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REGULATION OF LIPOPROTEIN(a) BY INTERLEUKIN-6 IN HUMANS

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ZUSAMMENFASSUNG

Kardiovaskuläre Erkrankungen sind immer noch die führende Todesursache bei Frauen und Männern in der westlichen Welt. Es gibt verschiedene Faktoren wie viszerale Adipositas, Hypertonie, Insulinresistenz und atherogene Dyslipidämie, welche zum Risiko des kardiovaskulären Todes substantiell beitragen. *Low density lipoprotein* (LDL) Moleküle initiieren die frühe Atherogenese. Eines der atherogensten Lipoproteine ist Lipoprotein(a) [Lp(a)], das aus einem LDL-ähnlichem Partikel und dem Apolipoprotein(a) [Apo(a)] besteht. Zirkulierende Lp(a) Serumspiegel jedoch sind bemerkenswerterweise gegen gewöhnliche lipidsenkende Therapien eher resistent. Eine Ausnahme ist Niazin, welches erhöhte Lp(a) Serumspiegel effektiv senken kann. Aufgrund von schweren Nebenwirkungen wurde Niazin allerdings in Europa bereits im letzten Jahr vom Markt genommen. Somit sind derzeit neben der sehr kostenintensiven, aufwändigen und zudem noch invasiven Lipid-Aphärese keine effektiven Therapiemöglichkeiten für die Reduktion von Lp(a) verfügbar. Aus diesem Grund werden alternative gut verträgliche und geeignete Therapieansätze benötigt, um erhöhte Lp(a) Serumspiegel zu senken. In einer jüngsten klinischen Studie wurde gezeigt, dass die Inhibition des Interleukin-6 (IL-6) Signalweges durch Tocilizumab (TCZ) die Lp(a) Serumspiegel in Patienten mit rheumatoider Arthritis (RA) bis zu 50% erniedrigt. Folglich könnte man davon ausgehen, dass TCZ als neuer, erfolgversprechender Ansatz für die Behandlung von erhöhtem Lp(a) betrachtet werden kann. Um auf Kausalität zu prüfen und mögliche Störfaktoren innerhalb der klinischen Studie zu exkludieren, intendierte diese Arbeit, die Apo(a)-induzierende Fähigkeit von IL-6 und die Apo(a)-inhibierende Fähigkeit von TCZ auf molekularer Ebene im humanen Model *in vivo* und *in vitro* weiter zu untersuchen. Um diese Fragestellung zu bearbeiten, wurden molekulare, zelluläre und physiologische Experimente sowie auch Assoziationsstudien kombiniert, um regulatorische Determinanten von Lp(a) im Menschen zu analysieren.

In der vorliegenden Arbeit wurde gezeigt, dass der Lp(a)-reduzierende Effekt durch TCZ spezifisch für IL-6 ist, da eine anti-TNF- α Therapie die Lp(a) Serumspiegel in RA Patienten nicht veränderte. Weiterhin wurden signifikante Assoziationen zwischen Determinanten wie IL-6, dem C-reaktivem Protein (CRP), dem Body Mass Index sowie auch dem Insulinresistenz-Index (HOMA) und Lp(a) in 1153 Probanden gefunden. Unter diesen waren IL-6 und Akut-Phase-Protein CRP am stärksten mit Lp(a) assoziiert. Mechanistische Zellkulturstudien zu IL-6 und Apo(a) bestätigten, dass IL-6 Apo(a) spezifisch induziert, und dass TCZ die IL-6-induzierte Apo(a) Expression auf Promoter, Transkript und Proteinebene in kultivierten Hepatozyten aufhebt. Da bekannt ist, dass der Promoter des Apo(a)-Gens sechs IL-6 responsive Elemente (IL-6-RE) besitzt, wurde der Apo(a) Promoter trunkiert, um explizit zu untersuchen, welche/s der sechs IL-6-RE für die Apo(a) Transkription verantwortlich ist/sind. Hierbei ergab sich, dass hauptsächlich IL-6-RE 6 die Apo(a) Transkription reguliert. Die IL-6-abhängige Bindung des Transkriptionsfaktors STAT3 an IL-6-RE 6 wurde ebenfalls in einem nachfolgenden EMSA Experiment verifiziert. Zusätzlich wurde demonstriert, dass nicht nur der Apo(a), sondern auch der LDL Metabolismus durch IL-6 und TCZ beeinflusst wird.

Zusammenfassend lässt sich sagen, dass diese Arbeit zahlreiche Beweise liefert, dass (1) IL-6 spezifisch Apo(a) und letztlich Lp(a) auf verschiedenen molekularen Ebenen *in vivo* und *in vitro* induziert und dass dadurch (2) eine IL-6 Rezeptor Blockierung durch TCZ eine vielversprechende zukünftige Therapieoption sein kann, um erhöhte Lp(a) Serumspiegel zu behandeln und um das kardiovaskuläre Risiko in betroffenen Patienten zu reduzieren. TCZ würde daraus folgend eine geeignete Alternative zur aufwendigen Lipid-Aphärese darstellen.

SUMMARY

Cardiovascular disease is still the leading cause of death in the western world in men and women. There are several factors such as visceral obesity, hypertension, insulin resistance and atherogenic dyslipidemia which substantially contribute to a risk of cardiovascular death. Low-density lipoprotein (LDL) molecules initiate early atherogenesis. One of the most atherogenic lipoproteins is lipoprotein(a) [Lp(a)], which consists of a LDL-like particle and apolipoprotein(a) [apo(a)]. However, circulating levels of Lp(a) are remarkably resistant to common lipid lowering therapies. One exception to effectively lower Lp(a) levels is niacin. However, due to severe side effects, niacin was recently withdrawn from the market in Europe. Thus, there are currently no effective treatments available for the reduction of Lp(a) apart from lipid aphaeresis which is costly and labor-intensive. For this reason, alternative well-tolerated and more convenient therapeutic approaches are required to decrease elevated Lp(a) serum levels. In a recent clinical study it was shown that inhibition of interleukin-6 (IL-6) signaling by tocilizumab (TCZ) lowers Lp(a) serum levels in rheumatoid arthritis (RA) patients by up to 50 %. Therefore, one may consider TCZ as a new promising approach for lowering Lp(a). In order to prove for causality and to exclude possible confounders within the clinical study this thesis aims to further examine the ability of IL-6 to induce hepatic apo(a) expression and the ability of TCZ to inhibit apo(a) on a molecular level in humans *in vitro* and *in vivo*. To address this question, molecular, cellular and physiological experiments as well as human association studies were combined to analyze regulatory determinants of Lp(a) in humans.

In the present thesis it was shown that the Lp(a) lowering effect of TCZ is specific to IL-6 signaling as anti-TNF- α therapy did not alter Lp(a) serum levels in RA patients. Moreover, significant associations between determinants such as IL-6, C-reactive protein (CRP), Body Mass

Index as well as homeostasis model assessment-estimated insulin resistance and Lp(a) in n=1153 human subjects were found. Amongst those, IL-6 and IL-6 response reactant CRP were strongest associated with Lp(a). Mechanistic cell culture studies on IL-6 and apo(a) revealed that IL-6 specifically induced apo(a) and that TCZ inhibited the IL-6-induced apo(a) expression on promoter, transcript and protein level in cultured hepatocytes. As it is known that the apo(a) promoter contains six IL-6 responsive elements (RE), the apo(a) promoter was truncated to explicitly investigate which IL-6 RE(s) is/are responsible for the apo(a) transcription. This analysis suggested that solely IL-6 RE 6 confers major apo(a) transcription. IL-6-dependent binding of transcription factor STAT3 to IL-6 RE was verified in subsequent EMSA experiments. In addition, not only apo(a) but also LDL metabolism were demonstrated to be affected by IL-6 and TCZ.

In conclusion, the present thesis provides several lines of evidence that (1) IL-6 specifically induces apo(a) and eventually Lp(a) on different molecular levels *in vivo* and *in vitro* and that therefore (2) IL-6 blockade by TCZ might be a promising future therapy to treat elevated Lp(a) serum levels and to reduce CVD risk in affected patients. Hence, TCZ might be a convenient alternative to the costly, labor-intensive and invasive lipid aphaeresis.

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LIST OF ABBREVIATIONS

°C	degree celsius
ADB	adalimumab
AHA	American Heart Association
ANOVA	Analysis Of Variance
apo(a)	apolipoprotein(a)
apoB	apolipoprotein B
BMI	Body Mass Index
Bp	base pairs
BSA	bovine serum albumin
CCL	chemokine (C-C motif) ligand
CCP	cyclic citrullinated peptide
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CID	chronic inflammatory diseases
CIP	Alkaline Phosphatase, Calf Intestinal
CO ₂	carbon dioxide
CRP	C-reactive protein
ctrl	control
CVD	cardiovascular diseases
DMARD	disease-modifying antirheumatic drug
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP's	deoxynucleotide triphosphates

DTT	DL-Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
FAS	fatty acid synthase
FCS	fetal calf serum
FFA	free fatty acids
HDL	high density lipoprotein cholesterol
HEPES	4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid
HOMA-IR	Homeostasis model assessment-estimated insulin resistance
HRP	horseradish peroxidase
hrs	hours
HSP	heat shock protein
IDF	International Diabetes Federation
IFN- γ	interferon gamma
IgG	immunoglobulin G
IL-6 RE	interleukin-6 responsive elements
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
JAK	Janus kinase
LB	Luria Bertani
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
Lp(a)	Lipoprotein(a)
MCP	monocyte chemoattractant protein

MCS	multiple cloning site
M-CSF	macrophage-colony-stimulating factor
MetS	metabolic syndrome
mRNA	messenger RNA
NAFLD	non-alcoholic fatty liver disease
NCEP/ATPIII	Third Report of the National Cholesterol Education Program's Adult Treatment Panel
NHANES	National Health and Nutrition Examination Survey
ns	not significant
oxLDL	oxidized LDL
P/S	penicillin/streptomycin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
RA	rheumatoid arthritis
RIPA	RadiolImmunoPrecipitation Assay
RLU	relative light units
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
r_s	Spearman's rank correlation coefficient
RT	room temperature
ScR	scavenger receptor
SEM	standard error of the mean
SMC	smooth muscle cells
STAT3	Signal transducer and activator of transcription 3

T2DM	type 2 diabetes mellitus
TCZ	tocilizumab
TEMED	Tetramethylethylenediamine
TG	triglycerides
Th1/2	T helper-1/2
TLR	toll-like receptor
TNF- α	tumor necrosis factor alpha
Treg	regulatory T cells
VCAM	vascular cell adhesion molecule
VLDL	very low density lipoprotein
WHO	World Health Organization

1. INTRODUCTION

Cardiovascular disease (CVD) is still the leading cause of death in the western world in men and women, despite recent progress regarding its treatment. It is accounting for one of three deaths in the United States today [1]. In the mid-20th century, the Framingham Heart Study revealed the epidemiology of atherosclerotic CVD and already identified smoking, obesity, glucose intolerance, high cholesterol levels and many more factors as substantially contributing to a risk of cardiovascular death [2].

All these factors, except for smoking, also represent the metabolic syndrome (MetS) (also referred to as syndrome X) and will be demonstrated in more detail in paragraph 1.1.

1.1 Metabolic Syndrome

1.1.1 Definition

The MetS is a complex disorder with high socioeconomic cost that is considered a worldwide epidemic. This is mostly due a modern lifestyle, namely the hypercaloric diet and the physical inactivity. MetS is defined by a combination of cardiovascular risk determinants, including obesity, insulin resistance, hypertension, and lipid abnormalities for example elevated triglycerides (TG) and apolipoprotein B (apoB) -containing lipoproteins, increased free fatty acids (FFA), and low high-density lipoprotein (HDL) cholesterol levels. The risk of developing CVD in that population is approximately doubled [3].

In 1988, Reaven first introduced the concept that insulin resistance clusters with glucose intolerance, dyslipidemia, and hypertension to increase cardiovascular risk [4]. In the 1990s, the so far called 'insulin resistance syndrome' changed into the clinical metabolic syndrome that has now taken hold in the medical literature. It has been defined and institutionalized, principally by the World Health Organization (WHO) [5], the Third Report of the National Cholesterol Education Program's Adult Treatment Panel (NCEP/ATP III) and the International Diabetes Federation (IDF) [3, 6-8]. As depicted in **table 1**, the criteria vary to some degree, but all definitions identify a population with increased risk for developing type 2 diabetes mellitus (T2DM) and CVD [5, 9, 10].

Table 1: Common definitions of the MetS

IDF (2005)	NCEP/ATP-III (2001-2005)	WHO (1998)
<ul style="list-style-type: none"> • Waist circumference ≥ 102 cm (men), ≥ 88 cm (women) • Plus 2 of: <ul style="list-style-type: none"> ↪ Blood pressure $\geq 130/85$ mmHg or treatment ↪ Triglycerides ≥ 150 mg/dL or treatment ↪ HDL < 40 mg/dL (men), < 50 mg/dL (women) ↪ Fasting glucose ≥ 100 mg/dL or treatment 	<p>At least 3 of the following:</p> <ul style="list-style-type: none"> • Waist circumference ≥ 102 cm (men), ≥ 88 cm (women) • Blood pressure $\geq 130/85$ mmHg or treatment • Triglycerides ≥ 150 mg/dL or treatment • HDL < 40 mg/dL (men), < 50 mg/dL (women) • Glucose ≥ 110 mg/dL or treatment 	<ul style="list-style-type: none"> • Insulin resistance or T2DM or impaired glucose tolerance or impaired fasting glucose • Plus 2 of: <ul style="list-style-type: none"> ↪ Body mass index ≥ 30 kg/m² or waist-to-hip-ratio > 0.9 (men) / > 0.85 (women) ↪ Blood pressure $\geq 140/90$ mmHg or treatment ↪ Triglycerides ≥ 150 mg/dL or treatment ↪ HDL < 35 mg/dL (men), < 39 mg/dL (women) ↪ Microalbuminuria

1.1.2 Epidemiology

The prevalence of the MetS varies and is dependent on the criteria in the different definitions of the MetS described above, as well as the composition (sex, age, race, ethnicity) of the population to be examined [11]. However, independent of the criteria, the prevalence of MetS is high and increasing in the western world, most likely due to the obesity epidemic [12-14]. For example, according to the National Health and Nutrition Examination Survey (NHANES) 2003-2006 [15], 34.0 % (age-adjusted) of the individuals having been studied met the NCEP/ATP III revised (2005) criteria for MetS. Males and females, 40-59 years of age, were about three times as likely as those 20-39 years of age to meet the criteria for MetS. Within the male and female groups with 60 years of age and over, the individuals were even four to six times as likely to meet the criteria suggesting that the prevalence of MetS increases with age.

1.1.3 Pathophysiology

MetS is the consequence of a complex interplay between genetic and environmental factors. The following paragraph exclusively focuses on the environmental determinants.

Several lines of evidence implicate visceral adiposity as a key component of the MetS. Visceral fat causes a decrease in insulin-mediated glucose uptake, thus is clearly related to insulin resistance. The mechanisms for that probably involve adipokines being produced by adipose tissue, that modulate the crosstalk between metabolism and vascular function [16]. These include tumor necrosis factor- α (TNF- α) [17, 18], interleukin-6 (IL-6) [19-21] and eventually C-reactive protein (CRP) [22, 23] as well as resistin [24-26] and leptin [27], which are pro-inflammatory and contribute to insulin resistance and vascular dysfunction. The renin-angiotensin system is also activated in adipose tissue and leads to hypertension and insulin

resistance [28]. In contrast, adiponectin is a protective adipokine that links insulin sensitivity with energy metabolism. Furthermore, adiponectin levels are decreased in obesity, T2DM and MetS [29] (**figure 1**).

In addition to these adipokines, FFAs, which are excessively released from visceral fat due to an imbalance between energy uptake and expenditure as well as impaired insulin signaling, are also closely related to the development of insulin resistance. In the liver, FFAs serve as substrate for the synthesis of TG. FFAs also stabilize the production of apoB, the main lipoprotein of very-low-density lipoprotein (VLDL) particles, resulting in more VLDL production. Moreover, the activity of lipoprotein lipase, the regulating factor of VLDL clearance, is decreased. Thus, hypertriglyceridemia in insulin resistance is the result of both an increase in VLDL production and a decrease in VLDL clearance. VLDL is metabolized to remnant lipoproteins and small dense LDL, both of which can promote atheroma formation due to a better susceptibility to be oxidized to oxLDL. The TGs in VLDL are transferred to HDL by the cholesterol ester transport protein in exchange for cholesteryl esters, resulting in TG-enriched HDL and cholesteryl ester-enriched VLDL particles. The TG-enriched HDL is a better substrate for hepatic lipase, so it is cleared rapidly from the circulation, leaving fewer HDL particles to participate in reverse cholesterol transport from the vasculature. All these lipid abnormalities form an atherogenic dyslipidemia triad of hypertriglyceridemia, low HDL levels, and small dense LDL particles [30].

In conclusion, visceral adipose tissue is, due to its high metabolic and inflammatory activity, the central player in MetS, which results in conditions such as hypertension, insulin resistance and finally atherogenic dyslipidemia, all leading to an increased risk to develop atherosclerosis [31, 32] (**figure 1**).

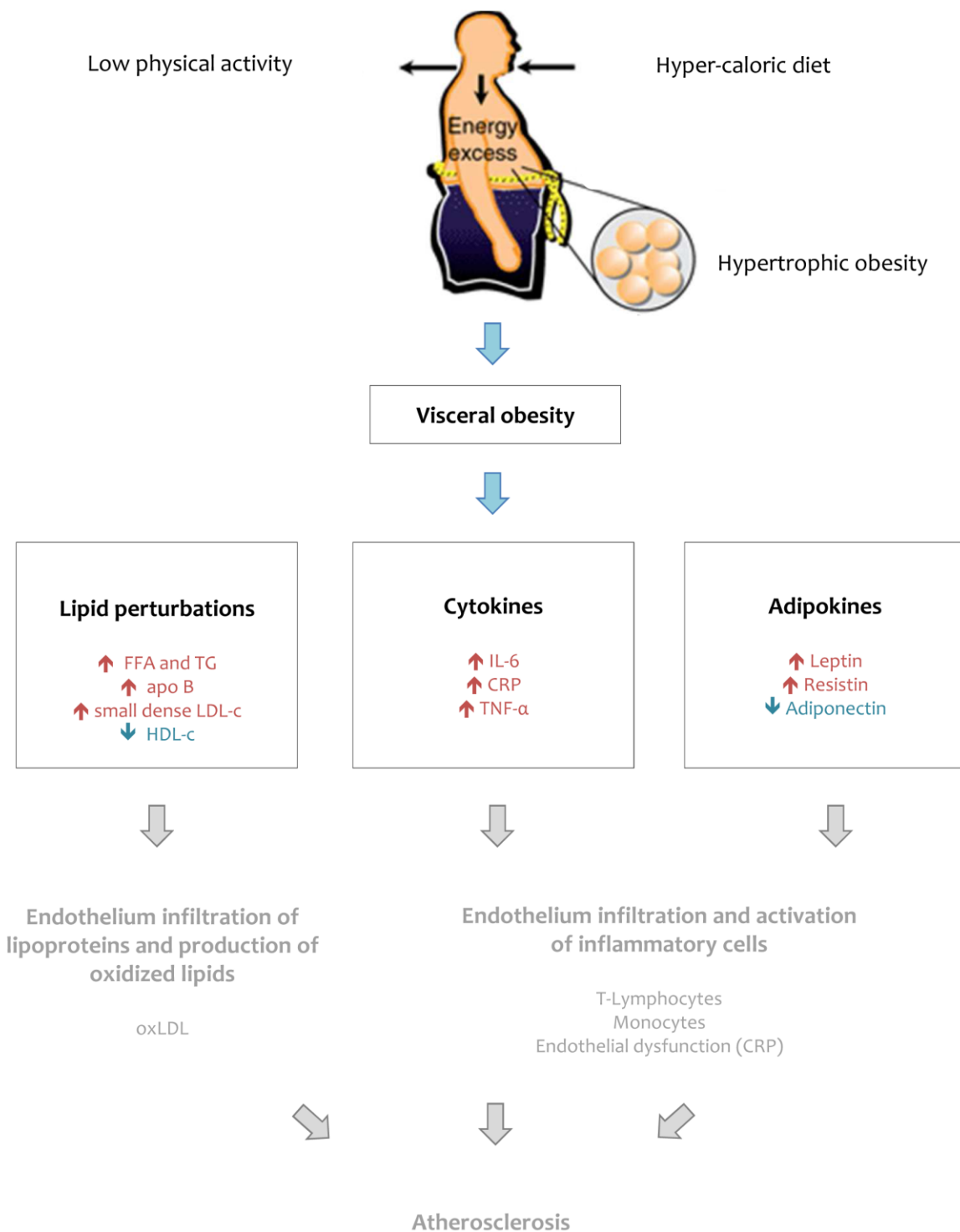


Figure 1: Pathophysiology of the MetS (modified according to [32]). Visceral obesity is the central player in the MetS resulting in lipid perturbations and an altered cytokine as well as altered adipokine secretion profile. All these conditions finally lead to the development of atherogenic dyslipidemia and endothelial dysfunction, the major components in atherogenesis. Abbreviations: FFA, free fatty acids; TG, triglycerides; apoB, apolipoprotein B, LDL-c, low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; IL-6, interleukin-6; CRP, C-reactive protein; TNF- α , Tumor necrosis factor- α ; oxLDL, oxidized LDL.

