-atherogenic. Notably, the LXR agonist GW3965 was shown to decrease lesion area approximately 50% in both ApoE and LDLR knockout mice [74]. For this reason the overall effect of t9,t11-CLA could be considered as beneficial. However, to confirm these supposed beneficial effects, the levels of cholesterol and triacylglycerides after t9,t11-CLA treatment of macrophages could be determined in a follow up of our study.

6 Conclusion

Conjugated linoleic acid (CLA) isomers are dietary fatty acids that modulate gene expression in many cell types. The aim of this work was to examine the function of the single c9,t11-, t10,c12- and t9,t11 CLA isomers on gene expression in human macrophages. Therefore, *in vitro* MCSF differentiated monocyte derived macrophages from three healthy donors and THP-1 macrophages were incubated with these CLA-isomers and whole genome transcripts were analyzed with DNA-microarrays and realtime RT-PCR . T9,t11-CLA, but not c9,t11- and t10,c12-CLA activated target genes of SREBP, SREBP-1c and ABCG1. This led to the hypothesis that ABCG1 is activated via SREBP-1c. Gene reporter assays with deletion constructs of the ABCG1 regulatory region and cotransfections with a SREBP-1c expression plasmid in RAW 264.7 macrophages confirmed that t9,t11-CLA activates ABCG1 via SREBP-1c. EMSAs showed that SREBP-1c binds to a SREBP responsive element in the promoter of ABCG1.

To answer the question by which mechanism this CLA isomer induces SREBP-1c, transcription of liver X receptor (LXR) α, which is a major regulator of SREBP-1c, and its target genes was analyzed in t9,t11-CLA stimulated macrophages with RT-PCR. A significant induction of LXRα and its target genes was found. Gene reporter assays with a plasmid containing the LXR regulatory region of the SREBP-1c promoter confirmed that SREBP-1c is activated via LXRα by t9,t11-CLA..

To determine the concentration range of t9,t11-CLA, human macrophages were treated with various doses of CLA and mRNA expression of target genes was analyzed. Incubation of human macrophages with 5 and 10µM t9,t11-CLA, which can be considered as physiological levels, led to a significant modulation of LXRα, SREBP-1c, ABCG1 and ABCA1. Finally the effects of 10µM t9,t11-CLA on ABCA1 and ABCG1 expression were found to parallel with the functional response of cholesterol efflux in human macrophages.

7 References

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8 Publications

J. Ecker, T. Langmann, C. Moehle, G. Schmitz, Isomer specific effects of Conjugated Linoleic Acid on macrophage ABCG1 transcription by a SREBP-1c dependent mechanism BBRC 352(3), (2007) 805-811

J. Ecker, H. Oberkoffler, T. Langmann, G. Liebisch, K. Leidl, W. Patsch, G. Schmitz, Trans-9,trans-11-conjugated linoleic acid activates SREBP-1c via liver X receptor and enhances cholesterol efflux in human macrophages Journal of Lipid Research, in revision

S. Heimerl, A.K. Bosserhoff, T. Langmann, J. Ecker, G. Schmitz, Mapping ATP-binding cassette transporter gene expression profiles in melanocytes and melanoma cells Melanoma Research 17(5), (2007) 265-273

9 Figures

Figure 12: LXR and its target genes in reverse cholesterol transport, from [57].

Figure 13: Experimental strategy for mRNA expression analysis of CLA treated primary macrophages from three healthy volunteers. MCSF, macrophage colony stimulating factor.

Figure 14: T9,t11-CLA induced protein expression of SREBP-1c and ABCG1. THP-1 macrophages were treated with 100µM CLA or EtOH as control for 24h.

Figure 15: T9,t11-, c9,t11, t10,c12-CLA induced mRNA expression of hABCG1-a in THP-1 cells and primary human monocyte derived macrophages from three probands. Cells were treated 100µM for 24h with the appropriate substance and ethanol as control. Gene expression was monitored using TaqMan^{TM} RT-PCR standardized to 18S rRNA as reference. $p<0.05$(*).

Figure 16: Correlated luciferase activity (X-fold; EtOH-Control=1) of RAW 264.7 cells transiently transfected with the indicated gene reporter reporter plasmids. Cells were treated with the appropriate concentration of t9,t11-CLA or EtOH as control for 36h.

Figure 17: Correlated luciferase activity of RAW 264.7 macrophages transiently transfected with a -957/+50bp-hABCG1-a luciferase reporter plasmid and expression plasmids for SREBP-1a or SREBP-1c. Cells were cotransfected for 36h. $p<0.01$(**).

Figure 18: Correlated luciferase activity (X-fold; Control=1) of RAW 264.7 macrophages transiently transfected with pTK-3xSRE and cotransfected with the indicated amount of pCMV-SREBP-1c. $p<0.05$(*); $p<0.01$(**).

10 Tables

Table 1: Genes, whose transcripts were increased in 3 probands after stimulation with 100µM t9,t11-CLA. for 24h. The expression signals are given for the untreated samples. The fold change (FC) of gene expression between samples and controls is indicated.

Table 2: t9,t11-, c9,t11-, t10,c12-CLA induced mRNA expression in THP-1 cells and primary human macrophages from three healthy donors. Cells were treated 100µM for 24h with the appropriate substance and EtOH as control. Gene expression was monitored using TaqMan™ RT-PCR standardized to 18S rRNA as reference. The fold change (FC) in THP-1 cells and the mean FC in primary human macrophages of gene expression between samples and controls is indicated.

Table 3: SRE binding sites of selected SREBP responsive genes and putative SRE sequence of ABCG1.

Table 4: T9,t11-CLA induced mRNA expression in human macrophages. Cells were treated 100µM t9,t11-CLA and ethanol as control for 24h. Gene expression was monitored using TaqManTM RT-PCR standardized to 18S rRNA as reference. FC, fold change.

11 Abbreviations

AACS	Acetoacetyl-CoA synthetase
ABC	ATP-binding cassette
Apo	Apolipoprotein
c9,t11	cis-9,trans-11
CETP	Cholesterol ester transfer protein
CLA	Conjugated linoleic acid
Cyp7a1	Cholesterol 7-α-hydroxylase
DHCR7	7-Dehydrochol. reductase
DR	Direct repeat
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmatic Reticulum
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
FASN	Fatty acid synthase
FC	Fold change
FDFT1	FDF-transferase 1
FDPS	FDF-synthase
h	human
HDL	High density lipoprotein
hHLH-LZ	basic helix loop helix leucine zipper
HMGCR	HMG-CoA reductase
HMGCS	HMG-CoA synthase 1
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
INSIG1	Insulin induced gene 1
LDLR	LDL receptor
LPL	Lipoprotein lipase
Luc	Luciferase
LXR	Liver X receptor
LXR-E	LXR binding element
MCSF	Macrophage colony stimulating factor
MIDIP1	MID1 interacting protein 1
n	nuclear
NFκB	Nuclear factor κ B
p	premature
PLTP	Phospholipid transfer protein
Pro	Proline
RXR	Retinoid X receptor
S1P	Site 1 protease
S2P	Site 2 protease
SAM	Significance analysis of microarrays
SCAP	SREBP cleavage activating protein
SCD	Stearoyl-CoA desaturase
SD	Standard deviation
Ser	Serine
SRE	SREBP response element
SREBP	Sterol regulatory element binding protein

t10,c12	trans-10,cis-12
t9,t11	trans-9,trans-11
TA	Trans activation
TGF	Transforming growth factor
TK	Thymidine kinase
VEGF	Vascular endothelial growth factor
ZNF	Zinc finger preotein

12 Eidesstattliche Erklärung

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