Diplomarbeit

Effects of PCSK9-Antibodies on Cholesterol Metabolism

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Abstract
Proprotein convertase subtilisin/kexin type 9 (PCSK9) antibodies were demonstrated in the large-scaled FOURIER study and the ODYSSEY OUTCOMES study to reduce cardiovascular endpoints. It is not completely understood how PCSK9-antibodies affect endogenous cholesterol biosynthesis, intestinal absorption of cholesterol and circulating PCSK9 levels. Furthermore, the implications of a previous therapy with statin drugs and or ezetimibe on treatment with PCSK9-antibodies are unclear.

In an observational cross-sectional and longitudinal clinical trial 245 patients with hypercholesterolemia received PCSK9-antibodies. At the beginning of therapy with PCSK9-antibodies and 4 - 8 weeks after starting therapy with PCSK9-antibodies blood samples were taken. We measured the non-cholesterol sterols campesterol and sitosterol which reflect the absorption rate of cholesterol and lathosterol which reflects the synthesis rate of cholesterol. 84 of the participants had no previous treatment with lipid lowering drugs, 26 were pretreated with ezetimibe, 38 were pretreated with statins and 97 with ezetimibe and statin drugs.

Intensified pretreatment revealed in a significant increase of circulating PCSK9. Therapy with PCSK9-antibodies resulted in a 760 ± 45 % increase of circulating PCSK9, a -38.8 ± 0.9 % decrease of total cholesterol, a -52.1 ± 1.7 % decrease of LDL-C, a 13.8 ± 1.5 % increase of HDL-C and a -17.1 ± 1.8 % decrease of total triglycerides (all \(p<0.001\)). Lathosterol was reduced by -30.4 ± 3.5 %, campesterol by -30.8 ± 3.1 % and sitosterol by -26.5 ± 3.0 % (all \(p<0.001\)). For the non-cholesterol sterols to cholesterol ratios no significant changes could be observed except for the ratio of sitosterol to cholesterol with a modest increase (\(p=0.009\)). Considering the effects of PCSK9-antibody therapy on the different pretreatments, no significant differences between the pretreatments were found for all analysed parameters.

There was a gradual increase in circulating PCSK9 with increased intensity of lipid lowering pretreatment like a counter-regulation, so that PCSK9-antibodies might have a strong effect in lowering cholesterol as an add on therapy by blocking this counter-regulation. We saw a significant decrease of the non-cholesterol sterols concentrations, but without significant changes in the equilibrium of cholesterol synthesis and absorption. So we conclude, that PCSK9-antibodies doesn’t have a strong effect on cholesterol absorption or synthesis. The different pretreatments have no impact on the effect of PCSK9-
antibodies. This also suggests that PCSK9-antibodies have no strong influence on lipid synthesis or absorption.
Zusammenfassung

In einer klinischen Querschnitts- und Longitudinalstudie erhielten 245 Patienten mit Hypercholesterinämie PCSK9-Antikörper. Vor Beginn der Therapie mit PCSK9-Antikörpern und 4 – 8 Wochen nach Start der Therapie mit PCSK9-Antikörpern wurden Blutproben entnommen. Wir haben die pflanzlichen Sterine Campesterin und Sitosterin als Marker für die Cholesterinabsorption und Lathosterin als Marker für die Cholesterinsynthese gemessen. 84 der Teilnehmer hatten keine Vorbehandlung mit lipidsenkenden Medikamenten, 26 mit Ezetimib, 38 mit Statinen und 97 mit Ezetimib und Statinen.

Unter Vortherapie mit Ezetimib und Statinen war das zirkulierende PCSK9 signifikant erhöht. Die Therapie mit PCSK9-Antikörpern resultierte in einem Anstieg des zirkulierenden PCSK9 um 760 ± 45 %, einer Abnahme des Gesamtcholesterins um - 38,8 ± 0,9 %, einer Abnahme des Low Density Lipoprotein Cholesterins um - 52,1 ± 1,7 %, einer Zunahme des High Density Lipoprotein Cholesterins um 13,8 ± 1,5 % sowie einer Abnahme der Gesamttriglyceride um - 17,1 ± 1,8 % (alle p <0,001). Lathosterin nahm um -30,4 ± 3,5 %, Campesterin um -30,8 ± 3,1 % und Sitosterin um -26,5 ± 3,0 % ab (alle p <0,001). Bei den Verhältnissen von Campesterin, Sitosterin und Lathosterin zu Cholesterin konnten keine starken Veränderungen beobachtet werden. Bei allen analysierten Parametern stellten wir zwischen den verschiedenen Vorbehandlungen mit Statinen oder Ezetimib oder Statinen + Ezetimib keine signifikanten Unterschiede unter Therapie mit PCSK9-Antikörpern fest.

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List of Abbreviations

ACS    acute coronary syndrome
aq. dest. distilled water
BMI    body mass index
BP     blood pressure
°C     degree Celsius
CHD    coronary heart disease
CKD    chronic kidney disease
CRP    c reactive protein
CVD    cardio vascular disease
dl    decilitre
DM     diabetes mellitus
e.g.   for example
EI+    electron ionization
ESC    european society of cardiology
EUR    euro
eV     electron-volt
GC     gas chromatograph
GCMS   gas chromatography and mass spectrometry
GFR    glomerular filtration rate
HDL    high density lipoprotein
IDL    intermediate density lipoprotein
kg     kilogram
L      liter
LDL    low density lipoprotein
LDL-C  low density lipoprotein-cholesterol
LP(a)  lipoprotein a
m      meter
MCP-1  monocyte chemotactic protein-1
mg     milligram
min    minute
ml     milliliter
mm     millimeter
mmHg  millimeter of mercury
mmol/l  millimoles per liter
m/z  mass to charge ratio
µA  micro-ampere
µl  microliter
MS  mass spectrometer
MSTFA  N-methyl-N-(trimethylsilyl)-trifluoroacetamide
ng  nanogram
nmol/l  nanomoles per liter
NYHA  new york heart association
PCSK9  proprotein convertase subtilisin/kexin type 9
rpm  rotation per minute
sc  subcutaneous
SCORE  systematic coronary risk estimation
SIM  single-ion-monitoring
TC  total cholesterol
TMCS  trimethylchlorosilane
VLDL  very low density lipoprotein
v:v  volume:volume
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1 Introduction

In 2016, over 40 % of total mortality in Austria was cardiovascular which represented the most frequent cause of death (1). This is similar to other industrial nations. Hyperlipidaemia is one of the main cardiovascular risk factors. Epidemiological investigations have demonstrated strong and consistent correlations between elevated low density lipoprotein cholesterol (LDL-C) and increased risk of myocardial infarction (2). In contrast, lowering LDL-C leads to a reduction of cardiovascular end points. Hence, the maxim "The lower, the better" is considered a fundament of cardiovascular prevention (3, 4).