1.2 Atherosclerosis

Atherosclerosis is the major cause of CVD such as myocardial infarction, stroke, and ischemic gangrene. This group of diseases represents the main cause of death in the western world today. In addition, due to the increasing prevalence of CVD in developing countries and Eastern Europe and an accumulation of metabolic risk factors, including obesity and diabetes in the western world, the WHO expects CVD to become the major killer even globally. These factors stress the need for intensified research in combination with increased preventive approaches against atherosclerotic diseases.

1.2.1 Initiation and early atherogenesis

Hypercholesterolemia and hypertension, two major criteria of the MetS and concomitantly risk factors for atherosclerosis cause endothelial activation in large- and medium-sized arteries. The atherosclerotic process is initiated when cholesterol-rich VLDL and LDL accumulate in the intima [33, 34]. Afterwards bioactive phospholipids released by modified (mostly by oxidation) LDL particles activate the endothelial cells [35] (**figure 2, left panel**). In the activated state, they express several types of leukocyte adhesion molecules (i.e. vascular cell adhesion molecule (VCAM-1)), which cause blood cells such as monocytes and lymphocytes to approach the vascular surface in order to adhere to the site of activation [36, 37]. Once adherent to the endothelium, leukocytes migrate into the underlying intima in response to chemoattractant stimuli including chemokines, e.g. monocyte chemoattractant protein-1 (MCP-1 or also referred to as chemokine (C-C motif) ligand 2 (CCL2)) [38, 39] (**figure 2, right panel**). There is even evidence that gene targeting of MCP-1 can inhibit disease development [38, 39].

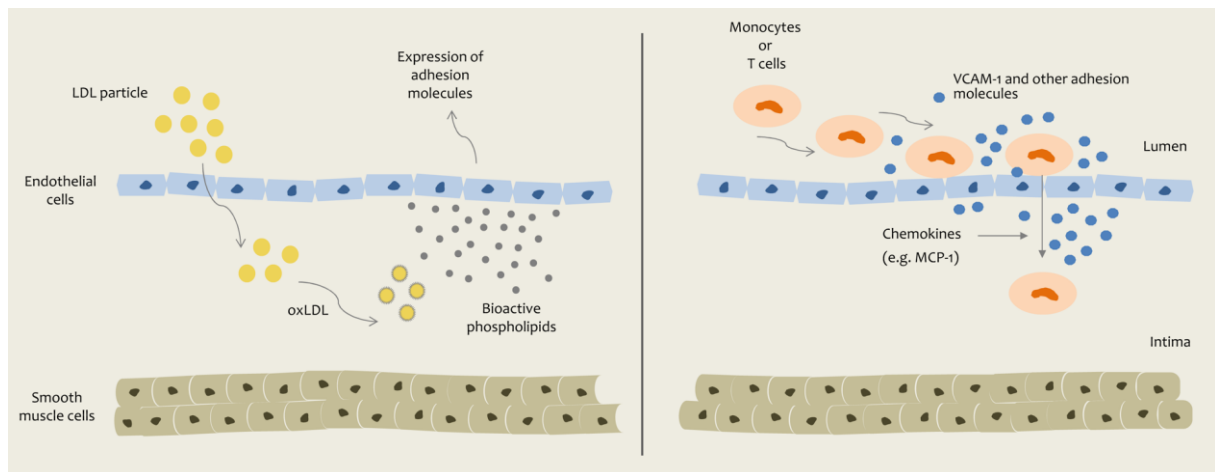


Figure 2: Initiation of atherosclerosis. LDL particles penetrate the endothelium and are retained in the intima where it undergoes oxidative modification. Bioactive phospholipids released from LDL stimulate endothelial cells to express adhesion molecules such as VCAM-1 (**left panel**). In consequence, circulating monocytes and/ or T cells adhere to the vascular surface and migrate into the intima in response to chemokines (e.g. MCP-1) (**right panel**). Abbreviations: LDL, low-density lipoprotein; oxLDL, oxidized LDL; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1.

1.2.2 Advanced plaque progression

The macrophage-colony-stimulating factor (M-CSF) induces the entering of monocytes and their differentiation into macrophages. Macrophage differentiation is necessary for atherosclerosis [40, 41], and up-regulates pattern recognition receptors, including scavenger receptors (ScRs) and toll-like receptors (TLRs) [41]. ScRs amongst others mediate oxLDL internalization by macrophages. If cholesterol derived from uptake of oxLDL cannot be sufficiently eliminated from the cell, it accumulates as cytosolic droplets. This is caused by pro-inflammatory cytokines and endotoxins which inhibit the expression of ATP-binding cassette transporters ABCA1 and ABCG1 that allow the export of cholesterol from the cell [42, 43]. As a consequence, the macrophage forms a foam cell, the prototypic cell of atherosclerosis. Unlike to ScRs, TLRs when bound by ligands with pathogen-like molecular patterns (such as bacterial toxins, stress proteins, DNA motifs, heat shock protein 60 (HSP60), oxLDL, etc.) [44-46], initiate a signal

cascade leading not only to the activation of macrophages, but also of TLR-expressing dendritic, mast and endothelial cells [47, 48]. Pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF- α can also activate macrophages. Macrophage activation leads to the release of reactive oxygen species, cytokines, vasoactive molecules such as nitric oxide, endothelins and eicosanoids [49, 50] and proteases which degrade matrix components leading to the destabilization of plaques and an increased risk for plaque rupture and thrombosis [51-54] (**figure 3, upper panel**).

T cells, equally to monocytes, adhere to the vascular surface via cell adhesion molecules and chemokines, and then migrate into the intima. T cells in lesions recognize local peptide antigens such as HSP60, and microbial antigens. T cell activation can lead to several types of effector responses, which of them are T helper-1 (Th1), -2 and regulatory T cells. However, the Th1 response involving the secretion of pro-inflammatory cytokines is most prevalent in atheroma. This finally contributes to local inflammation and growth of the plaque. Intensified inflammatory activation may lead to local proteolysis, plaque rupture, and thrombus formation, which cause ischemia and infarction [55] (**figure 3, lower panel**).

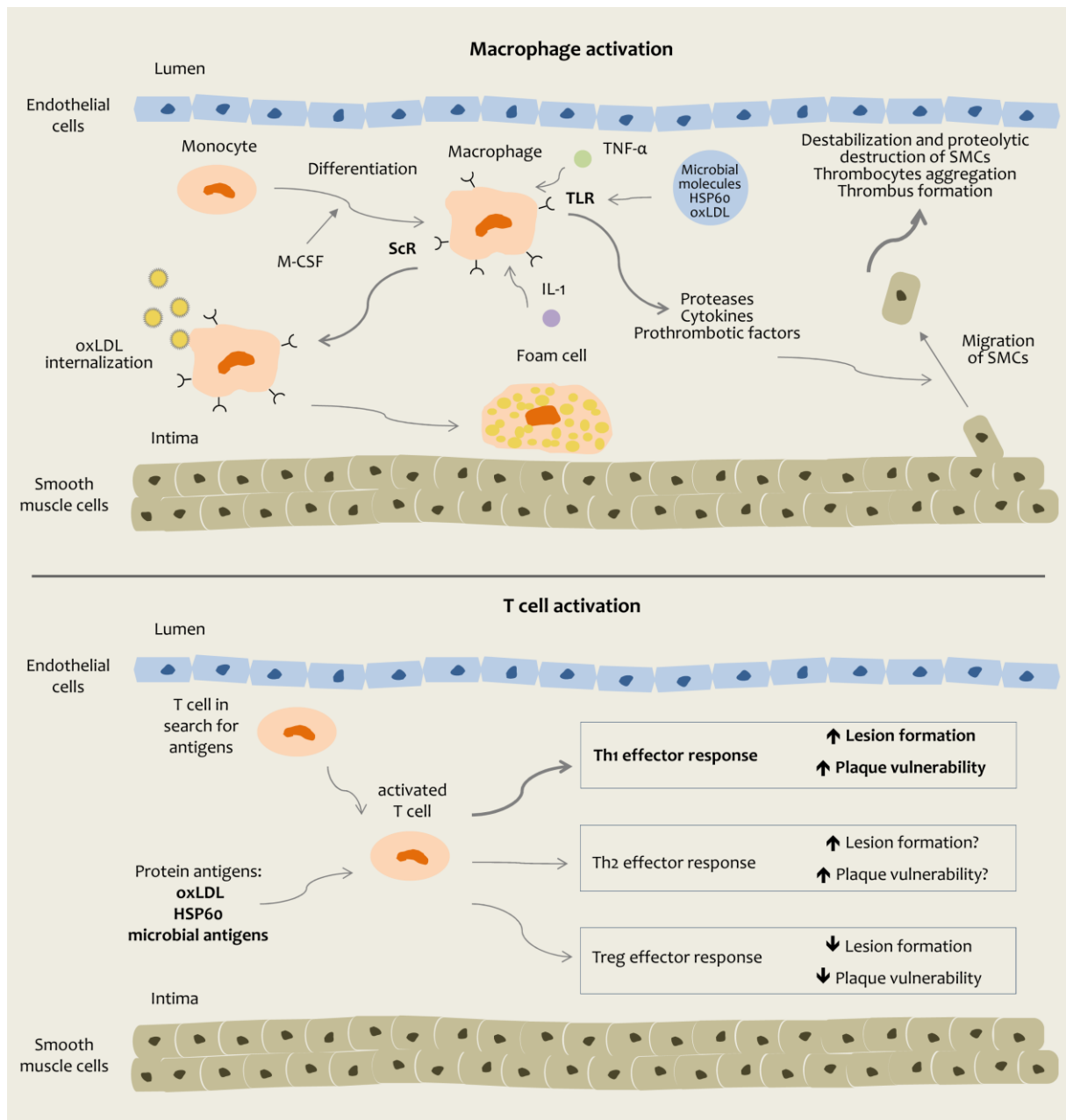


Figure 3: Macrophage activation, foam cell formation and T cell activation. Upper panel: Once the monocyte has migrated into the intima, the molecule M-CSF induces its differentiation into macrophages. During this process, ScRs and TLRs are upregulated. ScRs mediate the internalization of oxLDL by macrophages and cause the foam cell formation. TLRs ligated to microbial molecules, HSP60 or oxLDL as well as IL-1 and TNF- α can also activate these macrophages. Activated macrophages release many pro-inflammatory mediators responsible for SMC migration, thrombocytes aggregation and thrombus formation. **Lower panel:** Once the T cell has migrated into the intima, it searches for antigens. T cells respond to antigens such as oxLDL, HSP60 or microbial antigens. In consequence, T cells are activated and lead to three different effector responses, Th1, Th2 and Treg effector response. However, the Th1 response when compared with the others is the most prevalent in atheroma due to the secretion of several pro-inflammatory cytokines that promote lesion formation and plaque vulnerability. *Abbreviations: M-CSF, macrophage-colony-stimulating factor; ScR, Scavenger receptor; TLR, Toll-like receptor, oxLDL, oxidized LDL; IL-1, interleukin-1; TNF- α , tumor necrosis factor- α ; SMC, smooth muscle cells; HSP60, heat shock protein 60; Th1, T helper-1; Th2, T helper-2; Treg, regulatory T cell.*

1.3 Lipoprotein(a)

1.3.1 Biochemistry and mechanisms of atherogenicity of lipoprotein(a)

Lipoprotein(a) [Lp(a)], first discovered by Kåre Berg in 1963 [56], is a unique lipoprotein being formed through the extracellular association of an apoB-100-containing lipoprotein (e.g. LDL) to a highly glycosylated and polymorphic apolipoprotein(a) [apo(a); official gene symbol: *LPA*] secreted by hepatocytes, via a single disulfide bond [57]. The apo(a) gene further shares high homology with the gene of the plasminogen [58] being a protease involved in fibrinolysis.

Due to the atherogenic and pro-thrombotic character of Lp(a), raised levels of Lp(a) are associated with an increased incidence of a variety of CVD, combining atherosclerosis and thrombosis [59].

The mechanisms implicated in the atherogenicity of Lp(a) include self-aggregation and precipitation to the vascular wall, adhesion molecule production (VCAM and ICAM), avid binding to endothelial cells, proliferation and migration of smooth muscle cells and chemotaxis of monocytes [60-63]. Foam cell formation is also enhanced by Lp(a) [63]. Due to its unique structural homology to plasminogen, Lp(a) competes with plasminogen for binding to plasminogen receptors, fibrinogen and fibrin [64]. It also induces the production of plasminogen activator inhibitor 1 and inhibits the secretion of tissue-plasminogen activator by endothelial cells [65]. Lp(a) is further very susceptible to bind with oxidized phospholipids [65-68], which is then potentially taken up by the vessel wall and finally accelerates the development of atherosclerosis.

1.3.2 Regulation of lipoprotein(a)

Lp(a) concentrations are genetically determined by apo(a) size [69], with small isoforms at higher concentrations and large isoforms at lower concentrations [69-75]. Thus, Lp(a) concentrations vary substantially between individuals.

On the other hand, Lp(a) levels can be influenced by a limited number of physiological factors (such as estrogens [76], testosterone, growth or thyroid hormones), disease conditions (such as renal failure [77-79], or peroxisomal disorders [80]) or environmental agents (such as alcohol [81], HIV-1 protease inhibitors [82]).

1.3.3 Treatment of elevated lipoprotein(a)

Circulating levels of Lp(a) are remarkably resistant to common lipid lowering therapies [83-85]. One exception to effectively lower Lp(a) levels is niacin when given in high doses (2-3 g/day) [86, 87]. These doses of niacin, however, are lowering Lp(a) only approximately by 20-30 % and can be associated with headaches, flushing and liver toxicity and finally non-compliance. Also, due to severe side effects, a slow-release formulation of niacin (Tredaptive[®]) was recently withdrawn from the market in Europe (HPS2-THRIVE, EHJ Feb 2013). Aspirin at low doses was found to decrease Lp(a) serum levels only slightly [88] indicating rather minor importance.

Thus, there are currently no effective treatments available for the reduction of Lp(a) apart from plasma aphaeresis which is costly, labor-intensive and actually intended for patients with CVD progression and highly elevated Lp(a) levels [89, 90]. For this reason, alternative well-tolerated and more convenient therapeutic approaches are required to decrease elevated Lp(a) serum levels.

1.3.4 Chronic inflammatory disease and lipoprotein(a)

Chronic inflammatory diseases (CID) are associated with an increase in CVD risk. Interestingly, in a recent review it has been demonstrated nicely that in humans several CIDs are associated with an increase in Lp(a) levels [91]. Thus, elevated Lp(a) levels in CID patients might contribute to an increased CVD risk in these patients. Therefore, lowering Lp(a) levels might be beneficial in reducing the CVD risk not only in the general population but especially in CID patients. The finding that Lp(a) is elevated in CID leads to the suggestion that Lp(a) is a positive acute-phase reactant being triggered by IL-6 as major mediator of the acute-phase response [92]. That is consistent with six IL-6 responsive elements (IL-6 RE) present within the human apo(a) gene promoter [93, 94]. In 2009, a monoclonal antibody against the IL-6 receptor, referred to as tocilizumab (TCZ), was approved for the treatment of rheumatoid arthritis (RA) in Europe. In a recent clinical study of our group it was shown that inhibition of IL-6 signaling by TCZ indeed lowers Lp(a) serum levels in RA patients by up to 50 % [95].

1.4 Aim of the study

Due to the latter finding one may consider TCZ as a new promising approach for lowering Lp(a) levels in general and especially in CID patients. In order to prove for causality and to exclude possible confounders within the clinical study (e.g. co-medication) this thesis aims to further examine the ability of IL-6 to induce apo(a) expression and the ability of TCZ to inhibit apo(a) on a molecular level in humans *in vitro* and *in vivo*. To address this question, in the present thesis, molecular, cellular and physiological experiments as well as human association studies were combined to further analyze the relationship between IL-6, Lp(a) and TCZ in humans.