There are different approaches to reduce the LDL-C. The fundament of treating hyperlipidaemia is changing lifestyle. Increased physical activity, low-fat diet and weight loss go in parallel with a reduction of LDL-C (5). Lifestyle intervention has also other positive health effects, like lowering the incidence of cancer (6). However, this approach is highly dependent on the patient's compliance. Moreover, frequently it is not effective enough to achieve LDL cholesterol treatment targets. Consequently, drug therapy is essential. Statins are used as first line drug treatment in the primary and secondary prevention of cardiovascular disease by inhibiting cholesterol synthesis inside the liver, whereby they cause a very effective lowering of LDL-C (7). One-third of circulating cholesterol is absorbed in the intestine. Intestinal absorption of cholesterol can be blocked with ezetimibe. Again, studies have shown, that when reducing the cholesterol level with ezetimibe, cardiovascular risk will also be reduced. Ezetimibe may be used in the case of statin-intolerance or additionally to statins, if these alone are not sufficient (8). Recently, proprotein convertase subtilisin/kexin type 9 (PCSK9) antibodies were introduced for commercial use. They induce a 50 % reduction of LDL-C and have also shown in the large-scaled FOURIER study and in the ODYSSEY OUTCOMES study, that they also reduce cardiovascular endpoints (9, 10). The site of action of the PCSK9-antibodies is the liver, where they prevent the degradation of LDL receptors, so that LDL-C can be more efficiently absorbed from the blood into the liver (11). Although the mechanism of action is well known for PCSK9-antibodies, their impacts on cholesterol synthesis and absorption are still not sufficiently investigated. Moreover, it is unclear, whether previous treatment with statins or ezetimibe might affect the effectiveness of PCSK9-antibodies to reduce LDL-C.
1.1 Atherosclerosis

1.1.1 Definition
Atherosclerosis is a chronic disease of the arterial wall and affects the elastic arteries as well as the large and medium sized muscular arteries. It is a variable combination of changes within the intima through a focal accumulation of connective tissue, lipids, blood and blood components, complex carbohydrates and calcium deposits. Changes exist also in the media. This fibromatous remodelling of the vessel wall results in vessel wall thickening with loss of elasticity and lumen narrowing, which is a site of predilection for plaque rupture with following thrombus formation (12).

1.1.2 Pathogenesis
Atherosclerosis is a process that can be explained by the so-called “response to injury” hypothesis (13). In the initial phase, risk factors such as nicotine, hyperlipidaemia or hypertension cause damage of the vascular endothelium. This leads to a lipid influx into the intima, especially of LDL-C. In the inflammatory phase the immigrated LDL-C causes the release of chemokines such as MCP-1 (monocyte chemotactic protein-1). These chemokines lead to adhesion and immigration of monocytes, which convert into macrophages and phagocytise LDL-C. Cholesterol uptake by macrophages is mediated by the scavenger receptor. The scavenger receptor has a bigger tendency for oxidized LDL-C than for unmodified LDL-C. The phagocytizing macrophages then develop into foam cells (fat-storing cells). Accumulation of primarily cholesterol in the arterial wall leads to a lipid plaque. Further release of chemotactic substances like interleukine 6 and basic-fibroblast-growth-factor causes the immigration of smooth muscle cells from the media into the intima and their proliferation. Accumulation of connective tissue results in a fibrous plaque. This plaque may then become increasingly unstable due to thinning of the fibrous cap. This process is also caused by the secretion of gamma interferon during the inflammatory process. The plaques may also calcify caused by an active process involving the incorporation of calcium phosphate (12).

Plaque formation will cause vascular luminal narrowing, which leads to reduced blood flow, resulting in coronary heart disease and artery occlusive disease. Major complications by atherosclerosis include rupture especially of plaques with thin fibrous caps and following thrombosis. Plaque rupture often leads to complete occlusion of the vascular lumen. Such occlusions may cause myocardial infarction, stroke, or critical ischaemia of the lower extremities. Atherosclerotic plaques may also cause arterio-arterial
embolism. Moreover, atherosclerotic vessels are less resistant to strain induced by increased blood pressure. Consequently, atherosclerotic vessels predispose to aneurysm formation (14).

1.1.3 Morphology

The first signs of arteriosclerosis are lipid spots in the intima, which in time become confluent to so-called fatty streaks. Histologically, they consist of foam cells and extracellular lipid drops. This earliest lesion can even be found in adolescents. Over time, the fatty streaks develop into atheromatous plaques, which macroscopically appear as whitish-yellow spots, extending into the vessel lumen (15). Histologic analysis shows a hard fibrous cap located toward the vessel lumen. The deeper part is soft and can especially be found in larger plaques. This deeper part is constituted of yellowish fat tissue, from which the Greek name "Atherom" was derived. The main components of atheromatous plaques are:

- Cells (smooth muscle cells, monocytes, macrophages).
- Extracellular connective tissue matrix (collagen, elastin, proteoglycans).
- Intracellular and extracellular lipid deposits.

Advanced atherosclerotic lesions have the greatest clinical relevance. Features of advanced atherosclerotic lesions include:

- Calcifications with loss of vascular elasticity.
- Haemorrhage in an atheroma, which often affects the coronaries and may cause vascular stenosis or rupture of the plaque.

Complications of advanced atherosclerotic lesions include:

- Ulceration, which may expose highly thrombogenic fat mass to the blood stream, thereby causing arterial thrombosis or micro embolic events.
- Rupture is the most dangerous complication, as it can lead to acute vascular occlusion.
Aneurysmatic dilatation, especially of large arteries such as the abdominal aorta, since the media is often also affected by atrophy and loss of elastic fibre in the process of atherosclerosis (12).

1.1.4 Atherosclerosis in different vascular beds
Atherosclerosis essentially affects the middle-sized muscular arteries, especially the coronary arteries, carotids, basilar and vertebral arteries, and the peripheral arteries of the lower extremities. Less frequently, atherosclerosis may affect the renal and mesenterial arteries. Major consequences of atherosclerosis include coronary heart disease, heart attacks, transitory ischemic attacks, strokes, peripheral artery occlusive disease of the upper and lower extremities, renovascular hypertension, and mesenteric ischaemia. The aorta is also often affected (15).

1.1.5 Risk factors
Risk factors of first order:

- Hypertension: Excessive blood pressure causes endothelial lesions that favour arteriosclerosis.
- Lipid disorders (look at 1.1.6).
- Nicotine abuse: Effects on platelet function, lipoprotein levels, haemodynamics and endothelial integrity are discussed.
- Diabetes mellitus: Autoglycosylation of proteins leads to "advanced glycosylation endproducts", which favour lesions of the endothelium.
- Age: Already in the first decade, the intima of the aorta may show lipid deposits and already in the second decade the coronary arteries may show lipid deposits.
- Gender: Men more frequently and earlier are affected by atherosclerosis than women. Among 35 to 55 year-olds, four times more men than women die of heart attacks. However, postmenopausal cardiovascular mortality rates among women are increasing sharply, reaching those of men aged between sixty and eighty. This may be due to the loss of potential atheroprotective effects of estrogen.
Risk factors of second order:

Secondary risk factors include obesity, stress, sedentary lifestyle, hyperuricemia and hormonal factors (12).

1.1.6 Atherosclerosis and lipid metabolism

1.1.6.1 Lipoproteins

Lipoproteins are complexes required to transport hydrophobic lipids in plasma and lymph. They are essential for the uptake of cholesterol, fatty acids and fat-soluble vitamins, as well as their transport between liver and peripheral tissues. The plasma lipoproteins consist of lipids (cholesterol, triglycerides, phospholipids) and apolipoproteins. They main subfractions are named chylomicrons, VLDL (very low density lipoproteins), LDL and HDL (high density lipoproteins). They can be differentiated and separated according to their density classes with ultracentrifugation. They can also be separated by electrophoresis due to their different electric charges (table 1) (16).

Table 1: Classification of lipoproteins

<table>
<thead>
<tr>
<th>density classes</th>
<th>electrophoresis</th>
<th>main functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>chylomicrons</td>
<td>no movement in the electric field</td>
<td>Transport vehicle for exogenous triglycerides.</td>
</tr>
<tr>
<td>VLDL</td>
<td>pre-β-lipoprotein</td>
<td>Transport vehicle for endogenous triglycerides, precursors of LDL.</td>
</tr>
<tr>
<td>LDL</td>
<td>β-lipoproteins</td>
<td>Endproduct of the VLDL, transport vehicle for cholesterol to extrahepatic cells, regulator of cellular cholesterol homeostasis.</td>
</tr>
<tr>
<td>HDL</td>
<td>α-lipoproteins</td>
<td>Transport vehicle for cholesterol to the liver, regulator of cellular cholesterol homeostasis and lipolysis.</td>
</tr>
</tbody>
</table>

(16)

1.1.6.2 Cholesterol

Cholesterol can be synthesized by all body cells, especially by liver cells. It is an integral part of cell membranes and lipoproteins. Furthermore, it is necessary for the synthesis of steroid hormones and bile acids. Unlike the triglycerides and phospholipids, the cholesterol molecule cannot be catabolized due to its sterol ring. Cholesterol can be removed from the body directly or after catabolism into bile acids via the bile system. In plasma, 25 - 40 % of cholesterol is "free" (unesterified) and 60 - 75 % of cholesterol is esterified with fatty
acids. In routine diagnostics unesterified and esterified cholesterol are not differentiated but measured as total cholesterol. Since cholesterol in the plasma is only slightly soluble in water, it can only be transported in a complex with apolipoproteins. LDL contains most of the circulating cholesterol. The rest is in the HDL and VLDL fractions and only small amounts are in the chylomicrons (17).

1.1.6.3 Triglycerides
Triglycerides are esters of glycerol with three fatty acids. Human depot fat mainly consists of even-numbered, unbranched monocarboxylic acid, such as oleic acid and palmitic acid. In plasma, triglycerides are transported in lipoproteins because of their poor solubility in water. Apolipoproteins cover the hydrophobic core of the lipids and provide the required water solubility for transportation in plasma. The major triglyceride-rich lipoproteins are chylomicrons and VLDL. Chylomicrons carry exogenous triglycerides that have been absorbed via intestine and VLDL carry endogenous triglycerides that have been synthesized in the liver (17).

1.1.6.4 LDL-Cholesterol
The positive correlation between the plasma total cholesterol and LDL-C concentration and atherogenesis was demonstrated in numerous studies. If total cholesterol is lowered by 9 %, coronary events are lowered by 20 % (18). Oxidized LDL is considered highly atherogenic because it may cause inflammation and because it is promptly absorbed by macrophages through the scavenger receptor resulting in lipid deposits in arterial walls. Small, dense LDL are also regarded as highly atherogenic and are highly prevalent in patients with hypertriglyceridemia (18). LDL-C can be calculated by the Friedewald formula (LDL-C = total cholesterol - [triglycerides/5] – HDL-C) (17).

1.1.6.5 HDL-Cholesterol
The risk of atherosclerosis is reduced by a high level of HDL-C. When the concentration of HDL-C is increased by 1 mg/dl it correlates with a 2-3 % reduced cardiovascular risk (19). HDL is considered anti-atherogenic, mediating the transportation of cholesterol from peripheral tissues (including arterial walls) back to the liver (reverse cholesterol transport). They are also considered to have anti-oxidative and anti-inflammatory properties (18).
1.1.6.6 Apolipoproteins
Apolipoproteins are proteins that act as transport vehicles for lipids, as ligands of diverse receptors and as activators of lipolytic enzymes. The most important apolipoproteins are Apo B, Apo A-I and A-II. Apo A-I and A-II represent the major apolipoproteins of HDL, Apo B represents the main apolipoprotein of LDL. Apo B100 accounts for more than 95% of the protein content of LDL. Apo B100 is also present in VLDL and Lp(a). It has to be differentiated from Apo B48, which is only present in the lipoproteins of intestinal origin (20). Large clinical trials demonstrated a strong and positive correlation between Apo B and the risk of cardiovascular events (21). Hence, Apo B is also used for cardiovascular risk stratification.

1.1.6.7 Lipoprotein(a) [Lp(a)]
Lp(a) is also an important risk factor for atherosclerosis. However, it is often neglected in clinical practice. In addition to lipids Lp(a) incorporates Apo B100 and apolipoprotein(a) (22). Even isolated Lp(a) elevation (normal LDL-C) correlates with an elevated risk of atherosclerosis. There is a structural similarity between Lp(a) and LDL. The metabolism of Lp(a) is still not completely understood. The plasma Lp(a) concentration underlies strong genetic regulation and is hardly altered by diet (23).

1.2 LDL-C lowering

1.2.1 Recommended target values for LDL-C
LDL-C plays a prominent and causal role in the process of atherosclerosis. Therefore, it is the main therapeutic target for the reduction of cardiovascular risk. In the current ESC guidelines, the LDL-C target for people with very high cardiovascular risk is < 1.8 mmol/l. In patients with a baseline LDL-C value of 1.8 - 3.5 mmol/l, a reduction of at least 50% is recommended. The LDL-C target for patients with elevated cardiovascular danger is < 2.6 mmol/l or a reduction of at least 50% of the baseline value (table 2). In patients with little or modest cardiovascular risk LDL-C concentrations < 3.0 mmol/l should be achieved (5). The detailed guidelines can be seen in table 3.
1.2.2 Statins
Beyond lifestyle modifications, statins represent the first-line treatment option in patients with hypercholesterolemia. Statins effectively lower the LDL-C value and there is broad evidence that they also reduce cardiovascular risk (24). Statins block endogenous cholesterol biosynthesis by suppressing 3-hydroxy-3-methylglutaryl-CoA reductase. This leads to the decrease of intracellular cholesterol, especially in the liver, which then expresses more LDL receptors. An increased number of LDL receptors is correlated with a
higher hepatic uptake of LDL particles. Decreased synthesis and increased cholesterol uptake by the liver implicates a dose-dependent lowering of total and LDL-C by up to 46 - 60 % and a moderate increase of HDL-C by 5 - 10 % (25). It has also been shown that treatment with statins leads to increased fibrosis and calcification of the plaques. This will stabilize the plaques and probably account for the well-documented reduction of cardiovascular events (26). LDL-C already starts to decrease only a few days after the initiation of the treatment. In general, statins are well tolerated. Depending on the dose, muscular complaints may occur. In rare cases, severe myopathies and rhabdomyolyses may occur. Unfrequently, gastrointestinal side-effects such as abdominal pain and cramps, flatulence, constipation, vomiting and diarrhoea may occur at the beginning of treatment (25).