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Chemicals

Table 2: List of chemicals used in this thesis

	Product number	Manufacturer
10x DNase buffer	B0303S	New England Biolabs
2-propanol	1.09634.2500	Merck KGaA
5x Phusion® HF Reaction Buffer	F-518	Finnzymes
Acetic acid 100 % p.a.	1.00063.2500	Merck KGaA
Adalimumab (Humira®) (50 mg/mL)		Abbott Laboratories
Agarose	A9539	Sigma Aldrich
Agar-Agar	5210.3	Carl Roth
Aminocaproic acid	A2504	Sigma Aldrich
Ammoniumperoxodisulfat	9592.3	Carl Roth
Ampicillin sodium salt	A9518	Sigma Aldrich
Biotinylated Protein Ladder	7727	New England Biolabs
Boric acid	6943.1	Carl Roth
Bromophenol blue sodium salt	B8026	Sigma Aldrich
Bovine serum albumin (BSA)	01400.100	biomol
DL-Dithiothreitol (DTT)	43815	Sigma Aldrich
Dulbecco's Modified Eagle medium (DMEM)	E15-009	PAA Laboratories

DNase	M0303	New England Biolabs
ethylenediaminetetraacetic acid (EDTA)	1.08417.0250	Merck KgaA
Ethanol p.a. \geq 99.5 %	5054.1	Carl Roth
Fetal calf serum (FCS)	SO115	Biochrom
GelRed Nucleic Acid Stain	41003	Biotium
GeneRuler 1 kb Ladder	SM0313	Thermo Scientific
Glycerol	G5516	Sigma Aldrich
Glycine	3908.2	Carl Roth
4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES)	9105.2	Carl Roth
HiMark™ Pre-Stained Protein Standard	LC5699	novex® by Life technologies
Hydrochloric acid	1.00316.1000	Merck KgaA
Interleukin-6 (IL-6)	I1395	Sigma Aldrich
Luria Bertani (LB) medium	X968.2	Carl Roth
L-glutamine	M11-004	PAA Laboratories
Methanol p.a.	4627.2	Carl Roth
Milk powder	T145.2	Carl Roth
Nonidet P-40 Substitute (NP-40)	14060500	Roche Diagnostics
NuPAGE 3-8 % Tris-Acetate Gel	EA0378BOX	Invitrogen™
NuPAGE Antioxidant	NP0005	Invitrogen™
Opti-MEM®	51985-026	Gibco
PageRuler Plus Prestained Protein Ladder	#26619	Thermo Scientific
Phosphate buffered saline (PBS)	L1825	Biochrom
Penicillin/streptomycin (P/S)	P11-010	PAA Laboratories
PhosSTOP	04906845001	Roche Diagnostics

Phusion® Hot Start II DNA Polymerase [2U/μL]	F-549	Finnzymes
Potassium chloride	1.04936.0500	Merck KGaA
Protease inhibitor cocktail	P8340	Sigma Aldrich
Roti®-Fect	P001.3	Carl Roth GmbH
Rotiphorese® Gel 40 (37.5:1)	T802.1	Carl Roth
Roswell Park Memorial Institute (RPMI) 1640	F1215	Biochrom
Sodium chloride	9265.1	Carl Roth
Sodium deoxycholate	D6750	Sigma Aldrich
Sodium dodecyl sulphate (SDS)	04051.1	Biomol
β-mercaptoethanol	M3148	Sigma Aldrich
Taq DNA Polymerase [5U/μL]	18038-042	Invitrogen™
Tetramethylethylenediamine (TEMED)	2367.3	Carl Roth
Tocilizumab (RoActemra®) (20 mg/mL)		Roche Group
Tris Acetate Running Buffer	LA0041	Invitrogen™
Tris base	4855.2	Carl Roth
Trypsin/EDTA	L11-004	PAA Laboratories
Tumor necrosis factor alpha (TNF-α)	T6674	Sigma Aldrich
TWEEN 20®	P2287	Sigma Aldrich

2.1.2 Machines and labware

- **Blotting membranes**

Roti®-PVDF 0.45 µm, T830.1, Carl Roth

Nylon membrane Bodayne® B 0.45 µm, PALL Life Sciences

- **Filter paper**

Cat No. 1003-917, Whatman

- **Protein visualization**

Molecular Imager® ChemiDoc™ XRS+ Imaging System, Bio-Rad

- **Gel electrophoresis and blotting systems**

Mini Trans-Blot® Cell, Bio-Rad

XCell SureLock™ Mini-Cell, invitrogen

Power Pack P25 T, Biometra

Fastblot B34, Biometra

Compact M, Biometra

Safeshield Scalpel, Feather

- **Luminometer**

Mithras LB 940, Berthold

- **Spectrophotometer**

Nano drop® Spectrophotometer ND-1000, peqlab

Mithras LB 940, Berthold

- **Incubators**

Hera Cell 150, Thermo Electron Corporation

- **Microscope**

AE 31, Motic

- **Real-time PCR machine**

CFX Connect Real-Time PCR Detection System, Bio-Rad

- **pH meter**

FiveEasy™ FE20, Mettler Toledo

- **Pipettes**

Eppendorf research plus (2.5, 10, 20, 100, 200, 1000 µL)

Pipetboy acu, IBS Integra Biosciences

Pasteur pipettes, Assistant 567/2

- **Bench**

HeraSafe HS12, Thermo Electron Corporation

- **Water bath**

GFL 1003, Gesellschaft für Labortechnik mbH

- **Cell culture dishes and other plates**

24-well, 83.1836, Sarstedt

12-well, 3513, Costar

6-well, 140675, Nunc Thermo Scientific

Ø 10 cm dishes, 83.1802, Sarstedt

75 cm² culture flasks, 83.1813.002, Sarstedt

Microseal 'B' Adhesive Seals, MSB-1001, Bio-Rad Laboratories

Hard-Shell 96-well plates, clear well, white shell, HSP-9601, Bio-Rad Laboratories

Micro test protein plate, 82.1581, Sarstedt

Cell Scraper 25cm, 83.1830, Sarstedt

- **Centrifuges**

Heraeus Multifuge 15-R, Thermo Electron Corporation

Micro Star 17R, VWR

- **Analytical Machines**

COBAS INTEGRA 400, Roche Diagnostics

2.1.3 Antibodies

Table 3: Antibodies used for Western blot and EMSA experiments

	Product number	Manufacturer
<u>Primary Antibodies</u>		
apolipoprotein(a)	5402-1	Epitomics
HSP90 α / β	sc-7947	Santa Cruz Biotechnology
STAT3	sc-482X	Santa Cruz Biotechnology
<u>Secondary Antibodies</u>		
Anti-Rabbit IgG, HRP-linked Antibody	7074	Cell Signalling

2.1.4 Restriction, ligation and dephosphorylation enzymes

Table 4: Restriction enzymes, ligases and dephosphorylation enzymes used for cloning and truncation of apo(a) promoter

	Product number	Manufacturer
Asel	R0526S	New England Biolabs
BglII	R0144S	New England Biolabs
BlnI	R0585S	New England Biolabs
Bsu36I	R0524S	New England Biolabs
Alkaline Phosphatase, Calf Intestinal (CIP)	M0290S	New England Biolabs
EcoRV	R0195S	New England Biolabs
NlaIV	R0126S	New England Biolabs
SacI	R0156S	New England Biolabs
T4 DNA Ligase	M0202S	New England Biolabs

2.1.5 Commercial kits

Table 5: List of commercial reaction kits

	Product number	Manufacturer
AllPrep DNA/RNA Mini Kit	80204	Qiagen
Bio-Rad Protein Assay Dye Reagent Concentrate	#500-0006	Bio-Rad Laboratories
Precellys [®] Ceramic (1.4 mm) Beads Kit	10011152	Cayman Chemical
Clarity Western ECL Substrate	#170-5060	Bio-Rad Laboratories
Dual-Luciferase [®] Reporter Assay System	E1960	Promega
EndoFree [®] Plasmid Maxi Kit	12362	Qiagen
HuGene 1.1 ST array	901415	affymetrix
LightShift [®] Chemiluminescent EMSA Kit	89880	Thermo Scientific
Maxima First Strand cDNA Synthesis Kit for RT-qPCR	K1641	Thermo Fisher Scientific
Maxima SYBR Green/Fluorescein qPCR Master Mix	K0241	Thermo Fisher Scientific
NucleoSpin Extract II	740609.50	Macherey-Nagel
One Shot [®] TOP10 Chemically Competent <i>E.Coli</i>	C4040-03	Invitrogen
peqGOLD Total RNA Kit	12-6834-01	PEQLAB Biotechnologie
QIAprep [®] Spin Miniprep Kit	27104	Qiagen
Quick Blunting Kit	E1201S	New England Biolabs
Quick Ligation [™] Kit	M2200	New England Biolabs

2.1.6 Vector constructs

Table 6: Commercially purchased vector constructs used for cloning and subsequent luciferase reporter gene assays

	Product number	Manufacturer
pGL3 basic vector	E1751	Promega
phRL-TK		Promega

2.1.7 Buffers and solutions

Table 7: Buffer compositions

	Buffer composition
<u>RIPA lysis buffer</u>	50 mM Tris-HCl 150 mM sodium chloride 0.5 % sodium deoxycholate 0.1 % SDS 1 % NP-40
<u>Extraction buffer A pH 7.9</u>	10 mM HEPES 10 mM potassium chloride 0.2 mM EDTA Freshly add 1M DTT 1:10 PhosSTOP (10x) 1:100 protease inhibitor cocktail

<u>Extraction buffer B pH 7.9</u>	20 mM HEPES 0.4 M sodium chloride 0.2 mM EDTA Freshly add 1M DTT 1:10 PhosSTOP (10x) 1:100 protease inhibitor cocktail
<u>10x SDS running buffer</u>	60.4 g Tris base 288 g Glycine 20 g SDS H ₂ O ad 2000 mL
<u>Transfer buffer 1</u>	36.34 g Tris base 200 mL methanol H ₂ O ad 1000 mL
<u>Transfer buffer 2</u>	3.03 g Tris base 200 mL methanol H ₂ O ad 1000 mL
<u>Transfer buffer 3</u>	3.03 g Tris base 200 mL methanol H ₂ O ad 1000 mL 0.5 g aminocaproic acid per 100 mL transfer buffer 3
<u>2x Laemmli buffer pH 7.6</u>	128 mM Tris base 4.6 % SDS 10 % Glycerol
<u>4x SDS loading buffer</u>	0.005 % (0.01 g) bromophenol blue 2x Laemmli buffer ad 50 mL before use add 1:10 β-mercaptoethanol

<u>10x TBS buffer</u>	140 g sodium chloride 4 g potassium chloride 60 g Tris base pH 7.4 with hydrochloric acid H ₂ O ad 2000 mL
<u>1x TBS/T buffer</u>	100 mL 10x TBS 1 mL 100 % Tween® 20 H ₂ O ad 1000 mL
<u>Blocking buffer</u>	5 % milk powder or BSA (depending on antibody requirements) in TBS/T
<u>10x TBE buffer</u>	890 mM Tris-borate 20 mM EDTA
<u>10x TAE buffer</u>	400 mM Tris-acetate 10 mM EDTA
<u>Separating gel buffer pH 8.9</u>	1.875 M Tris base 10 % SDS
<u>Stacking gel buffer pH 6.7</u>	0.3 M Tris base 10 % SDS
<u>Stacking gel (per 2)</u>	3.3 mL H ₂ O 6.6 mL separating gel buffer 6.6 mL 30 % acrylamid 150 µL 10 % APS 15 µL TEMED
<u>Separating gel (per 2)</u>	4 mL H ₂ O 1.32 mL stacking gel buffer 1.12 mL 30 % acrylamid 60 µL 10 % APS 8 µL TEMED

Shift gel (EMSA) (per 2) 19.36 mL H₂O
 1.2 mL 10x TBE
 3.2 mL 30 % acrylamid
 240 µL 10 % APS
 24 µL TEMED

Agarose gel (1% or 2%, depending
on fragment size) 1 %
 1g agarose
 1x TAE buffer
 2 %
 2g agarose
 1x TBE buffer

LB-ampicillin agar plates (per L) 25g LB-medium
 15g agar-agar
 1 mL ampicillin [100 mg/mL]

2.1.8 Primers and oligonucleotides

Table 8: Oligonucleotides sequences

	Forward (5'→3')	Reverse (5'→3')
Cloning	ttgagctcTTGCCGAAAGATTGATACTATG CT [96]	ttagatCTTATGTTCCATTTTGGGACTGG [96]
Promoter studies		
Promoter truncation	<i>SmaI</i> _NlaIV_F2: GTGCTAGCCCCGCCACCAC	
	<i>SmaI</i> _AseI_F3: GCGTGCTAGCCCTAATTTTATTTGG	
	<i>SmaI</i> _Bsu36I_F4: GTGCTAGCCCTCAGGTGATCCATC	<i>SmaI</i> _BglII_F2-6: CAGATCTCGAGCCCCGATCTTATG
	<i>SmaI</i> _EcoRV_F5: CGCGTGCTAGCCCATCTTATAAC	
	<i>SmaI</i> _BlnI_F6: GCTAGCCCTGAGCCAGTGG	
EMSA	3'-biotin labelled	3'-biotin labelled
	IL-6 RE 1: CTAGTAGCTGGGATTACAGG	IL-6 RE 1: CCTGTAATCCCAGCTACTAG
	IL-6 RE 2: CCAAAGTGCTGGGATTACAG	IL-6 RE 2: CTGTAATCCCAGCACTTTGG
	IL-6 RE 3: TAGTAGCTGGGATGACTGGC	IL-6 RE 3: GCCAGTCATCCCAGCTACTA
	IL-6 RE 4: CCCAAATGCTGGGATTATAGGC	IL-6 RE 4: GCCTATAATCCCAGCATTTTGGG

	IL-6 RE 5: GGTGAGGGAAGGAGTAAGGAG	IL-6 RE 5: CTCCTTACTCCTTCCCTCACC
	IL-6 RE 6: GTCAACAATGTCCTGGGATTGGG	IL-6 RE 6: CCCAATCCCAGGACATTGTTGAC
real-time qRT-PCR	<i>apo(a)</i> : TGCTTGAAGAAGTCCTCAAGG	<i>apo(a)</i> : TATCTGCTTGTGTGGGCTCC
	<i>β-actin</i> : ACAGAGCCTCGCCTTTGC	<i>β-actin</i> : GAATCCTTCTGACCCATGCC

2.1.9 Human cell lines and bacterial cells

Human cell lines used for *in vitro*-experiments

- hepatocellular carcinoma cells (HEPG2), ACC-180, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
- hepatocellular carcinoma cells (HuH7), kindly provided by J. Hampe, Department of Internal Medicine I, University of Kiel

Bacterial cells used for transformation of vector constructs

- One Shot® TOP10 *E.coli* cells (similar to DH10B™ strain), Life technologies

2.1.10 Serum and biopsy samples of human studies

All studies were approved by the local ethics committees before the commencement of the studies. Furthermore, all patients provided written, informed consent.

TNF- α inhibition study population

Sera of 12 rheumatoid arthritis patients were taken before and three months after anti-tumor necrosis factor (TNF)- α therapy in order to study the influence of this intervention on serum Lp(a) levels. This study design is comparable to the study published in 2010 [95]. Weight, BMI, age and gender distribution of the treated subjects included into the study population were 78.3 ± 4.7 kg, 25.1 ± 1.3 kg/m², 52.1 ± 4.4 years and 52.9 % male subjects, respectively.

Liver biopsy study population

Liver samples were obtained percutaneously from patients undergoing liver biopsy for suspected non-alcoholic fatty liver disease (NAFLD) or intraoperatively in patients in whom an intraoperative liver biopsy was indicated on clinical grounds such as during scheduled liver resection, exclusion of liver malignancy during major oncological surgery or assessment of liver histology during bariatric surgery. Biopsies were immediately frozen in liquid nitrogen ensuring an ex-vivo time of less than 40 seconds in all cases. Patients with evidence for viral hepatitis, hemochromatosis or alcohol consumption higher than 20 g/day for women and 30 g/day for men were excluded.

FoCus cohort study population

As part of the Food Chain Plus (FoCus) project [<http://www.focus.uni-kiel.de>], a total number of n=1153 human subjects were examined in order to characterize their anthropometric and

metabolic parameter (**table 2**), to perform correlation analyses between all parameters measured in this study and eventually to compare Lp(a) serum levels in a normal and an elevated homeostasis model assessment-estimated insulin resistance (HOMA-IR), CRP, IL-6, TG and BMI group. Within the n=1153 human subjects, 500 subjects were enrolled from the obesity outpatient clinic of the Department of Internal Medicine I of the University of Kiel. The other 653 subjects were recruited from the regional registration offices as cross-sectional controls.

HOMA-IR was calculated using the following formula: fasting glucose (mg/dL) x fasting insulin (μ U/mL) / 405.

Cut-off levels

BMI cut-off was set according to the classification of normal-weight, overweight and obesity established by the WHO 2000. HOMA-IR cut-off was set at 2 knowing that the output of the model is calibrated to give normal β -cell function of 100% and normal IR of 1 [97]. CRP cut-off was set at 3.0 mg/L according to defined risk groups of CRP by the American Heart Association and the U.S. Centers for Disease Control and Prevention. Lp(a) cut-off was set at 300 mg/L. This is defined as high risk and at the same time represents a 3-fold of the value being connected to no or low cardiovascular risk. IL-6 cut-off (6 pg/mL) was chosen according to the reference ranges being established by the Institute of Clinical Chemistry of the University Medical Center in Kiel. Triglycerides cut-off was set at 200 mg/dL according to the guidelines of the National Cholesterol Education Program of the U.S. National Heart, Lung, and Blood Institute. Triglycerides serum levels above 200 mg/dL already represent high levels and are associated with an increased risk to develop CVD.

2.2 Methods

2.2.1 Cell culture

For luciferase reporter gene assays human hepato cellular carcinoma cells (HepG2) cells were maintained in RPMI1640 medium supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 1 % penicillin/streptomycin (P/S) at 5 % carbon dioxide (CO₂) and 37°C. For quantitative real-time RT-PCR, EMSA as well as for western blotting experiments, human hepato cellular carcinoma cells (Huh7), were maintained in DMEM with high glucose (4.5 g/L), supplemented with 2 mM L-glutamine, 10 % FCS and 1 % P/S at 5 % CO₂ and 37°C.

For passaging, adherent cells were washed with 1x PBS in order to remove traces of FCS-containing culture medium, afterwards detached using 1x trypsin/EDTA solution for approximately 5 min at 37°C and 5 % CO₂. The addition of culture medium stopped trypsinization. Cells were then centrifuged for 5 min at 1,200 rpm at room temperature (RT). Medium was aspirated and the cell pellet re-suspended in fresh culture medium before being seeded into new plates or flasks.

2.2.2 Cloning and truncation of apo(a) promoter

Molecular cloning is a set of experimental methods in molecular biology that are used to assemble recombinant DNA molecules and to direct their replication within host organisms.

The first step is the amplification of the DNA fragment (here: apo(a) promoter) to be cloned via polymerase chain reaction (PCR).

2.2.2.1 Amplification of apo(a) promoter by polymerase chain reaction

The human apo(a) promoter was first amplified from human genomic DNA. Therefore, a specific sense primer flanking -1293 to -1270 (according to the in reference [93] published sequence, see 2.1.8) as well as antisense primer flanking +153 to +131 of the apo(a) promoter were used to generate the full length promoter region.

The following PCR reaction (**table 9**) and cycling conditions (**table 10**) were used for this amplification.

Table 9: PCR reaction used for amplification of apo(a) promoter

Components	Volume
5x Phusion® HF Reaction Buffer	6 µL
10 mM dNTP's	0.6 µL
Forward Primer [100 µM]	0.15 µL
Reverse Primer [100 µM]	0.15 µL
Phusion Hot Start II DNA Polymerase [2 U/µL]	0.3 µL
Human genomic DNA [50 ng/µL]	2 µL
Nuclease-free water	ad 30 µL

Table 10: PCR cycling conditions for amplification of apo(a) promoter

	Temperature	Time	Number of Cycles
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	} 35
Annealing	65°C	20 s	
Extension	72°C	45 s	
Final Extension	72°C	10 min	1
Hold	4°C	∞	

2.2.2.2 Digestion with restriction enzymes

The amplified DNA sequence afterwards needs to be ligated into a vehicle vector in order to be replicated by the host organism (mostly *E.coli* bacterium).

Since primers were created including restriction sites for *SacI* and *BglIII*, PCR products were directly ligated into the multiple cloning site of pGL3 basic luciferase vector once digestion of both vector and insert with appropriate restriction enzymes has been performed. For digestion reaction, 1.5 µg purified PCR product was mixed with 10x Buffer X (depending on requirements of restriction enzymes) and 4 µL of *SacI* as well as 5 µL of *BglIII* (for Units/mL see **table 4**) in a 100 µL-reaction and incubated for 3h at 37°C.

For truncation, the sequence of the apo(a) promoter was analyzed for their existing restriction sites using the ApE (A plasmid Editor) software v2.0.30. Apo(a) promoter was digested after/behind IL-6 responsive element 1, 2, 3, 4 and 5 resulting in five new constructs with different lengths. pGL3-apo(a) (1451 bp) served as construct 1 containing all IL-6 responsive elements. The following restriction enzymes (**table 11**) have been used for the new constructs:

Table 11: Restriction enzyme combinations used for truncation of apo(a) promoter

	IL-6 responsive elements	restriction enzyme	size (bp) after digestion
Fragment 2	2, 3, 4, 5, 6	NlaIV+BglIII	1275
Fragment 3	3, 4, 5, 6	Asel+BglIII	1045
Fragment 4	4, 5, 6	Bsu36I+BglIII	795
Fragment 5	5, 6	EcoRV+BglIII	716
Fragment 6	6	BplI+BglIII	152

For truncation digestion reactions, 3 µg of plasmid DNA was mixed with 10x Buffer X (depending on requirements of restriction enzymes) and 3 µL of each enzyme (for Units/mL see **table 4**) in a 150 µL-reaction and incubated for 3h at 37°C.

2.2.2.3 Gel extraction

Digestion reaction was run on a 2 % agarose gel in order to explicitly pick the DNA fragment needed for analysis. Hence, relevant bands were cut using a scalpel and again purified using the NucleoSpin Extract II Kit according to the manufacturer's instructions.

2.2.2.4 Blunting

In case, in the truncation process, there was a restriction site for a restriction enzyme not being compatible with the Multiple Cloning Site (MCS) of the pGL3 basic luciferase vector and at the same time a restriction enzyme producing sticky/cohesive ends, those ends were blunted to allow the combination of this fragment with one of the established restriction sites (here: SmaI site) within the MCS of the pGL3 basic luciferase vector. For ligation of the new fragment to the vector, the pGL3 basic luciferase vector needed to be digested with SmaI. For blunting, the purified and cut DNA fragment was mixed with Blunting Enzyme Mix (according to the

manufacturer's recommendations), 1 mM dNTP's and 10x Buffer X in a 25 μ L-reaction and incubated for 15 min at RT, afterwards for 10 min at 70°C to inactivate the enzyme activity.

2.2.2.5 Dephosphorylation of vector DNA

For dephosphorylation, restriction enzymes in the reaction initially needed to be heat-inactivated for 20 min at 65°C. Afterwards, the digestion reaction including the cut and purified pGL3 basic luciferase vector was incubated with 5 U (sticky ends) or 15 U (blunt ends) of Alkaline Phosphatase (CIP) for 60 min at 37°C (sticky/cohesive) or 50°C (blunt) prior to gel extraction (see Gel extraction) and subsequent ligation to the insert. That was done in order to remove 5' phosphate groups from vector DNA. CIP-treated fragments lack the 5' phosphoryl termini required by ligases, so that they cannot self-ligate. Due to problems concerning the latter, dephosphorylation was used to efficiently ligate vector to insert and to decrease the vector background on agar plates.

2.2.2.6 Ligation

For cloning of apo(a) promoter, 100 ng cut, linearized and dephosphorylated vector was combined with a 3-fold molar excess of cut and purified PCR product (insert), supplemented with 400 U T4 DNA Ligase and 10x T4 Ligase Buffer in a 15 μ L-reaction and incubated overnight at 16°C.

For truncation of the apo(a) promoter, 50 ng cut, linearized and dephosphorylated vector was combined with a 3-fold molar excess of cut, purified and blunted insert fragment, supplemented with 2x Quick Ligase Buffer and 1 μ L Quick T4 DNA Ligase and afterwards incubated for 5 min at RT in a 20 μ L-reaction. Reactions were chilled on ice and subsequently used for transformation (see Transformation and colony PCR) or stored at -20°C.

2.2.2.7 Transformation and colony PCR

The newly generated recombinant DNA molecules were now inserted into *E.coli* bacterial cells via transformation. For each transformation, one vial of One Shot® TOP10 Chemically Competent *E.coli* was thawed on ice. 2 µL of the ligation reaction was added to the cell vial and mixed gently and afterwards incubated on ice for 30 min. Cells were heat-shocked for 30 sec at 42°C in a water bath, placed on ice for 2 min. Pre-warmed S.O.C. medium was aseptically added and tightly closed vials were shaken horizontally for 1 h at 37°C at 225 rpm in a shaking incubator. 150 µL of cell suspension was then plated out on agar plates prior to incubating them overnight at 37°C. Colonies were picked on the next day, plated out again on a fresh agar plate and incubated overnight at 37°C. Colony was picked and plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit in order to analyze for integrity of the insert via control digestion. Sequence of integrated insert for cloning of the apo(a) promoter was further confirmed by sequencing. Resulting plasmid is in the following referred to as pGL3-apo(a).

For truncation of cloned apo(a) promoter, all picked colonies were control-digested in order to check the fragment sizes. In addition, due to blunt end ligation, it was necessary to screen all colonies for sense and antisense directed inserts as only the sense-directed constructs can be used for luciferase reporter gene assays. Therefore, PCR primers flanking the region of pGL3 basic vector and promoter fragment linkage (see 2.1.8) were created and used for the sense/antisense-direction screening.

The following PCR reaction and cycling conditions were used for sense/antisense direction colony screening:

Table 12: Colony screen PCR reaction

Components	Volume
10x PCR Buffer	3 μ L
50 mM MgCl ₂	0.9 μ L
10 mM dNTP's	0.6 μ L
Forward Primer [100 μ M]	0.15 μ L
Reverse Primer [100 μ M]	0.15 μ L
<i>Taq</i> DNA Polymerase [5U/ μ L]	0.3 μ L
Re-suspended colony material	5 μ L
Nuclease-free water	ad 30 μ L

Table 13: Colony screen PCR cycling conditions

	Temperature	Time	Number of Cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	30 s	35
Annealing	57°C	30 s	
Extension	72°C	Depending on fragment length	
Final Extension	72°C	10 min	1
Hold	4°C	∞	

All established promoter constructs were afterwards analyzed using luciferase reporter gene assays (see 2.2.3).

2.2.3 Luciferase reporter gene assay

Luciferase Reporter Assays allow to investigate the promoter activity by measuring light output from luciferase enzyme that is expressed under the control of the promoter of interest. For transient transfection using lipofection agent Roti®-Fect, HepG2 cells were grown in 24-well plates to 60-70 % confluency. 0.5 µg of pGL3-apo(a) or pGL2-FAS was applied per well. To correct for transfection efficiency, 12.5 ng/well phRL-TK were co-transfected. Cells were subsequently incubated with transfection medium (Opti-MEM®, 10 % FCS, Roti®-Fect) for 24 h followed by a 12h-incubation in serum-reduced full medium (1 % FCS). 24 h after stimulation with 10 ng/mL IL-6 as well as 100 µg/mL tocilizumab or 100 ng/mL adalimumab (both antibodies 1h prior to IL-6 stimulation), cells were lysed by applying Passive Lysis Buffer, and luciferase activity was detected in a luminometer (Berthold Mithras LB 940) using the dual luciferase reporter assay kit.

2.2.4 Generation of RNA and two-step real-time RT-PCR

2.2.4.1 RNA isolation

RNA of Huh7 cells was isolated using the peqGOLD Total RNA Kit following the manufacturer's instructions. RNA concentration was measured using the spectrophotometer nanodrop®. RNA samples were only used for further analysis if 260/280 and 260/230 ratios were below 1.8 and 2.0, respectively.

2.2.4.2 DNase digestion

To remove residual contaminating genomic DNA from the RNA sample which was not able to be removed during the RNA isolation process described above, 5 µg ultrapure RNA was digested using DNase (2000 U/mL) supplemented with 10x DNase buffer in a 50 µL reaction for 10 min at 37°C. To heat-inactivate the enzyme activity of DNase, a final concentration of 5 mM EDTA was added and incubated for 10 min at 75°C.

2.2.4.3 cDNA synthesis

DNase-digested RNA was reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR. For this reaction 1 µg DNase-treated RNA supplemented with 5x Reaction Mix (containing reaction buffer, dNTP's, oligo(dT)₁₈ and random hexamer primers) and the Maxima Enzyme Mix (containing Maxima Reverse Transcriptase and RiboLock RNase Inhibitor) in a 20 µL-reaction was initially incubated for 10 min at 25°C, afterwards for 15 min at 55°C and finally for 5 min at 85°C. This step was performed in a thermal cycler to ensure more exact temperatures and to avoid excessive condensation. The 20 µL reaction was diluted 1:10 to obtain a final cDNA concentration of 5 ng/µL.

2.2.4.4 Primer design

Primer sequences were designed using Primer3 standard software (version 0.4.0; <http://frodo.wi.mit.edu/primer3/>), NCBI BLAST and NCBI PrimerBLAST following general recommendations for PCR primer design. Primer pairs were obtained from MWG Biotech AG. For primer sequences used in this thesis see 2.1.8.

2.2.4.5 Quantitative real-time PCR

For quantitative real-time PCR, 25 ng generated cDNA was used to amplify a specific target sequence being determined by corresponding primers. Therefore, cDNA was mixed with the 2x Maxima SYBR Green/Fluorescein qPCR Master Mix as well as with forward and reverse primers (0.3 μ M each) in a 25- μ L reaction. Reaction was run in a CFX Connect Real-Time Detection System using the following cycling conditions:

Table 14: Cycling conditions for real-time PCR

	Temperature	Time	Number of Cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	15 s	45
Annealing	depending on T_m	30 s	
Extension and Data collection	72°C	30 s	
<i>Denaturation</i>	95°C	60 s	1
<i>Starting temperature</i>	55°C	60 s	1
<i>Melting step</i>	55°C	10 s	80

2.2.5 Generation of proteins

In general, all steps being involved in protein isolation were performed on ice to prevent protein degradation.

2.2.5.1 Whole cell extracts

To obtain whole cell extracts, cells were washed with PBS and scraped into RIPA lysis buffer (see 2.1.7). After centrifugation at 4°C at 14,000 rpm for 30 min, supernatant was collected and frozen at -80°C until further analysis.

2.2.5.2 Nuclear extracts

Nuclear extracts of Huh7 cells were generated by scraping washed cells into extraction buffer A to separate the cytosolic fraction from the nuclear fraction and subsequently centrifuged for 1 min at 13,000 rpm and 4°C. Remaining pellet (nuclear fraction) was re-suspended in extraction buffer B, vortexed for 20 min at 4°C and afterwards centrifuged for 5 min at 13,000 rpm and 4°C. Supernatant (nuclear extract) was collected and stored at -80°C until further analysis.

2.2.5.3 Determination of protein concentration (Bradford)

Quantification of total protein was performed using the Bio-Rad Protein Assay being a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein.

For determination of protein concentration of whole cell and nuclear extracts, BSA standards as well as samples to be analyzed were thawed slowly on ice. BSA stock of 2 mg/mL was then serially diluted to obtain standards from 1 mg/mL to 0.030 mg/mL (1, 0.5, 0.25, 0.125, 0.060, 0.030 mg/mL). Samples were diluted appropriately as well, if needed. Bio-Rad Protein Assay reagent

(acidic dye) (diluted 1:5 with ultrapure distilled water) was mixed with diluted or undiluted protein solution (tested before) within the 96-well plate and measured at 620 nm in a spectrophotometer. Protein concentrations were calculated using the established standard curve and the protein's absorbances.

2.2.6 Western blotting

Western blotting (also called protein immunoblotting) is a widely accepted analytical technique used to detect specific proteins in a sample of tissue homogenate or extract.

For western blotting, 30 µg protein of whole cell extracts was added to 4x loading buffer, heated to 95°C for 5 min, and separated on a NuPAGE 3-8 % Tris-Acetate gel for 2 h at 150V in 1x Novex® Tris-Acetate SDS Running Buffer supplemented with 1:1600 NuPAGE® Antioxidant. HiMark™ Pre-Stained Protein Standard served as reference marker. Proteins were transferred to PVDF membranes for 1.5h at 30V in 1x NuPAGE® Transfer Buffer. On the PVDF membrane proteins were stained with the antibodies specific to apo(a) and HSP90α/β. Corresponding secondary antibodies were chosen according to the species the primary antibody was produced in (both anti-rabbit IgG, HRP-linked Antibody).

2.2.7 Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) is a common affinity electrophoresis technique used to study protein–DNA or protein–RNA interactions. This procedure determines whether a protein or mixture of proteins is capable of binding to a given DNA or RNA sequence, and sometimes indicate whether more than one protein molecule is involved in the binding complex. For EMSA experiments, 5 µg of nuclear protein extract were incubated with 1 pmol 3'-end biotin labelled double-stranded oligonucleotides (see 2.1.8) each containing one of the six IL-6 REs within the apo(a) promoter [93] and 1 µg poly dI·dC in supplied binding buffer (supplemented with 5 % glycerin and 1 mM EDTA) for 30 min at RT. Competitive reaction was performed under identical conditions adding 200fold of the amount of the respective unlabeled oligonucleotide. In supershift assays, 4 µg STAT-3 supershift antibody was incubated with 5 µg of nuclear extracts and 1 µg poly dI·dC in binding buffer for 30 min at 15°C. Afterwards, 1 pmol 3'-end biotin labeled double-stranded oligonucleotide was added and again incubated for 30 min at RT. Samples were separated in a 4 % PAGE until approx. two third of the gel. Final analysis was performed according to instructions of the LightShift® Chemiluminescent EMSA Kit.

2.2.8 Oligonucleotide liver microarray hybridization

In the Department for Internal Medicine I (Prof. Hampe) of the University hospital of Kiel, for homogenization of five to ten milligram frozen tissue and subsequent nucleic acid isolation, tubes with Precellys® 1.4 mm ceramic beads and the AllPrep DNA/RNA Mini Kit were used. mRNA expression analysis was performed using the Affymetrix® HuGene 1.1 ST array.

In the scope of this thesis, further correlation analysis as to which genes are correlated with the *LPA* gene was initially performed. Next, annotating of gene symbols being positively and significantly ($r \geq 0$, $p < 0.05$) correlated with the *LPA* gene was performed using GeneID Conversion Tool of the DAVID Bioinformatics Resources 6.7 software (National Institute of Allergy and Infectious Diseases (NIAID), NIH; <http://david.abcc.ncifcrf.gov/>). Converted gene list was further analyzed using the Functional Annotation Clustering Tool in order to select only genes being related to 'acute phase response', 'acute inflammatory response', 'inflammatory response', 'activation of plasma proteins involved in acute inflammatory response', 'regulation of inflammatory response', 'regulation of acute inflammatory response', and 'positive regulation of inflammatory response'.

2.2.9 Statistical analysis

All data are expressed as means \pm SEM. Statistical analysis was performed using GraphPad Prism Software Version 5.04. If the assumption of normal distribution of the data was not violated (tested using Shapiro-Wilk) Student's t-test or ANOVA was used. In case of not normal-distributed data the Mann-Whitney U test or Kruskal-Wallis test was applied. For correlation analyses, Spearman's rank correlation coefficient was applied as all data sets to be correlated were non-parametric. The significance level was set at 5 % and multiple comparisons were done using Bonferroni or Dunn's post test.

3. RESULTS

3.1 Lowering of Lp(a) serum levels is specific for IL-6 inhibition

Lipoprotein(a) [Lp(a)] as a highly atherogenic, pro-thrombotic lipid particle is elevated in several chronic inflammatory diseases. Recently, it was shown that tocilizumab (TCZ), an interleukin-6 receptor antibody, is able to substantially (by up to 50 %) decrease elevated Lp(a) serum levels in rheumatoid arthritis (RA) patients [95] suggesting that there may be a direct association between interleukin-6 (IL-6) and Lp(a). The aim was to investigate the specificity of the ability of TCZ to inhibit IL-6 signaling in order to decrease elevated Lp(a) serum levels. Therefore, Lp(a) was measured in n=12 sera of RA patients before and after three months of anti-tumor necrosis factor (TNF)- α therapy with adalimumab (ADB). This experiment however showed no significant change of Lp(a) serum levels in response to anti-TNF- α therapy (**figure 4**), indicating the Lp(a) lowering effect of TCZ to be specific for IL-6 inhibition.

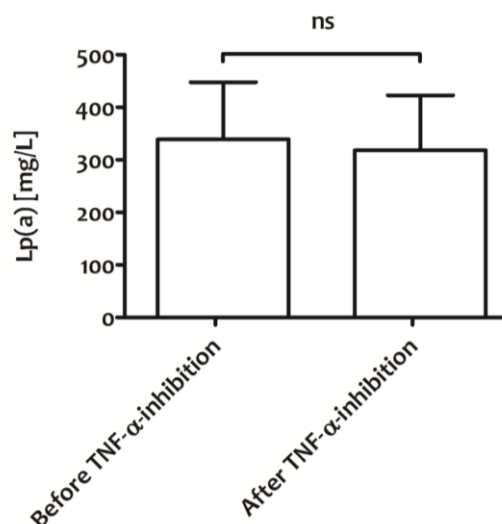


Figure 4: TNF- α -antibody treatment does not decrease Lp(a) serum levels in RA patients. Data are given as means+SEM of n=12 patients with RA treated with TNF- α -antibody for a time period of three months. Statistical significance ($\alpha=0.05$) was tested using Mann-Whitney U test. ns, not significant. Abbreviations: TNF- α , tumor necrosis factor- α ; Lp(a), Lipoprotein(a); RA, rheumatoid arthritis; SEM, standard error of the mean.

3.2 Elevated IL-6 and IL-6-response gene CRP are strongest associated with an increase of Lp(a) in humans *in vivo*

To investigate whether the direct IL-6/Lp(a) association shown in individuals receiving an anti-TNF- α therapy, can also be confirmed in a larger study population, a human cohort (n=1153), as part of the FoodChainPlus (FoCus) project (see 2.1.10), was consulted. To ensure a metabolically 'valid' cohort, this cohort was initially characterized regarding their metabolic and anthropometric parameters. Within this huge cohort, parameters such as Body Mass Index (BMI), blood glucose, insulin, triglycerides (TG), C-reactive protein (CRP), IL-6 as well as Lp(a) were measured. Homeostasis model assessment-estimated insulin resistance (HOMA-IR, calculation see 2.1.10) was taken to draw conclusions about grade of insulin sensitivity of the individuals included in the cohort.

Prevalence of elevated metabolic and anthropometric parameters

The cohort was next screened for their prevalence (**table 15**) of the above parameters when clinically evaluated as 'elevated' and as metabolic risk factor, respectively.

In the FoCus cohort (n=1153), 69.30 % exhibited a BMI above 25 kg/m², 60.97 % of the total number of individuals tended to be insulin resistant (set as HOMA-IR>2, see 2.1.10) rather than insulin sensitive (HOMA-IR<2). More than one third of the FoCus cohort study population had CRP serum levels above 3.1 mg/L (see 2.1.10), yet followed by 23.07 % which exhibited elevated Lp(a) serum levels (≥ 300 mg/L, see 2.1.10). 17.17 % individuals had elevated IL-6 serum levels (≥ 6 pg/mL, see 2.1.10). Elevated TG serum levels (≥ 200 mg/dL, see 2.1.10) were present in 12.75 % of human subjects.