1.2.3 Cholesterol absorption inhibitor
The second group of cholesterol-lowering drugs are absorption inhibitors. These include bile acid resins, which are hardly used in clinical practice because of gastrointestinal side-effects, and ezetimibe. Ezetimibe prevents the uptake of cholesterol and plant sterols in the small intestine. It binds to the Niemann-Pick C1-like protein 1, an important mediator of cholesterol absorption (16). Ezetimibe prevents intestinal cholesterol uptake by more than 50 % and reduces plasma total and LDL cholesterol by approximately 18 - 20 % and it induces a compensatory increase in cholesterol biosynthesis. There are only moderate effects on HDL-C and triglycerides. It shows an additive effect in combination with statins, so that even low statin doses together with ezetimibe have strong LDL-C lowering effects (25). In the large-scale IMPROVE-IT study, a decrease in cardiovascular end-points was observed, when statins and ezetimibe were combined compared to statins alone (27).

1.2.4 PCSK9 antibodies
Proprotein convertases represent a group of enzymes, which mediate the conversion of precursor secretory proteins like enzymes or hormones into their active form. PCSK9 was first reported in neuronal cells in 2003. However, the primary site of action appears to be in the liver, although PCSK9 appears also in the kidney, the colon as well as in the ileal epithelium (28). In 2003, rare gain-of-function variants in PCSK9 have been found to implicate hypercholesterolemia, whereas loss-of-function lead to decreased LDL-C concentrations. It was then found that PCSK9 acts as a regulator of LDL receptors (29). LDL binds to liver cells which is followed by endocytosis. This process is mediated by the LDL receptor. Subsequently, LDL is cleaved from the receptor and degraded. The free
receptor returns to the liver surface, where it can bind to new LDL. This process takes place up to 150 times and thus is of special importance in the control of LDL-C concentrations (30).

PCSK9 binds to the LDL receptor and apolipoprotein-B100, the main protein of LDL and ligand for the LDL receptor. Consequently, the hepatic LDL receptor is degraded together with LDL in the endosome and can no longer be recycled. So, LDL receptors bound to PCSK9 are no longer available for the hepatic uptake of LDL (figure 1) (31).

The plasma concentration of PCSK9 can vary widely from 80 – 4000 ng/ml (32).

Figure 1: Effect of PCSK9

LDL binds to the LDL receptor via Apo B100. Likewise, PCSK9 binds to the receptor and Apo B100, which tightly binds the receptor and LDL, thus breaking down the receptor in the liver along with LDL-C. (33)
PCSK9-antibodies now link to PCSK9. Thus, keeping it from linking to the LDL receptor. This enables the LDL receptor to be recycled into the liver and return to the liver surface for picking up LDL out of the blood. (33)

Legend

- LDL
- LDL receptor
- Apolipoprotein B 100
- Cholesterol
- Lanthasterol
- Campesterol
- Sitosterol
- PCSK9
- PCSK9-antibody
The therapeutic approach is based on monoclonal fully human PCSK9 antibodies which block the assembly of PCSK9 with the LDL receptor (figure 2). Since 2015, the two antibodies evolocumab (Repatha®) from Amgen and alirocumab (Praluent®) from Sanofi are authorized in Europe (figure 3). Both antibodies are injected subcutaneously. The recommended initiation dose of alirocumab is 75 mg once per two weeks. People who need greater (> 60 %) LDL-C reduction may be treated with 150 mg of alirocumab once per two weeks or 300 mg of alirocumab once per month. The recommended application of evolocumab is either 140 mg all fourteen days or 420 mg once a month (34). In addition to LDL-C, therapy with PCSK9-antibodies lowers Lp(a) by about 20 - 30 % (35).

LDL-C already decreases a few days after the first application of PCSK9-antibodies and this decrease reaches its maximum reduction after about 8 - 10 days. On average, an LDL-C decrease of 50 - 60 % can be expected, either as monotherapy or in addition to other therapeutics, such as statins and/or ezetimibe. It is also noteworthy, that the inhibition of endogenous cholesterol biosynthesis through statins causes an increased expression of LDL receptors. However, statins also increase PCSK9, that counteracts their LDL cholesterol lowering effects (36). This may on the other hand explain synergistic effects of statins and PCSK9-antibodies. Interestingly, the upregulation of LDL-C receptors by PCSK9-antibodies is independent of the concordant effects of statins (37). In other words, both therapeutic approaches seem to complement each other, so that a marked LDL-C reduction is achieved by PCSK9-antibodies in addition to statins. This seems to be particularly important in view of the fact that people suffering from familial hypercholesterolaemia have increased PCSK9 and increased cholesterol synthesis, so that the combination therapy with PCSK9-antibodies and statins has optimal LDL-C lowering effects (38).

The GLAGOV trial studied the effects of evolocumab in addition to statin treatment versus statin therapy plus placebo on coronary plaque volume in 846 participants. Over a period of 78 weeks, the plaque volume of the evolocumab cohort was significantly reduced in comparison to the placebo cohort. This was also true for participants with baseline cholesterol concentrations below 1.8 mmol/l (39). In addition, a further decrease of cardiovascular complications in high-risk patients was shown by reducing of LDL-C to markedly lower concentrations than the recommended level of 1.8 mmol/l (9).

The first endpoint study, the FOURIER study, was launched in March 2017. In this study, over 27,000 patients suffering from cardiovascular disease received either evolocumab or placebo in an add-on regimen to a statins therapy. The incidence of cardiovascular events
had been lowered by 15 % in the evolocumab cohort in comparison to the placebo cohort (9).

The ODYSSEY OUTCOMES study included almost 19,000 patients who had suffered acute coronary syndromes. They received the PCSK9-antibody alirocumab in addition to statins versus statins plus placebo. The endpoint results were presented in March 2018 and revealed that the risk of hard cardiovascular endpoints could be lowered by 15 % in the cohort that received PCSK9-antibodies and the maximum tolerated statin dose compared to the group receiving the highest tolerated dose of statins alone (10).

The antibodies seem to be very well tolerated. There has been a debate whether very little LDL cholesterol amounts could cause adverse neurocognitive side effects. However, the EBBINGHAUS study investigated the neurocognitive consequences of an evolocumab medication in nearly 2,000 participants of the FOURIER study with standardised tests and found no such effects (40). Otherwise, in a meta-analysis with almost 11,000 participants receiving PCSK9-antibodies, more neurocognitive disorders were detected compared to the placebo cohort. Nevertheless, this result was based on subjective testimony of the study participants (41). There may indeed be no adverse effects of treatment with PCSK9-antibodies relating to the metabolism of fat-soluble vitamins and on steroid hormone synthesis (42). However, it is important to collect data on potential long-term side-effects of PCSK9-antibodies which are not available to date.

In Germany, the annual therapy with alirocumab costs about 9,300,- EUR. For comparison, the annual treatment with statins costs about 77,- EUR. Therefore, precise selection of patients for treatment with PCSK9-antibodies is required. In Germany, reimbursement of treatment with evolocumab and alirocumab is available for defined groups of patients. These groups include people suffering from homo- and heterozygous familial hypercholesterolemia or non-familial hypercholesterolemia or mixed dyslipidaemia, that cannot reach LDL cholesterol targets despite dietary intervention and maximum tolerated drug therapy (statins and/or further lipid-reducing treatment in case of statin intolerance) (43).