Table 15: Prevalence of elevated BMI, HOMA-IR, CRP, Lp(a), IL-6 and TG serum levels within the FoCus cohort. Shown is the percentage of individuals exhibiting a BMI \geq 25 (30) kg/m², a HOMA-IR above 2, CRP serum levels above 3.1 mg/L, Lp(a) serum levels above 300 mg/L, IL-6 serum levels above 6 pg/mL and TG serum levels above 200 mg/dL of the FoCus cohort. In addition, mean (\pm SEM), minimum, 25 % percentile, median, 75 % percentile and maximum are demonstrated. *Abbreviations: BMI, Body Mass Index; HOMA-IR, homeostasis model assessment-estimated insulin resistance; CRP, C-reactive protein, IL-6, interleukin-6; TG, triglycerides; SEM, standard error of the mean.*

n=1153		Prevalence	Mean (\pm SEM)	Minimum	25 % Percentile	Median	75 % Percentile	Maximum
	Cut-off	%						
BMI	≥ 25 (30) kg/m ²	69.30 (44.93)	31.96 \pm 0.31	16.10	23.95	28.63	38.85	78.04
HOMA-IR	≥ 2	60.97	5.28 \pm 0.31	0.04	1.50	2.484	4.83	147.60
Fasting insulin			17.27 \pm 0.67	0.20	6.70	10.50	19.40	403.80
Fasting glucose			103.90 \pm 1.07	49.00	88.00	95.00	105.00	448.00
CRP	≥ 3.1 mg/L	40.16	5.02 \pm 0.25	0.90	0.90	2.10	5.70	147.80
Lp(a)	≥ 300 mg/L	23.07	224.00 \pm 6.83	23.30	95.00	95.00	266.00	1850.00
IL-6	≥ 6 pg/mL	17.17	4.65 \pm 0.24	1.50	1.65	3.20	5.10	201.90
TG	≥ 200 mg/dL	12.75	132.60 \pm 2.92	11.00	76.00	109.00	153.50	1097.00

Correlation analyses of metabolic and anthropometric parameters of the FoCUS cohort

To investigate the cohort for associations between the selected parameters, correlation analyses were performed (**figure 5-7**). All performed correlation analyses revealed positive, significant relationships and correlation strength was interpreted using the given correlation coefficients r_s as moderate ($r_s=0.5-0.75$), weak ($r_s=0.25-0.5$) and negligible ($r_s<0.25$). Correlation analyses of anthropometric with metabolic parameters and metabolic with metabolic parameters showed that HOMA-IR and BMI ($r_s=0.617$, $p<0.0000001$) (**figure 5A**) were moderately correlated followed by CRP and BMI ($r_s=0.614$, $p<0.0000001$) (**figure 5B**) and CRP and IL-6 ($r_s=0.532$, $p<0.0000001$) (**figure 5C**).

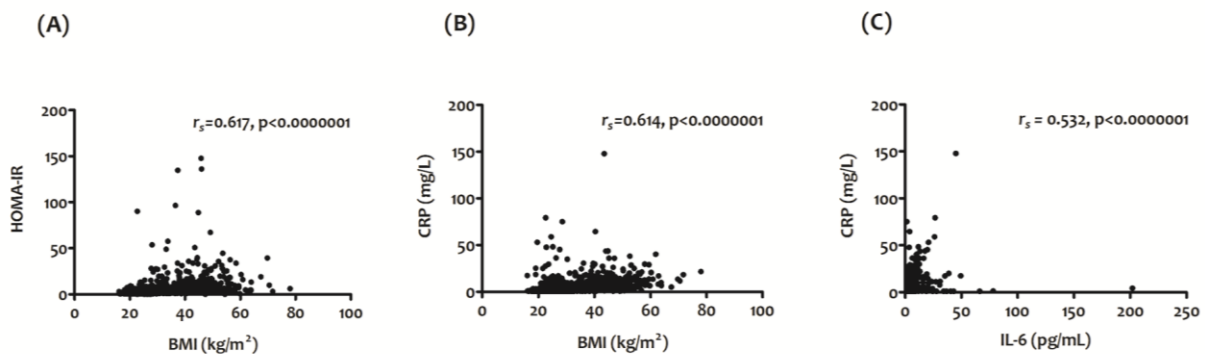


Figure 5: Moderate correlations between anthropometric and metabolic parameters of the FoCUS cohort (n=1153). Shows scatterplots of Spearman correlation (r_s , p) between (A) BMI and HOMA-IR ($r_s=0.617$, $p<0.0000001$), (B) BMI and CRP serum levels ($r_s=0.614$, $p<0.0000001$) and (C) between IL-6 and CRP serum levels ($r_s=0.532$, $p<0.0000001$). Abbreviations: BMI, Body Mass Index; HOMA-IR, homeostasis model assessment-estimated insulin resistance; CRP, C-reactive protein; IL-6, interleukin-6.

Weak correlations were found between HOMA-IR and TG ($r_s=0.493$, $p<0.0000001$) (**figure 6A**), HOMA-IR and CRP ($r_s=0.454$, $p<0.0000001$) (**figure 6B**), BMI and IL-6 ($r_s=0.445$, $p<0.0000001$) (**figure 6C**), BMI and TG ($r_s=0.434$, $p<0.0000001$) (**figure 6D**), HOMA-IR and IL-6 ($r_s=0.379$, $p<0.0000001$) (**figure 6E**) as well as between TG and CRP ($r_s=0.333$, $p<0.0000001$) (**figure 6F**).

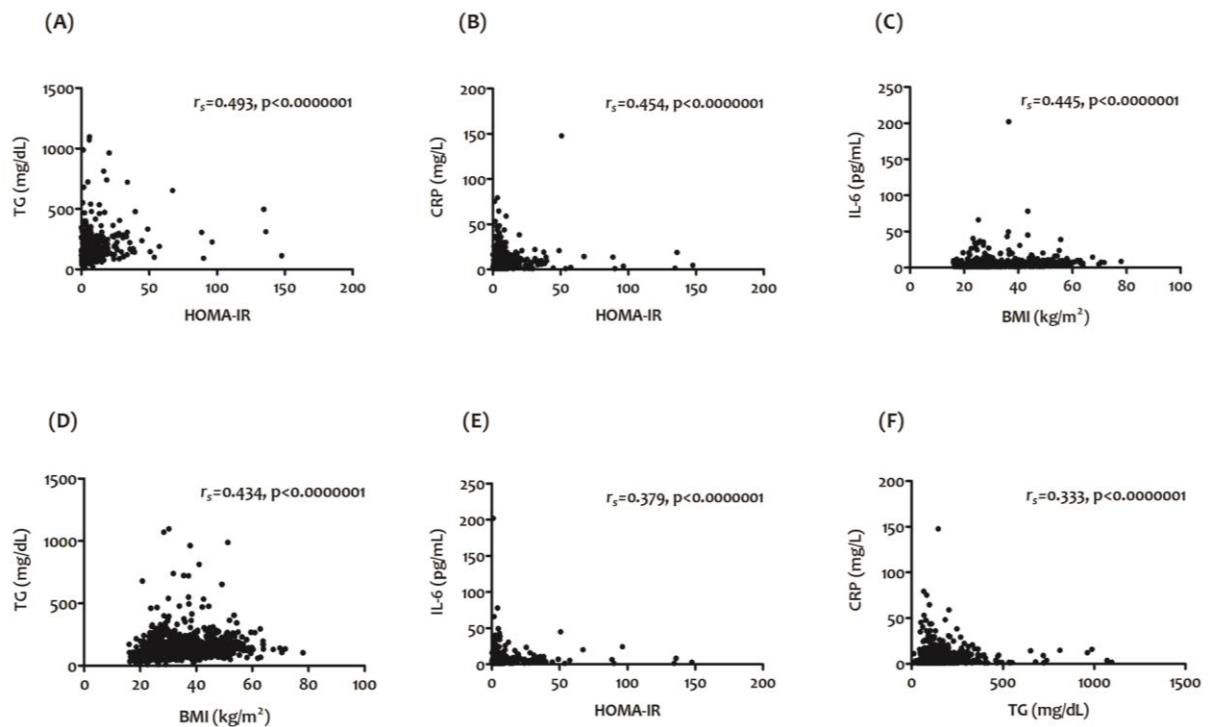


Figure 6: Weak correlations between anthropometric and metabolic parameters of the FoCUS cohort (n=1153). Shows scatterplots of Spearman correlation (r_s , p) between (A) HOMA-IR and TG serum levels ($r_s=0.493$, $p<0.0000001$), (B) HOMA-IR and CRP serum levels ($r_s=0.454$, $p<0.0000001$), (C) BMI and IL-6 serum levels ($r_s=0.445$, $p<0.0000001$), (D) BMI and TG serum levels ($r_s=0.434$, $p<0.0000001$), (E) HOMA-IR and IL-6 serum levels ($r_s=0.379$, $p<0.0000001$) and (F) between TG and CRP serum levels ($r_s=0.333$, $p<0.0000001$). Abbreviations: HOMA-IR, homeostasis model assessment-estimated insulin resistance; TG, triglycerides; CRP, C-reactive protein; BMI, Body Mass Index; IL-6, interleukin-6.

IL-6 was negligibly correlated with TG serum levels ($r_s=0.258$, $p<0.0000001$) (**figure 7A**). Also, IL-6, CRP, BMI, HOMA-IR and TG were negligibly correlated with Lp(a) (**figure 7B-F**). Amongst those, IL-6 ($r_s=0.102$, $p=0.0005$) (**figure 7A**) and CRP ($r_s=0.107$, $p=0.0003$) (**figure 7B**) at least were highest correlated to Lp(a) when compared to BMI, HOMA-IR and TG (**figure 7C-E**).

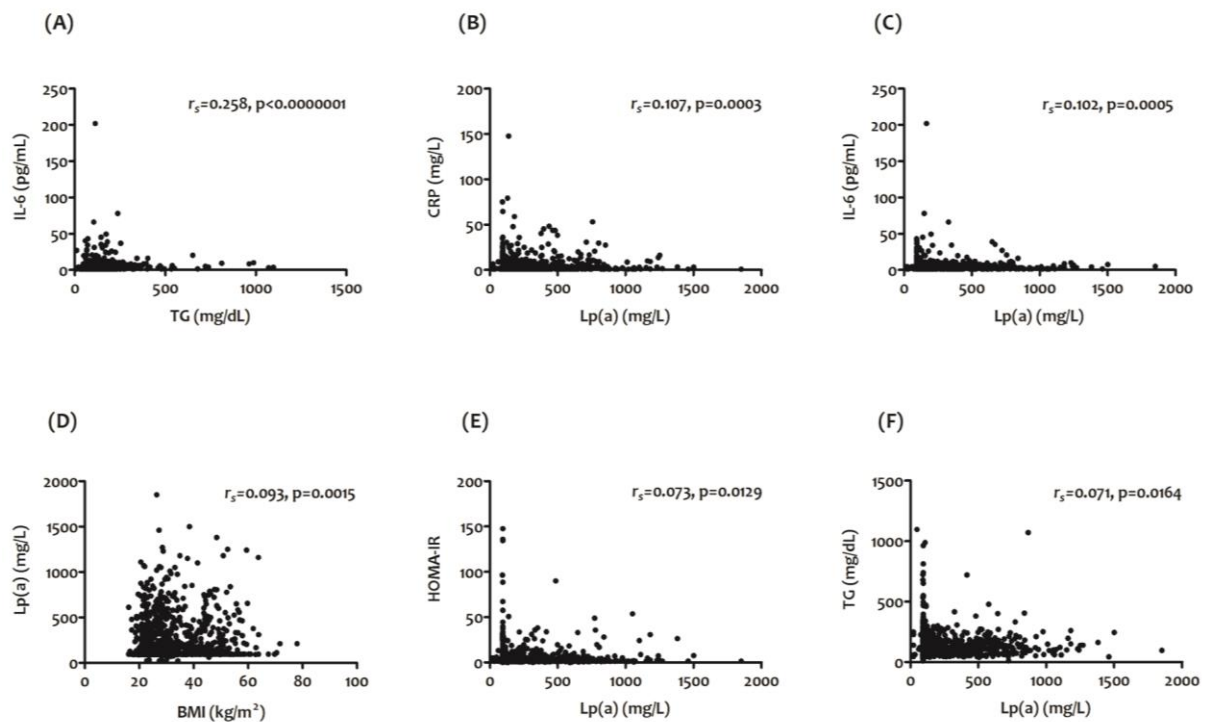


Figure 7: Negligible correlations between anthropometric and metabolic parameters of the FoCUS cohort (n=1153). Shows scatterplots of Spearman correlations (r_s , p) between (A) TG and IL-6 ($r_s=0.258$, $p<0.0000001$), (B) Lp(a) and CRP serum levels ($r_s=0.107$, $p=0.0003$), (C) Lp(a) and IL-6 serum levels ($r_s=0.102$, $p=0.0005$), (D) BMI and Lp(a) serum levels ($r_s=0.093$, $p=0.0015$), (E) Lp(a) and HOMA-IR ($r_s=0.073$, $p=0.0129$) and (F) between Lp(a) and TG serum levels ($r_s=0.071$, $p=0.0164$). Abbreviations: TG, triglycerides; IL-6, interleukin-6; Lp(a), Lipoprotein(a); CRP, C-reactive protein; BMI, Body Mass Index; BMI, HOMA-IR, homeostasis model assessment-estimated insulin resistance.

As this thesis aims to specifically investigate the regulation of hepatic apo(a) and eventually Lp(a), the following analyses will focus on Lp(a) although it is rather negligibly correlated with all parameters measured. However, Lp(a) serum levels have been reported to be genetically determined. Thus, the cohort was classified into individuals exhibiting normal and elevated levels of different metabolic parameters in order to exclude the genetic determinant and to explicitly study whether Lp(a) may be associated with one of these factors.

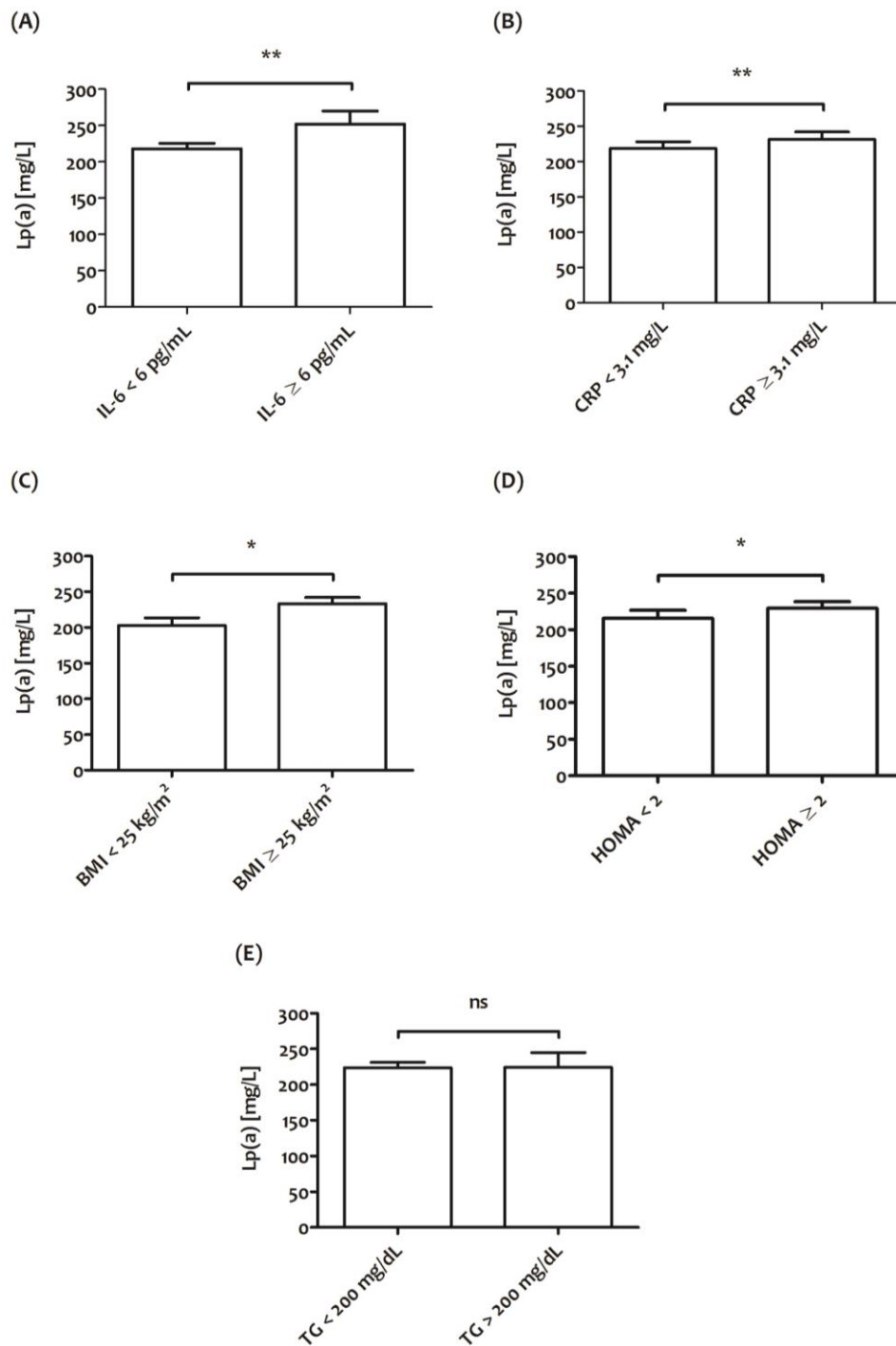


Figure 8: Elevated IL-6 and CRP serum levels and individuals exhibiting BMIs above 25 and HOMA-IR above 2 are associated with an increase of Lp(a) in humans in vivo. This comparison analysis represents significant differences of Lp(a) serum levels in groups with normal (n=955) and elevated (n=198) IL-6 serum levels (p=0.0015) (A), in groups with normal (n=698) and elevated (n=464) CRP serum levels (p=0.0041) (B), in groups with BMIs below 25 kg/m² (n=354) and above 25 kg/m² (n=799) (p=0.0234) (C) and in groups with normal (n=450) and elevated (n=703) HOMA-IR (p=0.0475) (D). Lp(a) serum levels are not significantly different between normal and elevated TG serum levels (E). Statistical significance ($\alpha=0.05$) was tested using Mann-Whitney U test. *, p<0.05; **, p<0.01; ns, not significant. Abbreviations: IL-6, interleukin-6; CRP, C-reactive protein; BMI, Body Mass Index; HOMA-IR, homeostasis model assessment-estimated insulin resistance; Lp(a), lipoprotein(a); TG, triglycerides.

This analyses revealed significantly higher Lp(a) serum levels in subjects with elevated IL-6 and CRP compared to individuals with normal IL-6 and CRP serum levels (**figure 8A+B**). In addition, individuals with BMIs above 25 kg/m² and with HOMA-IR above 2 also showed significantly higher Lp(a) serum levels when compared to their counterparts (**figure 8C+D**). Lp(a) serum levels were unchanged between TG<200 mg/dL and TG>200 mg/dL (**figure 8E**).

3.3 Expression of typical IL-6 response genes correlates with apo(a) expression in human liver *in vivo*

With the analyses of the FoCus cohort, it was found, with respect to Lp(a) pathophysiology, that elevated IL-6 and CRP serum levels as well as BMI above 25 kg/m² and HOMA-IR above 2 were significantly associated with Lp(a) serum levels. However, IL-6 as inducer of acute phase response gene CRP [98] is strongest associated with Lp(a). Therefore, the following molecular and genetic experiments focus on IL-6 but not on CRP, BMI or HOMA-IR, as IL-6 is the initial or direct inducer of apolipoprotein(a) [apo(a)], the determining moiety of Lp(a) serum levels.