Table 4 shows an algorithm to reduce lipids in patients with acute coronary syndromes. First, highly effective statins are used. If the LDL-C target cannot be achieved with statins only, ezetimibe and PCSK9-antibodies must be added.
Figure 3: Timeline of development of PCSK9-antibodies

Table 4: Lipid lowering in acute coronary syndrome

<table>
<thead>
<tr>
<th>Step 1</th>
<th>High dose highly effective statin on the first day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control of LDL-C values after 3-6 weeks</td>
<td></td>
</tr>
<tr>
<td>Step 2 (if target is not reached)</td>
<td>Addition of ezetimibe</td>
</tr>
<tr>
<td>Control of LDL-C values</td>
<td></td>
</tr>
<tr>
<td>Step 3 (if target is not reached)</td>
<td>Addition of PCSK9-antibodies</td>
</tr>
</tbody>
</table>
1.3 Sterols as markers for cholesterol synthesis and absorption

To assess cholesterol metabolism, different markers are used. These markers are sterols that represent cholesterol absorption and cholesterol synthesis. Sterols are a group of naturally occurring steroids, that have a 3β-hydroxyl group and a cholestane skeleton. These are polycyclic, hydroaromatic compounds, that are precursors of membrane lipids, hormones and bile acids.

Sterols are classified according to their origin:

- Zoosterols: sterols of animal origin, e.g. cholesterol (lathosterol is a precursor of cholesterol)
- Phytosterols: sterols of plant origin, e.g. campesterol and sitosterol
- Mycosterols: fungal sterols, e.g. ergosterol (provitamin D2, calciferols) (45)

The most abundant sterol in the human body is cholesterol. In healthy patients, the plasma total cholesterol concentration should be <200 mg/dl.

Lathosterol represents a cholesterol precursor that is synthesized from squalene (46, 47). Cholesterol has a double bond at position 5 - 6. In contrast, lathosterol has a double bond at position 7 - 8 (46). The normal range for the plasma concentration of lathosterol is 0.0 – 3.0 mg/l (48). The plasma concentration of lathosterol positively correlates with 3-hydroxy-3-methylglutaryl-CoA reductase activity (49). Hence, circulating lathosterol reflects the cholesterol synthesis rate in the human body (50).

Campesterol and sitosterol have a chemical structure that is hardly different from cholesterol. The only difference is that these plant sterols have an additional methyl or ethyl side-chain (figure 4). Contrary to cholesterol, it is not possible for the human organism to synthesize phytosterols, but they are only absorbed through the intestine. In agreement with cholesterol, the alimentary absorption of these plant fats is mediated by the Niemann-Pick C1-Like 1 membrane carrier. However, there is also an efflux system in the enterocyte, which resecretes unesterified cholesterol, particularly plant sterols, back from the enterocyte in the intestine (46, 51). The ATP binding cassette transporters G5 and G8 mediate this process which prevents the vast majority of phytosterols from entering the human body (52). Consequently, intestinal plant sterol absorption is markedly lower than
intestinal cholesterol absorption. As a result, phytosterols only make up <1 % of the total cholesterol concentration (53, 54). The normal range for circulating campesterol and sitosterol are 0.0 – 7.0 mg/l and 0.0 – 5.0 mg/l, respectively (48). To assess the absorption of cholesterol the plasma concentrations of the plant sterols campesterol and sitosterol can be quantified.

Figure 4: Different sterols

**Cholesterol**

![Cholesterol](image)

Molecular Formular $C_{27}H_{46}O$
Molecular Weight 386.65

**Lathosterol**

![Lathosterol](image)

Molecular Formular $C_{27}H_{46}O$
Molecular Weight 386.65
1.4 Study Aims

This research was meant to further elucidate the impact of PCSK9-antibodies on cholesterol synthesis and absorption and on circulating PCSK9 in an observational cross-sectional and longitudinal clinical study. Another aim was to investigate whether pretreatment with statin drugs and/or ezetimibe has an influence on effectiveness of PCSK9-antibodies to reduce the plasma LDL-C concentration.
2 Methods

2.1 Participants

We recruited patients who were at least 18 years of age and had the indication to be treated with PCSK9-antibodies. In addition, they had to comprehend the study plan and follow the study process. Patient recruitment took place in 2016 and 2017. A total of 245 patients were enrolled at the Outpatient Lipid Clinic of the Charité Berlin (Berlin, Germany) and the Department of Cardiology at the University Clinics Homburg Saar (Homburg, Germany).

The participants received PCSK9-antibody therapy with either evolocumab 140 mg sc. once every two weeks or evolocumab 420 mg sc. once every four weeks or alirocumab 75 mg or 150 mg sc. once every two weeks.

At baseline, participants had to fill out a questionnaire about their medical history. Previous cardiovascular diseases, hypertension and diabetes mellitus were diagnosed using patient records.

There were two visits at the study site. The first visit was before treatment initiation. The second visit was four to eight weeks after treatment initiation. Blood samples were taken at both visits (33).

The study was performed in accordance with the Declaration of Helsinki 1975 and was approved by the ethical committees of the Charité Berlin and the University of the Saarland. All patients gave written informed consent.

2.2 Laboratory procedures

2.2.1 Sample collection, storing and standard laboratory methods

We took fasting blood samples at the Outpatient Lipid Clinic of the Charité Berlin and the Department of Cardiology at the University Clinics Homburg Saar. Since there was a period of several months between taking blood and measuring lipids, PCSK9 and sterols, the plasma was saved at -80 °C in the meantime. Cholesterol and triglycerides were quantified enzymatically with an Olympus AU640 automatic analyzer and with reagents from DiaSys (Holzheim, Germany). The lipoproteins have been measured by β-quantification which includes an ultracentrifugation precipitation procedure. For the measurement of PCSK9 we used the Quantikine ELISA Kit (R&D, Minneapolis, MN, USA) (33).
2.2.2 Analysis of lathosterol, campesterol and sitosterol

Gas chromatography, combined with mass spectrometry, is a common method used to measure sterols. After separation of the different sterols in the gas chromatograph they were identified and quantified in the mass spectrometer.

2.2.2.1 Materials

Epicoprostanol was purchased from Sigma, C7578. Pyridine, TS-27530 and trimethylchlorosilane (TMCS), 88530 were from Pierce, N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) has been purchased from ABCR, AB110060. Silica gel was supplied by Macherey Nagl, 60,815330.1 and aq. dest. from Fresenius. All other reagents and solvents were from Merck. These were potassium hydroxide, 1.05033.1000; ethanol, 1.00983.1011; methanol, 1.06009.1011; N-hexane, 1.04367.1000; and isopropanol, 1.00995.1000.

Epicoprostanol with a molecular mass of 388.67 g/mol was used as internal standard. 3.88 mg of epicoprostanol are diluted to 10 ml with methanol. From this stock solution, 1 ml is taken and mixed with 2 ml of 2 propranol to a solution with a concentration of epicoprostanol with 200 nmol/ml. This solution is now further diluted 1:10 and the working solution is prepared with epicoprostanol in a concentration of 2 nmol/100 μl. The derivatization reagent of the MSTFA solution is prepared from 10 ml of MSTFA mixed with 5 ml pyridine and 100 μl TMCS.