The aim of the next experiment was to investigate the relationship between IL-6 and Lp(a) in the liver, the origin of apo(a) synthesis, instead of in serum samples. Therefore, a microarray experiment using liver biopsies obtained during elective surgical procedures was performed. In this study human subjects with BMI≥28 kg/m² were included as it is known that those individuals have higher hepatic IL-6 levels due to conditions such as obesity and/or non-alcoholic fatty liver disease (NAFLD) [99]. When correlating the hepatic apo(a) gene with inflammatory response-associated genes, associations with several typical IL-6 acute phase response genes were found [100]. For example complement C9 ($r=0.61677$), complement C4-binding protein ($r=0.60312$) and three serpin peptidase inhibitors (**table 16**) were correlated with the apo(a) gene expression clearly indicating a relationship of the IL-6 activity and apo(a) expression in human liver *in vivo*.

Table 16: (Acute) inflammatory response-associated genes showing a significant positive correlation with the expression of the hepatic apo(a) gene. Gene symbols displayed in bold type are those being significantly correlated with apo(a) after adjusting the significance level α . Underlined gene symbols represent typical hepatic IL-6 acute phase response genes [100].

Gene symbol	GeneID	Encoded protein	p-value	Correlation coefficient r
<u>BMI < 28 kg/m² (n=13)</u>				
<u>$\alpha_{adj} = 0.006$</u>				
CD40	958	CD40 molecule, TNF receptor superfamily member 5	0.00529	0.74176
NUPR1	26471	nuclear protein, transcriptional regulator, 1	0.00632	0.73077
CCL17	6361	chemokine (C-C motif) ligand 17	0.01713	0.65934
APOA2	336	apolipoprotein A-II	0.02229	0.63736
TIRAP	114609	toll-interleukin 1 receptor (TIR) domain containing adaptor protein	0.03029	0.60989
IL6R	3570	interleukin 6 receptor	0.03603	0.59341
KLKB1	3818	kallikrein B, plasma (Fletcher factor) 1	0.03603	0.59341
CCL16	6360	chemokine (C-C motif) ligand 16	0.04254	0.57692
TNFRSF4	7293	tumor necrosis factor receptor superfamily, member 4	0.04734	0.56593
<u>BMI ≥ 28 kg/m² (n=44)</u>				
<u>$\alpha_{adj} = 0.0009$</u>				
<u>C9</u>	735	complement component 9	0.00001	0.61677
<u>C4BPA</u>	722	complement component 4 binding protein, alpha	0.00001	0.60312
<u>C6</u>	729	complement component 6	0.00009	0.56448
<u>ITIH4</u>	3700	inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	0.00014	0.55095
<u>SERPINC1</u>	462	serpin peptidase inhibitor, clade C (antithrombin), member 1	0.00024	0.53347
<u>CLU</u>	1191	clusterin	0.00036	0.52037

<u>SERPINA1</u>	5265	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	0.00038	0.51868
<u>SERPINA3</u>	12	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	0.00057	0.50416
MASP2	10747	mannan-binding lectin serine peptidase 2	0.00058	0.50388
IGF2	3481	insulin-like growth factor 2 (somatomedin A); insulin; INS-IGF2 readthrough transcript	0.00076	0.49401
C8A	731	complement component 8, alpha polypeptide	0.00102	0.48344
<u>SERPING1</u>	710	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	0.00123	0.47611
KLKB1	3818	kallikrein B, plasma (Fletcher factor) 1	0.00137	0.47202
SERPINF2	5345	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	0.00187	0.45990
CFHR5	81494	complement factor H-related 5	0.00196	0.45821
C2	717	complement component 2	0.00294	0.44158
C5	727	complement component 5	0.00327	0.43707
BLNK	29760	B-cell linker	0.00395	0.42903
IL1RAP	3556	interleukin 1 receptor accessory protein	0.00395	0.42903
PLA2G2A	5320	phospholipase A2, group IIA (platelets, synovial fluid)	0.00479	0.42058
IL17D	53342	interleukin 17D	0.00514	0.41748
NFATC3	4775	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	0.00547	0.41466
CFB	629	complement factor B	0.00561	0.41353
PRDX2	7001	peroxiredoxin 2	0.00689	0.40409
CDO1	1036	cysteine dioxygenase, type I	0.00732	0.40127
C1S	716	complement component 1, s subcomponent	0.00848	0.39436
CFI	3426	complement factor I	0.01089	0.38224
<u>LBP</u>	3929	lipopolysaccharide binding protein	0.01133	0.38027
CLU	1191	clusterin	0.01166	0.37886
C1R	715	complement component 1, r subcomponent	0.01166	0.37886
C1RL	51279	complement component 1, r subcomponent-like	0.01166	0.37886
PRKCA	5578	protein kinase C, alpha	0.02150	0.34579
CD46	4179	CD46 molecule, complement regulatory protein	0.02185	0.34616
CYP4F11	57834	cytochrome P450, family 4, subfamily F, polypeptide 11	0.02573	0.33714
C8B	732	complement component 8, beta polypeptide	0.02712	0.33418
CFH	3075	complement factor H	0.02726	0.33390

<u>C4BPB</u>	725	complement component 4 binding protein, beta	0.03084	0.32685
ORM1	5004	orosomuroid 1	0.03293	0.32304
CXCR2	3579	interleukin 8 receptor, beta	0.03350	0.32206
<u>C3</u>	718	similar to Complement C3 precursor; complement component 3; hypothetical protein LOC100133511	0.03350	0.32206
<u>MBL2</u>	4153	mannose-binding lectin (protein C) 2, soluble (opsonic defect)	0.03523	0.31910
NGF	4803	nerve growth factor (beta polypeptide)	0.03785	0.31411
TF	7018	transferrin	0.03993	0.31163
CX3CL1	6376	chemokine (C-X3-C motif) ligand 1	0.04003	0.31149
IL27	246778	interleukin 27	0.04022	0.31121
TFRC	7037	transferrin receptor (p90, CD71)	0.04145	0.30937
<u>IL1RN</u>	3557	interleukin 1 receptor antagonist	0.04272	0.30754
AOX1	316	aldehyde oxidase 1	0.04302	0.30712
APCS	325	amyloid P component, serum	0.04302	0.30712
NFE2L1	4779	nuclear factor (erythroid-derived 2)-like 1	0.04392	0.30585
CCL16	6360	chemokine (C-C motif) ligand 16	0.04422	0.30543
F2	2147	coagulation factor II (thrombin)	0.04834	0.29993
F12	2161	coagulation factor XII (Hageman factor)	0.04834	0.29993
KL	9365	klotho	0.04966	0.29824

3.4 Tocilizumab inhibits IL-6-induced expression of apo(a) in cultured human hepatocytes

To further study the association between IL-6 and apo(a) and eventually Lp(a) mechanistically on different molecular levels in hepatocytes – the origin of apo(a) synthesis – cultured human hepatocytes were treated with IL-6 and IL-6-receptor-antibody TCZ.

The exposure of human hepatocytes to IL-6 for 12 and 48hrs resulted in a significant upregulation of hepatic apo(a) at mRNA (**figure 9**) and protein levels (**figure 10**), respectively. To confirm that the effect of IL-6 on apo(a) is specific, the cells were co-incubated with IL-6 and IL-6 receptor antibody TCZ. This indeed led to a significant inversion of the effect caused by IL-6 stimulation on apo(a) mRNA (**figure 9**) and protein expression (**figure 10**) indicating the effect of IL-6 on apo(a) to be specific.

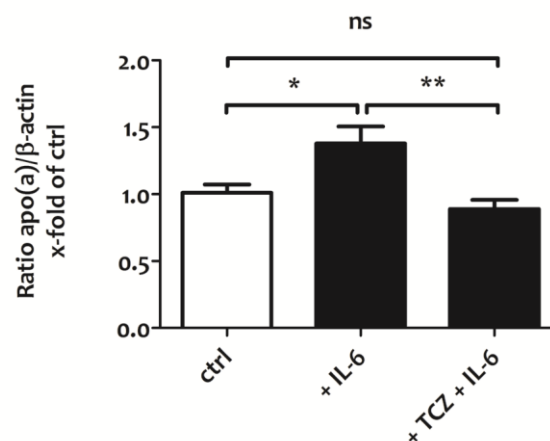


Figure 9: TCZ inhibits IL-6-induced mRNA expression of apo(a) in cultured human hepatocytes. Shown are apo(a) mRNA expression following IL-6 and IL-6 combined with TCZ exposure for 12 hrs in whole cell extracts of cultured human hepatocytes, respectively. β -actin expression served as housekeeping gene in real-time RT-PCR. Data are expressed in ratios of apo(a)/ β -actin and are given as means+SEM of three independent experiments, each performed in duplicate. Significance ($\alpha=0.05$) was tested using ANOVA, *, $p<0.05$; **, $p<0.01$, ns, not significant. Abbreviations: TCZ, tocilizumab; IL-6, interleukin-6; mRNA, messenger RNA; apo(a), apolipoprotein(a); ctrl, control; hrs, hours; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean.

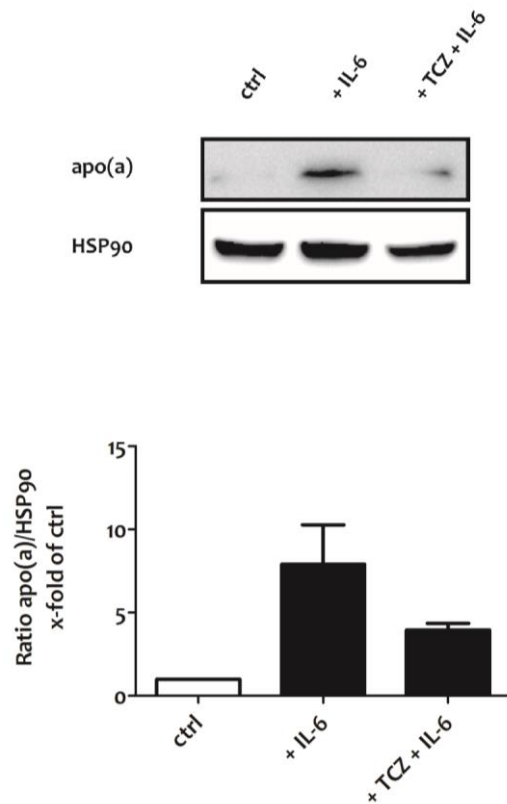


Figure 10: TCZ inhibits IL-6-induced protein expression of apo(a) in cultured human hepatocytes. Shown are apo(a) protein expression following IL-6 and IL-6 combined with TCZ exposure for 48 hrs in whole cell extracts of cultured human hepatocytes. Detection of HSP90 expression served as loading control. The shown immunoblot is one representative out of three independent experiments. Densitometry graphs data in this figure are shown as means+SEM. Abbreviations: TCZ, tocilizumab; IL-6, interleukin-6; apo(a), apolipoprotein(a); ctrl, control; hrs, hours; HSP90, heat shock protein 90; SEM, standard error of the mean.

3.5 Tocilizumab specifically inhibits apo(a) and LDLR promoter activity in cultured human hepatocytes

In order to characterize the effects of IL-6 on apo(a) promoter activity, the full length promoter of apo(a) was cloned into the pGL3 basic plasmid and subsequently luciferase reporter gene assays in human hepatocytes were performed. In this experiment, it was found that IL-6 is able to activate the apo(a) promoter (**figure 11A**). When co-incubated with TCZ and IL-6, IL6-induced apo(a) promoter activity decreased down to the baseline (without stimulation) (**figure 11B**). However, having co-stimulated the cells with TNF- α inhibitor ADB, apo(a) gene promoter activity did not change (**figure 11B**) indicating the effect to be specific for IL-6.

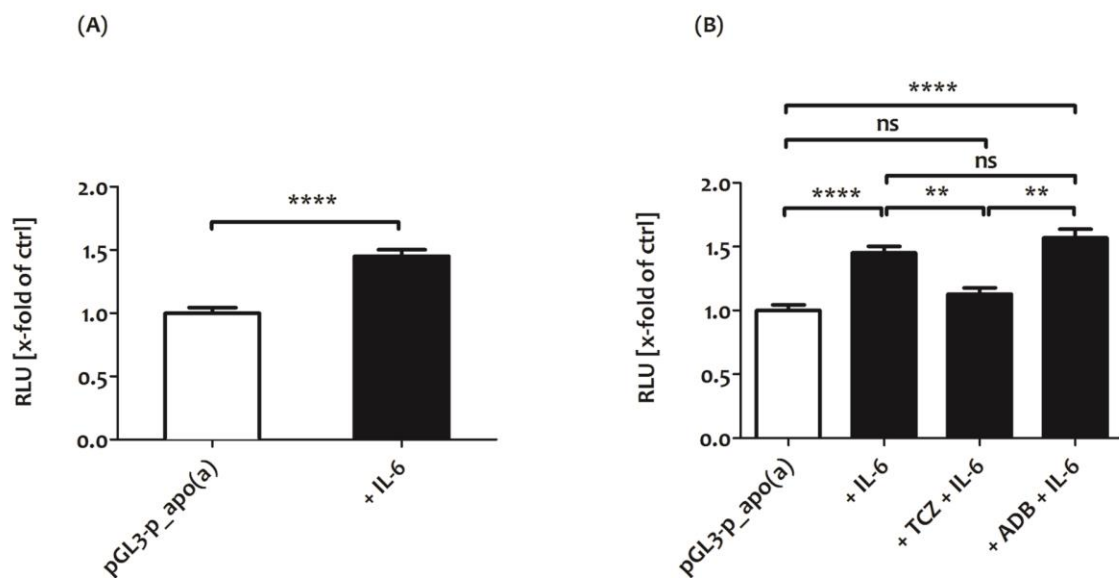


Figure 11: TCZ specifically inhibits apo(a) promoter activity in cultured human hepatocytes. Shown is apo(a) promoter activity stimulated with IL-6 (11A+11B), TCZ or ADB (11B) for 24 hrs in cultured human hepatocytes. Data are expressed in RLU [x-fold of ctrl] and are given as means+SEM of five independent experiments, each performed in duplicate. Statistical significance ($\alpha=0.05$) was tested using Mann-Whitney U or Kruskal-Wallis test. **, $p < 0.01$; ****, $p < 0.0001$; ns, not significant. Abbreviations: TCZ, tocilizumab; ADB, adalimumab; IL-6, interleukin-6; apo(a), apolipoprotein(a); hrs, hours; RLU, relative light units; SEM, standard error of the mean.

In addition, it was investigated whether IL-6 only influences the determining moiety of Lp(a) – apo(a) – or whether also the low density lipoprotein (LDL) cholesterol metabolism which is being involved in Lp(a) synthesis. Therefore, the LDLR promoter cloned into a luciferase construct was examined as described above. In this assay, it was shown that IL-6 is able to induce the LDLR promoter to the same extent as the apo(a) promoter (**figure 12A**). When co-incubated with TCZ and IL-6, IL-6-induced LDLR promoter activity decreased down to the baseline (without stimulation) (**figure 12B**). However, having co-stimulated the cells with ADB, LDLR gene promoter activity did not change (**figure 12B**) indicating the effect to be specific for IL-6.

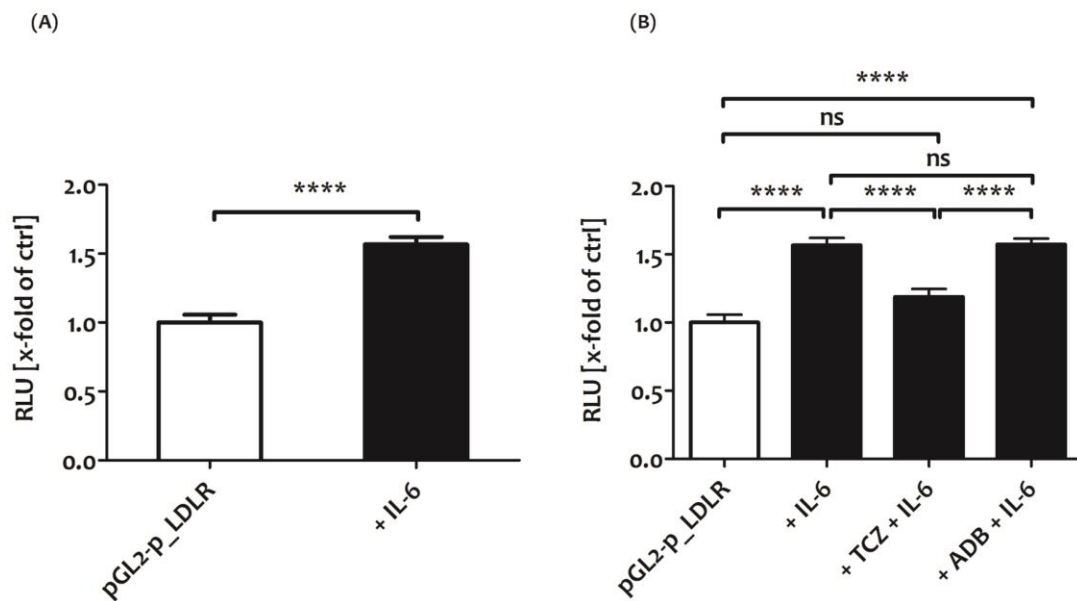


Figure 12: TCZ specifically inhibits LDLR promoter activity in cultured human hepatocytes. Shown is LDLR promoter activity stimulated with IL-6 (12A+B), TCZ or ADB (12B) for 24 hrs in cultured human hepatocytes. Data are expressed in RLU [x-fold of ctrl] and are given as means+SEM of five independent experiments, each performed in duplicate. Statistical significance ($\alpha=0.05$) was tested using student's t-test or ANOVA. ****, $p<0.0001$; ns, not significant. Abbreviations: TCZ, tocilizumab; ADB, adalimumab; IL-6, interleukin-6; LDLR, low density lipoprotein receptor; hrs, hours; RLU, relative light units; SEM, standard error of the mean.

Next, it was examined whether the promoter of the fatty acid synthase (FAS) as an additional gene of lipid metabolism may also be activated by IL-6 in order to exclude unspecific metabolic effects. This experiment revealed that IL-6 did not activate the FAS promoter (**figure 13**), again indicating specificity of the effect of IL-6 on the apo(a) and LDLR promoter.

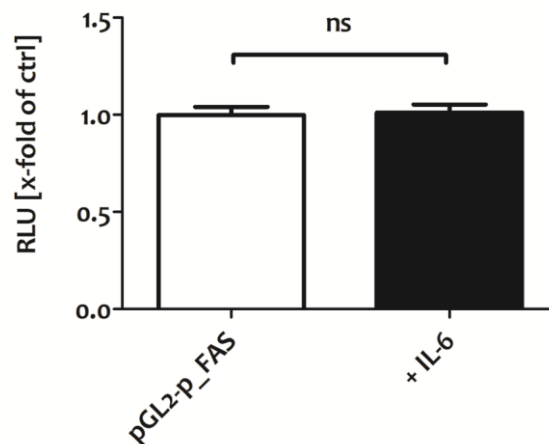


Figure 13: IL-6 does not activate FAS promoter activity in human hepatocytes. Shown is FAS promoter activity stimulated with IL-6 for 24 hrs in cultured human hepatocytes. Data are expressed in RLU [x-fold of ctrl] and are given as means+SEM of five independent experiments, each performed in duplicate. Statistical significance ($\alpha=0.05$) was tested using student's *t*-test. ns, not significant. Abbreviations: IL-6, interleukin-6; FAS, fatty acid synthase; hrs, hours; RLU, relative light units; SEM, standard error of the mean.

3.6 Tocilizumab regulates apo(a) promoter activity via a responsive element at position -46 to -40

The apo(a) promoter contains six established IL-6 responsive elements (IL-6-RE) which is why the full length apo(a) promoter was truncated and six constructs generated, the first containing the fragment including all six IL-6-REs, the sixth only containing IL-6-RE 6 (**figure 14**).

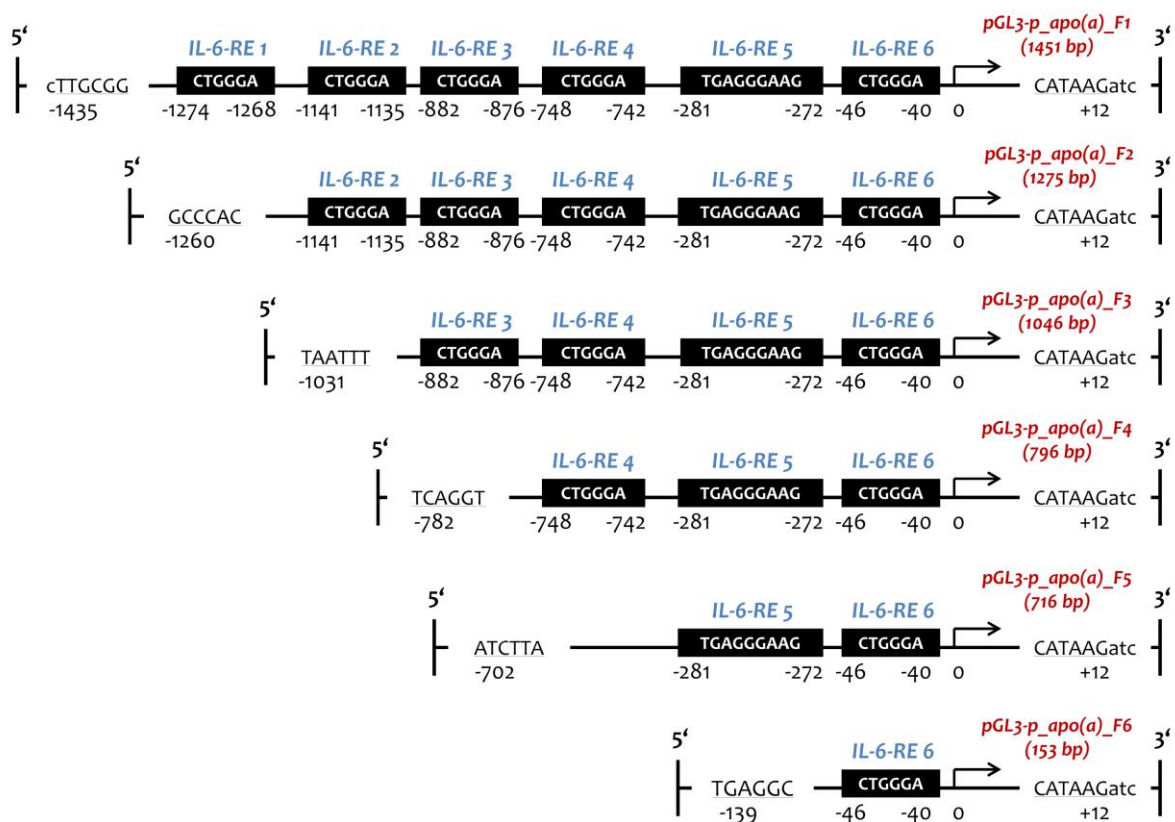


Figure 14: Schematic design of truncated apo(a) promoter constructs. Shown are all six generated constructs (5'-3' direction), the first containing the fragment including all six IL-6-REs (1451 bp), the sixth only containing IL-6-RE 6 (153 bp). Base sequences and positions of each IL-6 responsive element are displayed in each promoter construct pGL3-p_apo(a)_F1-F6. Start of transcription is designated as 0 (ATG start codon). Abbreviations: IL-6 RE, interleukin-6 responsive element; bp, base pair.

Next, luciferase reporter gene assays were performed by incubating transfected human hepatocytes with IL-6 for 24 hrs in order to examine which IL6-RE/s is/are responsible for the transcriptional activity. The results indicated that IL6-REs 1-5 may not be important for the expression of the apo(a) gene whereas IL-6-RE 6 at -46 to -40 seems to be the major regulatory element when bound to transcription factor Signal transducer and activator of transcription 3 (STAT3) (**figure 15A**). Since it was shown that only IL-6-RE 6 regulates the transcription, cells transfected with the promoter construct containing IL-6-RE 6 (designated as pGL3-p_apo(a)_F6 (**figure 14**)) were further co-stimulated with TCZ and IL-6 showing that the effect of IL-6 is inhibited by TCZ (**figure 15B**).

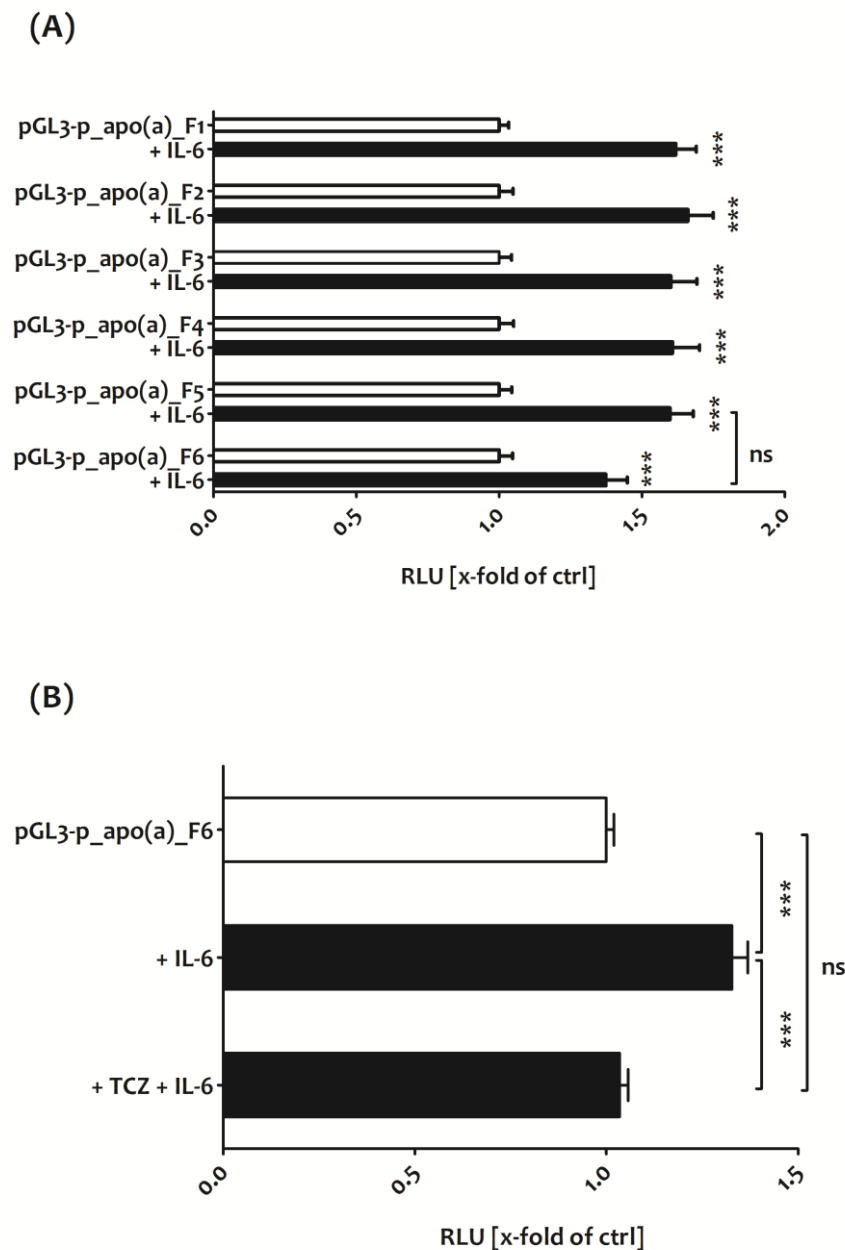


Figure 15: IL-6 responsive element 6 of apo(a) promoter confers major transcriptional activity following IL-6-dependent binding of transcription factor STAT3. Shown are IL-6-induced apo(a) promoter activities (in RLU [x-fold of ctrl]) (15A) of truncated apo(a) promoter in cultured human hepatocytes (24 hrs). (15B) Shows inhibition of IL-6-induced promoter activity of truncated apo(a) promoter containing IL-6 responsive element 6 by TCZ. Data are expressed in x-fold of control and are given as means+SEM of five independent experiments, each performed in duplicate. Statistical significance ($\alpha=0.05$) was tested using student's t-test or ANOVA. ***, $p<0.001$; ns, not significant. Abbreviations: TCZ, tocilizumab; IL-6, interleukin-6; hrs, hours; RLU, relative light units; SEM, standard error of the mean.

To further analyze TCZ effects on apo(a), electrophoretic mobility shift assays (EMSA) were additionally carried out for IL-6-RE 6. This analysis showed that IL-6 specifically activated STAT3 consequently binding to IL-6-RE 6 (**figure 16**). To confirm specificity of the binding of STAT3 to IL-6-RE 6, a STAT3 antibody was used in a supershift experiment. As shown in **figure 16**, pre-incubation with that antibody abolished binding of STAT3 to the IL-6-RE 6, proving specificity of the effect.

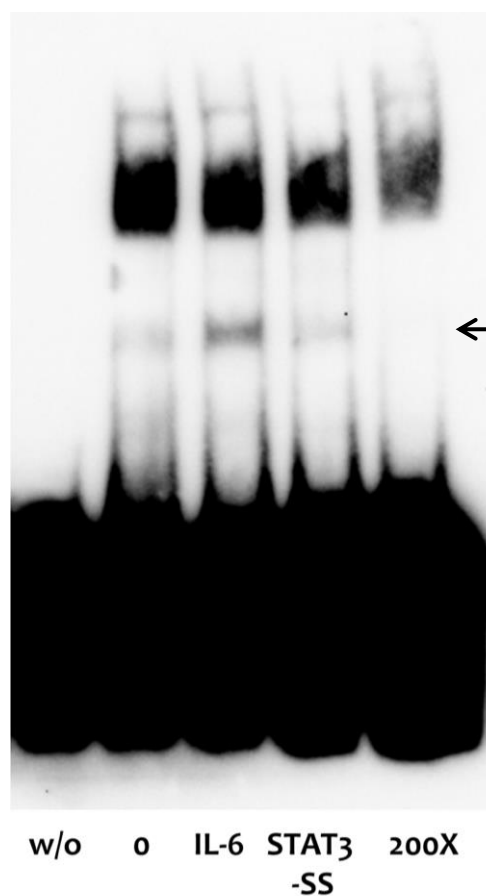


Figure 16: IL-6-dependent binding of STAT3 to IL-6 responsive element 6 of apo(a) promoter for 3 hrs. Shown is one representative EMSA out of at least three independent experiments. STAT3 supershift antibody specifically blocks the DNA binding domain resulting in disappearance of the STAT3 shift (lane IL-6). *Abbreviations: IL-6, interleukin-6; STAT3, Signal transducer and activator of transcription 3; min, minutes; 200X, 200-fold excess of non-labelled oligonucleotide, DNA, deoxyribonucleic acid.*

4. DISCUSSION

Targeting cardiovascular risk factors is more and more considered to be important in the prevention of cardiovascular diseases (CVD). Modifiable cardiovascular risk factors basically comprise those of the metabolic syndrome such as hypertension, overweight/obesity, raised blood glucose and disturbances in lipid metabolism but also physical inactivity, tobacco use and unhealthy diet. All these factors in the long term contribute to the pathogenesis of atherosclerosis, the major cause of CVD. Generally lipids but specifically the LDL-cholesterol containing particles, amongst those lipoprotein(a) [Lp(a)], are most essential for initiating the atherosclerotic process (see 1.2.1).

Lp(a) is a structurally and functionally unique lipoprotein consisting of the glycoprotein apolipoprotein(a) which is covalently bound to a LDL-like moiety [57]. The latter is composed of a central core of cholesteryl esters and triglycerides (TG) surrounded by phospholipids, free cholesterol and a single molecule of apoB [101]. The apo(a) moiety on the other hand shares a high degree of sequence homology (~90 %) with the corresponding sequence of plasminogen [58]. Both properties can potentially increase CVD risk a) via prothrombotic/anti-fibrinolytic effects, b) via activating monocyte adhesion via monocyte chemoattractant protein-1 [102] and c) via accelerated atherogenesis as a result of intimal deposition of Lp(a), cholesterol, or both [103] and oxidized phospholipids [104] (see 1.3). Hence, Lp(a) is thought to combine activation of the immune system, atherogenesis and local thrombosis; therefore being highly atherogenic.

Up to date, there are no effective treatment options available except for the costly, labor-intensive and invasive lipid aphaeresis. Niacin, when given in high doses (2-3 g/day) [86, 87], was

considered as approved drug to effectively lower Lp(a) levels. However, these doses of niacin can be associated with headaches, flushing and liver toxicity and finally non-compliance. Also, due to unfavorable side effects, a special slow release formulation of niacin (Tredaptive®) was recently withdrawn from the market. Aspirin at low doses was further shown to decrease Lp(a) serum levels slightly [88] indicating rather minor importance. For this reason, alternative well-tolerated and more convenient therapeutic approaches are required to decrease elevated Lp(a) serum levels.

In a recent clinical study by our group it was shown that a single dose of intravenous (i.v.) IL-6 receptor antibody tocilizumab (TCZ) (8 mg/kg body weight) is able to induce a profound and significant reduction in serum Lp(a) by up to 50 % in rheumatoid arthritis (RA) patients [95]. Interestingly, this effect was even more pronounced than that of niacin [105], indicating TCZ to be the most powerful pharmacological agent to lower Lp(a). In addition, due to the mode of action of TCZ to block the IL-6 receptor site, this leads to the assumption that IL-6 may be a major regulator of Lp(a) *in vivo*.

To further clarify the above hypothesis, in the present thesis, Lp(a) serum levels were determined in RA patients (n=12) before and after receiving a tumor necrosis factor (TNF)- α -antibody (adalimumab (ADB)) treatment for a time period of three months. This clinical trial served to test whether apo(a) secretion and thus Lp(a) assembly may be enhanced via any kind of pro-inflammatory cytokine or whether specifically IL-6 activates apo(a). Since ADB did not affect serum Lp(a) serum levels, this experiment clearly showed that lowering of Lp(a) due to TCZ is not the result of a reduction in general inflammatory activity but is a specific effect of IL-6 on Lp(a) metabolism (**figure 4**).

To strengthen this conclusion in a larger sample size, n=1153 human subjects were enrolled in the scope of the FoodChainPlus (FoCus) project (see 2.1.10). This cohort was initially characterized regarding their metabolic and anthropometric parameters to ensure a metabolic validity of the study population.

Analyzing the prevalence of overweight and obesity ($\text{BMI} \geq 25 \text{ kg/m}^2$), insulin resistance using HOMA-IR, C-reactive protein (CRP), IL-6, TG and Lp(a) serum levels (**table 15**), it was shown that more than half of the study population (69.93 %) above 18 years of age exhibited a BMI above 25 kg/m^2 (**table 15**). This nicely fits with the data from the National Health and Nutrition Examination Survey (NHANES), a nationally representative cross-sectional survey of the U.S. Department of Health and Human Diseases (Centers for Disease Control and Prevention (CDC) and National Center for Health Statistics), in which it was demonstrated for the time period 2009-2010 that 69.2 % of the study sample (n=5926) above 20 years of age were overweight or obese [106]. 44.93 % of the FoCus cohort had a BMI above 30 kg/m^2 (**table 15**). In the NHANES cohort however, only 35.9 % of the study sample were obese [106] suggesting in the FoCus cohort obesity to be basically more present than in the NHANES study. To compare the prevalence of obesity with NHANES in the time period the FoCus cohort was conducted, the prevalence in NHANES did not change between 2009-2010 and 2011-2012 [107] indicating that the increase by 9.03 % between the FoCus cohort and NHANES is most likely not due to general changes in western life style but rather to the different individuals included into the cohort.

When examining the FoCus cohort for the occurrence of impaired insulin signaling or insulin resistance, this analysis showed that 60.97 % of the study sample exhibited a HOMA-IR of ≥ 2 , (**table 15**). HOMA-IR is calculated of fasting insulin and glucose serum concentrations. The median insulin serum concentration in the FoCus cohort was 10.50 $\mu\text{U/mL}$ (**table 15**). Compared with NHANES data of 1999-2002 (n=2993 aged 20 and above) the median of fasting insulin concentrations was 8.95 $\mu\text{U/mL}$ [108]. This fact indicates that the prevalence of disturbances in

insulin signaling may have increased during the last ten years. Another cause for the higher median in the FoCus cohort may be the higher prevalence of obesity that is associated with increased occurrence of insulin resistance due to low-grade inflammation. Moreover, the NHANES cohort was restricted to non-diabetic adults whereas in the FoCus cohort diabetics were not explicitly excluded. This may be an explanation for the lower insulin median in the NHANES study. However, since the median of fasting insulin concentrations has already risen by 0.93 $\mu\text{U/mL}$ from NHANES III 1988-1994 ($n=7926$) to NHANES 1999-2002 (8.02 and 8.95 $\mu\text{U/mL}$, respectively) [108], this change may be realistic.

More than one third (40.16 %) of the study population of the FoCus cohort exhibited elevated serum levels of CRP (**table 15**), a marker of inflammation and identified as a risk factor for CVD and mortality. In the US population NHANES IV (1999-2002; $n=7634$) 37.2 % human subjects aged 20 years and older had serum CRP levels above 3.1 mg/L [109] indicating a similar prevalence of CRP serum levels in both cohorts. Differences may be reasoned by time changes (1999-2002 vs. 2011-now) or the higher prevalence of obesity in the FoCus cohort.

Analyzing Lp(a) prevalence, in the FoCus cohort the mean Lp(a) serum level was 22.4 mg/dL being similar with data from the NHANES III study ($n=2631$, ≥ 18 years of age) of non-hispanic whites which reflects the ethnicity studied in the FoCus cohort. In this study mean Lp(a) serum levels were 20.3 mg/dL [110]. In addition, the prevalence of Lp(a) serum levels higher than 300 mg/L representing a high risk to develop CVD (also see 2.1.10) was 23.07 % in the FoCus cohort. This fits nicely with the Lp(a) prevalence of a study examining $n=720$ healthy middle-aged Caucasian employees [111].

The prevalence of elevated serum IL-6 (≥ 6 pg/mL, see 2.1.10) within the FoCus cohort was 17.17 % (**table 15**). IL-6 is just as CRP an inflammatory protein. It induces CRP through activating Janus kinases suggesting related roles for both proteins. Signal transducers and activators of transcription subsequently switch on the CRP gene expression, leading to most of the CRP

production [98]. This leads to the hypothesis that the IL-6 prevalence may be in a proportional relation to this one of CRP suggesting a similar IL-6 prevalence in the respective NHANES cohort.