2.2.2.2 Sample preparation

50 μl are taken from the thawed plasma sample and mixed with 50 μl aq. dest. To this mixture 100 μl of internal standard were added. Furthermore, 250 μl of a 50 % potassium hydroxide solution and 800 μl of ethanol are added. The ampule is sealed and stored at 75 °C for one hour in the incubator for saponification. The mixture is then cooled to room temperature and 1 ml aq. dest. and 2 ml of hexane are added. For extraction, the sample is left for 10 min., placed in a 360° shaker and then centrifuged for 2 min. at 1.500 rpm. The supernatant is transferred to new vials and from there conveyed to a silica gel column. The silica gel column is equilibrated with 4 ml of hexane. Under reduced pressure, the sample is applied on the column, washed with 2 ml of hexane and then eluted with 4 ml hexane and isopropanol (v:v, 70:30). The solution is now vaporized by nitrogen. 50 μl MSTFA solution are added to the dried sample and incubated for 30 min. at room temperature for incubation. Then the sample is dried again. Finally, the samples are dissolved with 100 μl of hexane, filled into autosampler vials, and sealed (56).
2.2.2.3 GCMS Analysis

The purified samples were analysed on a Thermo Trace 1300 gas chromatograph (GC) combined with a Thermo ISQlt quadrupole mass spectrometer (MS). The injection volume is 3 μl. The separation column was an HT5 fused silica capillary column from SGE, 25 m / 0.22 mm with a film thickness of 0.1 μm. Helium with a continual flux of 1.0 ml/min is required as transport gas. The GC’s column was directly linked to the MS. The oven at injection was heated to 200 °C and this temperature was kept for 1 min. Then the temperature was increased by 15 °C/min to 300 °C, which was then kept for seven minutes. The transfer line is heated to 310 °C. Ionization is achieved by electron impact ionization in the mode EI+ at 70 eV with an emission current of 150 μA. A quantitative analysis of trace components was performed in single-ion-monitoring mode. The specific masses and ions of lathosterol were m/z 458.5, of campesterol m/z 382.4, of sitosterol m/z 357.3 and of epicoprostanol m/z 370.4. In the corresponding chromatogram, the non-cholesterol sterols were checked qualitatively (figure 5). Subsequently, the individual non-cholesterol sterols were quantitatively measured by integration (figures 6, 7, 8). For the analysis the software Xcalibur 3.1.6610 from Thermo Fisher Scientific was used (56).

Figure 5: Sample analysis Qual-Browser, Chromatogram
Figure 6: Quantitative analysis: Integration of lathosterol

Figure 7: Quantitative analysis: Integration of campesterol
2.3 Statistical analysis

The baseline data for clinical and biochemical characteristics are given as means with standard errors for continuous parameters and as numbers and percentages for categories. In addition, ratios of non-cholesterol sterols to cholesterol and the ratios of the plant sterols to lathosterol were calculated. For the relationships among the ratios of non-cholesterol sterols to cholesterol, Pearson correlation was calculated. We also provided a separate listing of the baseline levels of PCSK9, lipids, and ratios after each. ANalysis Of VAriance was performed to calculate p-values for differences among four pretreatment cohorts. The effect of PCSK9-antibodies on circulating PCSK9, lipids and non-cholesterol sterols was reported as absolute and percent change. For the comparison between pretreatment and posttreatment, paired samples t-tests were calculated. The impact of PCSK9-antibodies on circulating PCSK9 and lipids according to the pretreatment was also reported as absolute and percent change. P-values for differences in changes according to pretreatment were calculated with ANalysis Of VAriance of percent variations. Data that were not normally distributed were transformed logarithmically. The statistical evaluation is carried out with SPSS 23 (IBM, Armonk, NY, US) (33).
3 Results

3.1 Patients

At study entry, the study participants were characterized by an average age of about 61 ± 0.7 years and by a pre-adipose mean body mass index of 28.2 ± 0.3 kg/m². Hypertension and cardiovascular disease were highly prevalent, with 64.6 % and 81.8 % of the participants, respectively. 23.1 % of the study participants suffered from diabetes mellitus and 25.3 % of the participants were smokers. 47.4 % of the participants had complete statin intolerance, 27 % had partial intolerance and 25.6 % had no statin intolerance. Lipid-lowering pretreatment was received by a total of 65.7 % of the study participants. 55.1 % of the participants were treated with statins and 50.2 % with ezetimibe. In the entire cohort, mean circulating PCSK9 was 356 ± 17 ng/ml. Total cholesterol (6.31 ± 0.11 mmol/l), LDL-C (3.87 ± 0.10 mmol/l) and total triglycerides (1.98 ± 0.09 mmol/l) were increased. Lathosterol, campesterol and sitosterol concentrations were 13.02 ± 0.79 μmol/l, 13.89 ± 0.69 μmol/l and 16.64 ± 0.67 μmol/l respectively (table 5).

3.2 Correlations

At baseline, there were highly significant (all p < 0.001) positive correlations between cholesterol and campesterol (r = 0.37), sitosterol (r = 0.23), and lathosterol (r = 0.45). The campesterol to cholesterol ratio (r = -0.14, p = 0.024) was inversely related to the lathosterol to cholesterol ratio. The sitosterol to cholesterol ratio (r = -0.20, p < 0.001) was also negatively correlated to the lathosterol to cholesterol ratio. In contrast, the campesterol and sitosterol to cholesterol ratios were significantly positively correlated (r = 0.80, p < 0.001) (33).
Table 5: Characteristic of the patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>61.3 (0.7)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>28.2 (0.3)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>153 (84.6)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>56 (23.1)</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>198 (81.8)</td>
</tr>
<tr>
<td>Smoking</td>
<td>60 (25.3)</td>
</tr>
<tr>
<td>Statin intolerance</td>
<td>55 (25.6)</td>
</tr>
<tr>
<td>Partial</td>
<td>58 (27.0)</td>
</tr>
<tr>
<td>Complete</td>
<td>102 (47.4)</td>
</tr>
<tr>
<td><strong>Lipid lowering therapy</strong></td>
<td></td>
</tr>
<tr>
<td>Statin</td>
<td>135 (56.1)</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>123 (50.2)</td>
</tr>
<tr>
<td>PCSK9, ng/ml</td>
<td>356 (17)</td>
</tr>
<tr>
<td><strong>Lipids</strong></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>6.31 (0.11)</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/l</td>
<td>3.87 (0.10)</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/l</td>
<td>1.19 (0.03)</td>
</tr>
<tr>
<td>Total triglycerides, mmol/l</td>
<td>1.98 (0.09)</td>
</tr>
<tr>
<td>Lathosterol, µmol/l</td>
<td>13.02 (0.79)</td>
</tr>
<tr>
<td>Campesterol, µmol/l</td>
<td>13.89 (0.69)</td>
</tr>
<tr>
<td>Sitosterol, µmol/l</td>
<td>16.64 (0.67)</td>
</tr>
<tr>
<td>Lathosterol: cholesterol, µmol/mmol</td>
<td>2.00 (0.11)</td>
</tr>
<tr>
<td>Campesterol: cholesterol, µmol/mmol</td>
<td>2.21 (0.11)</td>
</tr>
<tr>
<td>Sitosterol: cholesterol, µmol/mmol</td>
<td>2.74 (0.12)</td>
</tr>
<tr>
<td>Campesterol: lathosterol</td>
<td>2.32 (0.22)</td>
</tr>
<tr>
<td>Sitosterol: lathosterol</td>
<td>3.01 (0.27)</td>
</tr>
</tbody>
</table>

**Legend:** Values are means with standard error in cases of continuous data or numbers and percentages in cases of categorical data; for hypertension, diabetes mellitus, cardiovascular disease, smoking, statin intolerance, and PCSK9, data were available in 237, 242, 242, 237, 215, and 47 patients. (33)
3.3 Impact of pretreatment on baseline characteristics

More potent lipid-reducing pretreatment is associated with increased circulating PCSK9. Thus, circulating PCSK9 is lowest in untreated patients with 279 ± 80 ng/ml and increased in patients treated with ezetimibe 296 ± 87 ng/ml, with statins 327 ± 115 ng/ml, and with ezetimibe + statin to 442 ± 100 ng/ml. Total cholesterol is reduced in patients receiving potent lipid-lowering pretreatment. Thus, it is highest in patients without pretreatment and lower in patients who received both, ezetimibe and statins. Likewise, LDL-C is lower in patients who received both, ezetimibe and statins, than in those without pretreatment. Patients, receiving a single therapy with statins have lower total cholesterol and LDL-C levels compared to patients receiving a single therapy with ezetimibe. For HDL-C, no significant differences were found according to pretreatment. Regarding triglycerides, a decrease with more potent pretreatment was observed. However, patients receiving a single therapy of statins had similar triglyceride concentrations as those treated with the combination therapy of ezetimibe and statins.

In comparison to the untreated group lathosterol was increased in patients treated with ezetimibe and lower in patients treated with statins. In patients receiving combination therapy of ezetimibe and statins lathosterol was similarly low as in the cohort receiving statins. In comparison to the untreated cohort, campesterol was lower in the cohort treated with ezetimibe and higher in the cohort treated with statins. Patients receiving the combination therapy of ezetimibe and statins had higher campesterol than those in the group with ezetimibe treatment. Sitosterol was also highest in the group with statin treatment and lowest in the cohort with ezetimibe therapy. In comparison to the untreated cohort, the lathosterol to cholesterol ratio was higher in the ezetimibe cohort and lower in the statin cohort. In contrast, the plant sterol to cholesterol ratios were lowest in the cohort treated with ezetimibe and highest in the cohort treated with statins. The plant sterol to lathosterol ratios were lowest in the ezetimibe cohort and highest in cohort treated with statins (table 6).
Table 6: Impact of pretreatment

<table>
<thead>
<tr>
<th>Number</th>
<th>No Treatment</th>
<th>Ezetimibe</th>
<th>Statin</th>
<th>Ezetimibe + Statin</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCSK₉, ng/ml†</td>
<td>279 (80)</td>
<td>296 (87)</td>
<td>327 (115)</td>
<td>442 (100)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>7.27 (0.19)</td>
<td>6.62 (0.39)</td>
<td>6.18 (0.23)</td>
<td>5.46 (0.15)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/l</td>
<td>4.57 (0.17)</td>
<td>4.17 (0.38)</td>
<td>3.88 (0.19)</td>
<td>3.37 (0.12)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/l</td>
<td>1.17 (0.04)</td>
<td>1.21 (0.07)</td>
<td>1.34 (0.07)</td>
<td>1.16 (0.04)</td>
<td>0.101</td>
</tr>
<tr>
<td>Total triglycerides, mmol/l</td>
<td>2.45 (0.18)</td>
<td>2.02 (0.21)</td>
<td>1.67 (0.20)</td>
<td>1.69 (0.11)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lathosterol µmol/l</td>
<td>18.9 (1.2)</td>
<td>24.8 (3.2)</td>
<td>6.3 (0.8)</td>
<td>7.4 (1.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Campesterol µmol/l</td>
<td>16.3 (1.2)</td>
<td>6.8 (0.8)</td>
<td>19.2 (1.6)</td>
<td>11.6 (1.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sitosterol µmol/l</td>
<td>17.2 (1.0)</td>
<td>10.4 (1.3)</td>
<td>21.5 (1.7)</td>
<td>15.9 (1.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lathosterol: cholesterol, µmol/mmol</td>
<td>2.6 (0.2)</td>
<td>3.8 (0.4)</td>
<td>1.0 (0.1)</td>
<td>1.3 (0.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Campesterol: cholesterol, µmol/mmol</td>
<td>2.2 (0.1)</td>
<td>1.1 (0.1)</td>
<td>3.1 (0.2)</td>
<td>2.2 (0.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sitosterol: cholesterol, µmol/mmol</td>
<td>2.4 (0.1)</td>
<td>1.6 (0.2)</td>
<td>3.6 (0.3)</td>
<td>3.0 (0.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Campesterol: lathosterol</td>
<td>1.3 (0.2)</td>
<td>0.6 (0.2)</td>
<td>5.4 (1.0)</td>
<td>2.5 (0.3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sitosterol: lathosterol</td>
<td>1.4 (0.2)</td>
<td>0.9 (0.3)</td>
<td>5.9 (0.9)</td>
<td>3.8 (0.5)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Legend: Values are means with standard error; * calculated with ANalysis Of VAriance; † numbers: 14/4/10/19 (33)
3.4 Effects of PCSK9-antibodies in the entire cohort

Therapy with PCSK9-antibodies resulted in a very strong increase of circulating PCSK9 by 2459 ± 115 ng/ml, which corresponds to 760 ± 45 %. Total cholesterol was significantly lowered by -2.44 ± 0.08 mmol/l, corresponding to -38.8 ± 0.9 %. LDL-C was reduced by -2.02 ± 0.06 mmol/l or -52.1 ± 1.7 %. HDL-C significantly increased by 0.14 ± 0.01 mmol/l or 13.8 ± 1.5 % and total triglycerides significantly decreased by -0.48 ± 0.05 mmol/l, which is -17.1 ± 1.8 %. Significant reductions by PCSK9-antibodies were also observed for the non-cholesterol sterols. Lathosterol was strongly reduced by -5.57 ± 0.55 µmol/l, which corresponds to -30.4 ± 3.5 %. Campesterol significantly decreased by -4.78 ± 0.54 µmol/l which corresponds to -30.8 ± 3.1 % and sitosterol significantly decreased by -5.22 ± 0.59 µmol/l which corresponds to -26.5 ± 3.0 %. Considering the ratios of non-cholesterol sterols to cholesterol no significant changes could be observed except from the sitosterol to cholesterol ratio which showed a modest increase (table 7).