When analyzing the prevalence of elevated or high serum TG concentrations it was shown that 12.75 % of the individuals exhibit TG serum levels above 200 mg/dL (**table 15**). This is considered as hypertriglyceridemia and connected to an increased risk for CVD. In comparison, in the NHANES study cohort 16.2 % of adults exhibited TG serum levels above 200 mg/dL with no appreciable change between NHANES 1988-1994 and 1999-2008 [112]. It is to mention at this point that the prevalence of high risk Lp(a) serum levels was higher than this of high risk TG serum levels in the FoCus cohort. Due to the higher atherogenicity of Lp(a), and also due to its 'double' character of also being pro-thrombotic when compared to TG, this suggests that high risk Lp(a) serum levels should be paid even more attention to with respect to its pathophysiology and consequent treatment options alternative to lipid aphaeresis.

In the FoCus cohort, moderate correlations were found for HOMA-IR and BMI ($r_s=0.617$, $p<0.0000001$), CRP and BMI ($r_s=0.614$, $p<0.0000001$) as well as CRP and IL-6 ($r_s=0.532$, $p<0.0000001$) (**figure 5**).

The correlation between HOMA-IR and BMI is in concordance with a study from Trirogoff *et al.* [113] revealing a correlation coefficient of 0.496 between HOMA-IR and BMI in $n=95$ non-diabetic chronic kidney disease patients but also in $n=36$ matched control subjects. These differences in the strength of correlation between HOMA-IR and BMI may be due to different mean BMIs within both cohorts (28 kg/m² vs. 31.96 kg/m² in FoCus (**table 15**)) or to the lower sample size in this study.

Furthermore, the significant positive association between CRP and BMI was for example verified in a cross-sectional European Study from Copenhagen ($n=21836$) [114] and in a study using NHANES data of $n=16616$ individuals [115]. Both studies showed that CRP levels were higher with

higher BMI providing evidence, especially due to the huge sample size, for CRP to be a marker of elevated adiposity. Since the cytokine IL-6 is released by human adipose tissue, and IL-6 stimulates the production of acute phase proteins such as CRP, this might explain the observed associations between CRP and BMI.

Since CRP is then released by the liver into the circulation one may assume a direct correlation between serum IL-6 and CRP. The association was not only confirmed in the present study (**figure 5**) but also in a British study consisting of 107 human subjects in whom a significant relationship between CRP and IL-6 was shown as well [116].

In this study, weak correlations were found between HOMA-IR and TG ($r_s=0.493$, $p<0.0000001$), HOMA-IR and CRP ($r_s=0.454$, $p<0.0000001$), BMI and IL-6 ($r_s=0.445$, $p<0.0000001$), BMI and TG ($r_s=0.434$, $p<0.0000001$), HOMA-IR and IL-6 ($r_s=0.379$, $p<0.0000001$) and between CRP and TG ($r_s=0.333$, $p<0.0000001$) (**figure 6**).

An imbalance between energy expenditure and uptake results in free fatty acids (FFAs) excessively released from visceral fat and the up-regulation of insulin signaling-impairing adipocytokines. FFAs in the next step serve as substrate for the synthesis of TG and VLDL hence leading to hypertriglyceridemia (also see 1.1.3). According to this pathophysiology one might expect a direct relation between HOMA-IR and TG serum levels. That was confirmed in the present study ($r_s=0.493$) (**figure 6**). The Korean NHANES data from 2007-2009 however showed that TG and HOMA-IR are significantly but rather negligibly correlated ($r_s=0.195$ for men and $r_s=0.191$ for women) in 7623 participants [117]. It is possible that the ethnicity has a substantial influence with respect to this correlation. That there is indeed provided evidence for ethnicity differences regarding this correlation was nicely confirmed by a study of Sumner *et al.* [118]. They suggested that TG serum levels are not markers of insulin resistance in African Americans. This was repeatedly shown in a study from the University of Yale in which an association

between the TG/HDL ratio and HOMA-IR in African Americans compared to white youths was not found [119]. Therefore, it is possible, that Korean individuals do not exert such strong correlations between TG and insulin resistance when compared to Caucasians, as well. However, clarifying studies are required with regard to this hypothesis.

CRP has not only been reported to be positively correlated with HOMA-IR ($r_s=0.468$) but also with TG ($r_s=0.111$) in an Italian study population of $n=201$ [120] being in concordance with the data presented in this work (**figure 6**). However, in this study the association between CRP and TG was far stronger which might be due to the generally higher prevalence of obesity in the FoCus cohort (**table 15**).

IL-6 is similarly correlated with HOMA-IR and with BMI in the FoCus study (**figure 6**). A very simple explanation might again be the release of excessive FFAs by adipose tissue and subsequently of many adipocytokines, e.g. IL-6, which then impair insulin signaling and induce the development of insulin resistance finally resulting in increased HOMA-IR. Additionally, very similar correlations were repeatedly shown in a study by Leinonen *et al.* [121] revealing a correlation coefficient of 0.324 (HOMA-IR) and 0.336 (BMI) in $n=135$ Finnish individuals.

Inversely, as TGs were shown to be weakly correlated with HOMA-IR, BMI should also be correlated with TGs according to the described pathophysiology of insulin resistance. Indeed, this was demonstrated not only in the FoCus cohort (**figure 6**) but also in a study of Turcato *et al.* examining $n=146$ women and $n=83$ men [122].

Rather negligible but significant correlations were found between TG and IL-6 ($r_s=0.258$, $p<0.0001$), Lp(a) and CRP ($r_s=0.107$, $p=0.0003$), Lp(a) and IL-6 ($r_s=0.102$, $p=0.0005$), Lp(a) and BMI ($r_s=0.093$, $p=0.0015$), Lp(a) and HOMA-IR ($r_s=0.073$, $p=0.0129$) and Lp(a) and TG ($r_s=0.071$, $p=0.0164$) (**figure 7**).

The correlation between TG and IL-6 is in line with a study of Cardellini *et al.* [123] showing a similar correlation coefficient of 0.170 in a sample size of $n=470$ Italian Caucasians.

In the FoCus cohort, a positive significant but negligible correlation was shown between IL-6 and Lp(a) (**figure 7**). As it is well-known that apo(a) is under strict genetic control [69], and as it was also shown that the correlation between IL-6 and Lp(a) ($r=0.276$, $p=0.0065$) alters when dividing into individuals having high or low apo(a) isoforms [124], the FoCus cohort was divided into a normal IL-6 group and an elevated IL-6 group in order to show an impact of IL-6 on Lp(a) *in vivo*, independently of the apo(a) genotype. This analysis showed, that Lp(a) is significantly different in both groups (**figure 8**) indicating a role for IL-6 in Lp(a) metabolism not only in a small study population (**figure 4**).

IL-6 is a direct inducer of CRP. Therefore, it seems obvious, that a similar correlation and association between Lp(a) and CRP serum levels will be found accordingly. Indeed, in the FoCus cohort an almost equal correlation coefficient ($r_s=0.107$ for CRP vs. $r_s=0.102$ for IL-6) for the correlation between Lp(a) and CRP serum levels was evident. Just as for IL-6 and Lp(a), Lp(a) serum levels were determined in a normal and an elevated CRP group. In that analysis, significantly higher Lp(a) serum levels were found in the elevated CRP group (**figure 8**). This is in line with a study showing that Lp(a) serum levels are significantly increased in hemodialysis patients exhibiting elevated CRP levels [125].

BMI in the FoCus cohort was significantly associated but rather not correlated with Lp(a) serum levels. In contrast, for example, it was shown in a study of Lamon-Fava *et al.* [126] that BMI is not associated with Lp(a) serum levels indicating not fully elucidated data concerning that relationship. However, since a higher BMI is correlated with higher IL-6 (as shown in **figure 6**) and since there is now a reliable proof for the relationship between IL-6 and Lp(a), this argues for a positive association between BMI and Lp(a), as shown in the present thesis.

Also, the relationship of Lp(a) and insulin resistance is controversial and unproven. For example, decreased Lp(a) levels in response to hyperinsulinemia [127] and a negative relationship between Lp(a) levels and the incidence of type 2 diabetes mellitus (T2DM) have been reported previously [128, 129]. In contrast, some studies have shown a strong positive association between T2DM and elevated Lp(a) levels [130, 131]. Similar Lp(a) concentrations in subjects with T2DM and healthy controls have been reported in one study [132]. However, in the FoCUS cohort a positive significant but low correlation was found (**figure 7**). Due to the strict genetic control of the apo(a) gene [69], the cohort was also classified into a group exhibiting HOMA-IR below 2 and one group with HOMA-IR above 2 (**figure 8**) in order to eliminate the genetic influence. In this analysis though, a significant difference of Lp(a) serum levels between individuals having an HOMA-IR below or above 2 was found (**figure 8**). A cross-sectional population-based study (n=1030) showed that an inverse association between HOMA-IR and Lp(a) serum levels [133]. However, with respect to previous performed correlations in the FoCUS cohort (**figure 5-7**), it has to be admitted that IL-6 was found to significantly correlate with HOMA-IR (**figure 6**) and Lp(a) serum levels to be significantly higher with higher IL-6 serum levels (**figure 8A**). Therefore, one might speculate that the positive association between HOMA-IR and Lp(a) serum levels found in the scope of this cohort seems realistic.

Data on relationships between TG and Lp(a) revealed a negative significant correlation ($r=-0.120$, $p<0.001$) in 1200 hyperlipidemic individuals [134]. This is contradictory to the FoCUS data showing that TG and Lp(a) are positively, even if negligibly, correlated with TG serum levels ($r_s=0.071$, $p=0.0164$) (**figure 7**). However, it has to be taken into account that the FoCUS cohort is not only composed of hyperlipidemic individuals but also of normolipidemic individuals (**table 15**). On the other hand, as this only seems to be a slight shift, the genetic variability of the apo(a) gene might be responsible for that, as well.

In conclusion, using a very representative study population including many thousands of human subjects (NHANES) and many other smaller human studies, the Focus cohort reflects a metabolically valid study population regarding the prevalence of elevated or high-risk parameters and also regarding the correlations between different metabolic and anthropometric parameters. One exception is the metabolic parameter Lp(a) which partly revealed contradictory data that is, as mentioned, likely due to genetic variability of the apo(a) gene. However, subsequent association studies between Lp(a) and the above mentioned parameters showed that IL-6 and IL-6 acute phase reactant CRP were strongest associated with Lp(a) (**figure 8A+B**). However, BMI and HOMA-IR were also significantly related to Lp(a) serum levels. Interestingly, Lp(a) serum levels rather seem to be related to inflammatory parameters (IL-6, CRP, BMI and HOMA-IR), as Lp(a) serum levels were not significantly different between the TG groups (below or above 200 mg/dL) (**figure 8F**). The strength of those associations is also reflected in the corresponding correlation coefficients of those parameters to Lp(a) (**figure 7B-F**). In addition, this nicely confirms the hypothesized link between IL-6 and Lp(a) that was shown already in two independent experiments [95, 135] (**figure 4**).

Therefore, in the following this thesis aimed to further examine the effect of IL-6 on hepatic apo(a) – the determining moiety of Lp(a) synthesis. A microarray experiment using human liver biopsies confirmed what was found in the aforementioned *in vivo* studies, since five typical IL-6 target genes were positively and significantly correlated with the apo(a) gene expression (**table 16**).

The found association between IL-6 and apo(a) [69] was further investigated mechanistically on different molecular levels in hepatocytes – the origin of apo(a) synthesis.

In these experiments human hepatocytes were treated with IL-6 and IL-6 receptor antibody TCZ. This experiment suggested apo(a) mRNA and protein expression to be significantly induced by IL-6 exposure, being in agreement with the data of Kagawa *et al.* [136] and Ramharack *et al.* [137]. In addition to these previous reports, in the present study it was shown that IL-6-induced apo(a) expression is significantly inhibited by TCZ in cultured hepatocytes (**figure 9, figure 10**) supporting previous findings [95].

To further study the apo(a) gene promoter the full length apo(a) promoter was cloned to investigate effects of IL-6 and TCZ as well as TNF- α and TNF- α -antibody ADB on apo(a) promoter activity. As shown in **figure 11A+B**, IL-6 did significantly induce apo(a) promoter activity and the IL-6 effect described in **figure 11** was abolished by TCZ. The anti-TNF- α antibody ADB did not neutralize the IL-6-induced apo(a) promoter activity again suggesting TCZ to specifically decrease IL-6-triggered apo(a) expression. This is in line with the human *in vivo* data of **figure 4** in which Lp(a) serum levels remained unaffected following TNF α -antibody treatment.

To show that IL-6 does not unspecifically activate other hepatic genes associated with lipid metabolism, cultured hepatocytes were additionally transfected with the fatty acid synthase (FAS) promoter and treated with IL-6. This, however, did not lead to an induction of the FAS promoter activity (**figure 13**) suggesting that the effect of IL-6 on lipid metabolism is specific for Lp(a) rather than an unspecific alteration of liver function.

Promoter truncation experiments of the six established IL-6 responsive elements (RE) of the apo(a) promoter ranging from position -1435 to +12 (**figure 14**) revealed that IL-6 RE 6 confers major transcriptional activity (**figure 15**) following IL-6-dependent binding of transcription factor STAT3. According to Wade *et al.* the major transcription start site appears to occur at 141 bases before the translation start ATG [93]. Those 141 bases do only comprise IL-6 RE 6 (-46 to -40) but not 1, 2, 3, 4 or 5 and might therefore be an explanation why only IL-6 RE 6 seems to confer

major transcriptional activity of apo(a) (**figure 15**). IL-6-induced transcriptional activity mediated via IL-6 RE 6 was inhibited in the presence of TCZ (**figure 15**).

It is known for IL-6 to induce/mediate acute phase reactions. This takes place via the JAK/STAT (Janus kinase (JAK)/ Signal Transducer and Activator of Transcription (STAT)) signaling. IL-6 signals through its receptor IL-6R/IL6ST (IL-6 signal transducer or gp130), activates Janus kinases which in turn phosphorylate and activate STATs (signal transducer and activator of transcription), including STAT3. Phosphorylated Stat3 dimerize and translocate into the nucleus to regulate target gene transcription. For this reason, EMSAs using complementary oligonucleotides to each IL-6 RE and STAT3 as supershift antibody were performed in which the luciferase assay data were confirmed (**figure 16**).

In conclusion, all these data at different levels of molecular examination suggest that IL-6 is indeed a major regulator of apo(a) and eventually Lp(a), and that increased apo(a) expression can be decreased by TCZ.

However, TCZ has also been found to increase LDL-cholesterol concentrations *in vivo*, which is why some authors argued that IL-6 inhibition might negatively influence CVD risk [138]. Indeed, in the scope of this thesis, it was shown on a molecular level that the LDL-receptor expression in cultured hepatocytes is up-regulated by IL-6 and blunted by the additional exposure of TCZ indicating a LDL-cholesterol elevation (**figure 12**) [139]. However, so far all long-term studies on TCZ-treated patients failed to demonstrate increased CVD mortality. In this respect, it is important to mention that the MEASURE study [140] clearly showed that the increase of LDL-cholesterol in TCZ-treated human subjects is mostly due to an increase in high- and normal dense LDL-particles whereas small-dense LDL particles being most important for atherosclerosis-progression, are not affected by IL-6 inhibition due to TCZ [140]. In addition, paraoxonase being an enzyme principally responsible for protecting LDL against oxidation was significantly

increased in the TCZ-treated group compared to the placebo group. This might explain the insignificant change of oxidized LDL, well known to promote atheroma formation though (also see 1.2), in the TCZ-treated group compared to placebo. Therefore, from the data reported so far it cannot be concluded that TCZ is pro-atherogenic or is increasing CVD risk. In complete contrast, the data on TCZ and Lp(a) reported in two clinical trials [95, 135] and the present thesis suggest that TCZ indeed seems to transfer anti-atherogenicity by reducing the most pro-atherogenic lipid particle, Lp(a).

The data observed here may suggest that TCZ might be a future treatment option in patients with progressive CVD due to elevated Lp(a). In addition to CVD in general, these results may be of major importance for example in the differential therapy of RA patients for whom TCZ is approved. As several pharmaceutical agents are available for RA, elevated Lp(a) serum levels thus may be a pivotal additional indication in order to decide for the use of TCZ infusions instead to use e.g. TNF- α -inhibitors. In the past, the decision for the use of a biological agent was made mainly on disease-specific parameters. For example, in subjects with high titers of cyclic citrullinated peptide (CCP) antibodies rituximab is often suggested [141] and in patients who do not tolerate methotrexate, TCZ might be most effective [142]. The data reported here suggested that in the future the decision for a specific biological agent might not only be made on immunological disease-specific parameters but also on metabolic co-morbidities. From this point of view the previous two clinical trials [95, 135] and the present thesis suggest that for a patient suffering from both, RA and profoundly increased Lp(a) concentrations, TCZ should be the first choice as a first line biological agent if disease-modifying antirheumatic drug (DMARD) therapy is not sufficient in these subjects.

Up to date, TCZ is only approved as a biological drug for the treatment of RA, Castleman's disease and systemic juvenile idiopathic arthritis. Therefore, there is need for further clinical

trials evaluating the efficacy and safety of TCZ in individuals not suffering from RA. Indeed, it was reported for Lp(a) to be elevated in many autoimmune and chronic inflammatory diseases (CID) such as Systemic Sclerosis, Systemic Lupus Erythematosus, Antiphospholipid Syndrome and RA or Crohn's disease [91, 143]. In those, it has been shown that IL-6 production is implicated in the diseases' pathogenesis [144-146] suggesting IL-6 as likely to be responsible for even higher Lp(a) serum levels in these patients. Thus, if TCZ in these diseases would be considered for treatment, this concomitantly would mean another step forward in the treatment of elevated Lp(a) and hence in reducing the CVD risk in even more affected patients. In addition, it would be interesting whether TCZ will also be able to reduce elevated Lp(a) serum levels and hence CVD risk, in individuals not suffering from CID.

In summary, this thesis presents several lines of evidence that (1) IL-6 specifically induces apo(a) and eventually Lp(a) on different molecular levels *in vivo* and *in vitro* and that therefore (2) IL-6 blockade by monoclonal antibodies might be a promising future therapy to treat elevated Lp(a) serum levels and to reduce CVD risk in affected patients. TCZ might therefore be a convenient alternative to the costly, labor-intensive and invasive lipid aphaeresis.

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Congresses, Presentations and Posters

- DZD Diabetes Research School together with the XII International Symposium on Insulin Receptors and Insulin Action, Barcelona, November 2013
- 15th Annual Meeting of YARE joint with 1st EYES meeting in Rotterdam, October 2013
Diabetes Congress in Leipzig, May 2013
- Interdisciplinary Symposium of the Cluster of Excellence ‘Inflammation at Interfaces’ in Hamburg, February 2013
- 14th Annual Meeting of YARE in Dresden, October 2012
- Diabetes Congress in Stuttgart, May 2012

Awards

05/2012	Poster Award, Diabetes Congress in Stuttgart
11/2013	Attendance and accommodation grant, Diabetes Research School and XII International Symposium on Insulin Receptor and Insulin Action in Barcelona
05/2013	Travel grant, Diabetes Congress in Leipzig
02/2013	Travel grant, Interdisciplinary Symposium of the Cluster of Excellence ‘Inflammation at Interfaces’ in Hamburg
10/2012	Travel grant, 14 th Annual Meeting of YARE in Dresden
05/2012	Travel grant, Diabetes Congress in Stuttgart