Table 7: Effects of PCSK9-antibodies

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Absolute change</th>
<th>% change</th>
<th>( p^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCSK9, ng/ml*</td>
<td>2459 (115)</td>
<td>760 (45)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>-2.44 (0.08)</td>
<td>-38.8 (0.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/l</td>
<td>-2.02 (0.06)</td>
<td>-62.1 (1.7)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/l</td>
<td>0.14 (0.01)</td>
<td>13.8 (1.5)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total triglycerides, mmol/l</td>
<td>-0.48 (0.05)</td>
<td>-17.1 (1.8)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lathosterol µmol/l</td>
<td>-5.57 (0.55)</td>
<td>-30.4 (3.5)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Campesterol µmol/l</td>
<td>-4.78 (0.54)</td>
<td>-30.8 (3.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sitosterol µmol/l</td>
<td>-5.22 (0.59)</td>
<td>-26.5 (3.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lathosterol: cholesterol, µmol/mmol</td>
<td>-0.14 (0.08)</td>
<td>12.7 (6.0)</td>
<td>0.744</td>
</tr>
<tr>
<td>Campesterol: cholesterol, µmol/mmol</td>
<td>0.08 (0.11)</td>
<td>12.6 (4.8)</td>
<td>0.819</td>
</tr>
<tr>
<td>Sitosterol: cholesterol, µmol/mmol</td>
<td>0.28 (0.13)</td>
<td>20.1 (4.4)</td>
<td>0.009</td>
</tr>
<tr>
<td>Campesterol: lathosterol</td>
<td>-0.16 (0.11)</td>
<td>18.2 (7.0)</td>
<td>0.597</td>
</tr>
<tr>
<td>Sitosterol: lathosterol</td>
<td>0.09 (0.15)</td>
<td>27.8 (7.9)</td>
<td>0.010</td>
</tr>
</tbody>
</table>

Legend: * calculated with paired samples t-test; † number: 46 (33)
3.5 *Effects of PCSK9-antibodies according to pretreatment*

The changes of all analyzed parameters in response to PCSK9-antibodies did not significantly differ among the four pretreatment cohorts. An increase in circulating PCSK9 was about the same in the cohort with no pretreatment and in the cohorts treated with ezetimibe, statins, and ezetimibe plus statins. For total cholesterol, LDL-C and total triglycerides a similar reduction was also observed in all four groups. For HDL-C a similar increase was found in all four groups. The decrease in the non-cholesterol sterols was similar in the no pretreatment, ezetimibe, statin, and ezetimibe plus statin groups. Also, the ratios of lathosterol, campesterol and sitosterol to cholesterol and the ratios of the plant sterol to lathosterol similarly responded to PCSK9-antibodies in all four pretreatment groups (*table 8*).
Table 8: Effects of PCSK9-antibodies according to pretreatment

<table>
<thead>
<tr>
<th>Number</th>
<th>No Treatment</th>
<th>Ezetimibe</th>
<th>Statin</th>
<th>Ezetimibe + Statin</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCSK9†</td>
<td>2120 (163)</td>
<td>2592 (354)</td>
<td>2315 (145)</td>
<td>2772 (224)</td>
<td>0.263</td>
</tr>
<tr>
<td>%</td>
<td>814 (80)</td>
<td>971 (235)</td>
<td>770 (73)</td>
<td>867 (59)</td>
<td>0.265</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.305</td>
</tr>
<tr>
<td>Absolute, mmol/l</td>
<td>-2.73 (0.13)</td>
<td>-2.44 (0.17)</td>
<td>-2.24 (0.22)</td>
<td>-2.27 (0.12)</td>
<td>0.118</td>
</tr>
<tr>
<td>%</td>
<td>-37.4 (1.4)</td>
<td>-37.5 (2.1)</td>
<td>-38.8 (3.4)</td>
<td>-41.1 (1.5)</td>
<td>0.990</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.428</td>
</tr>
<tr>
<td>Absolute, mmol/l</td>
<td>-2.18 (0.10)</td>
<td>-2.07 (0.15)</td>
<td>-1.90 (0.21)</td>
<td>-1.90 (0.09)</td>
<td>0.655</td>
</tr>
<tr>
<td>%</td>
<td>-48.6 (1.9)</td>
<td>-51.9 (3.0)</td>
<td>-50.5 (5.4)</td>
<td>-55.9 (3.2)</td>
<td>0.278</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.158</td>
</tr>
<tr>
<td>Absolute, mmol/l</td>
<td>0.14 (0.02)</td>
<td>0.22 (0.06)</td>
<td>0.13 (0.04)</td>
<td>0.11 (0.02)</td>
<td>0.912</td>
</tr>
<tr>
<td>%</td>
<td>14.0 (1.9)</td>
<td>23.8 (8.7)</td>
<td>10.4 (2.7)</td>
<td>12.1 (2.3)</td>
<td>0.946</td>
</tr>
<tr>
<td>Total triglycerides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.562</td>
</tr>
<tr>
<td>Absolute, mmol/l</td>
<td>-0.63 (0.11)</td>
<td>-0.47 (0.18)</td>
<td>-0.41 (0.14)</td>
<td>-0.37 (0.07)</td>
<td>0.278</td>
</tr>
<tr>
<td>%</td>
<td>-18.0 (3.0)</td>
<td>-16.3 (6.4)</td>
<td>-16.6 (4.9)</td>
<td>-16.9 (2.8)</td>
<td>0.428</td>
</tr>
<tr>
<td>Lathosterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.658</td>
</tr>
<tr>
<td>Absolute, µmol/l</td>
<td>-7.96 (0.91)</td>
<td>-9.96 (1.88)</td>
<td>-2.00 (0.71)</td>
<td>-3.72 (0.91)</td>
<td>0.562</td>
</tr>
<tr>
<td>%</td>
<td>-26.5 (8.4)</td>
<td>-31.2 (6.2)</td>
<td>-21.8 (8.7)</td>
<td>-37.0 (3.3)</td>
<td>0.278</td>
</tr>
<tr>
<td>Campesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.719</td>
</tr>
<tr>
<td>Absolute, µmol/l</td>
<td>-4.42 (1.04)</td>
<td>-2.30 (0.63)</td>
<td>-6.40 (1.19)</td>
<td>-5.12 (0.91)</td>
<td>0.912</td>
</tr>
<tr>
<td>%</td>
<td>-26.3 (5.2)</td>
<td>-30.8 (7.0)</td>
<td>-28.8 (7.9)</td>
<td>-35.4 (5.5)</td>
<td>0.946</td>
</tr>
<tr>
<td>Sitosterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.658</td>
</tr>
<tr>
<td>Absolute, µmol/l</td>
<td>-3.79 (1.00)</td>
<td>-3.45 (1.13)</td>
<td>-6.64 (1.68)</td>
<td>-6.39 (0.94)</td>
<td>0.562</td>
</tr>
<tr>
<td>%</td>
<td>-21.7 (5.1)</td>
<td>-18.3 (12.2)</td>
<td>-21.5 (9.2)</td>
<td>-34.0 (3.6)</td>
<td>0.719</td>
</tr>
<tr>
<td>Lathosterol: cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.719</td>
</tr>
<tr>
<td>Absolute, µmol/mmol</td>
<td>-0.15 (0.11)</td>
<td>-0.24 (0.23)</td>
<td>0.05 (0.12)</td>
<td>-0.17 (0.15)</td>
<td>0.912</td>
</tr>
<tr>
<td>%</td>
<td>13.9 (11.5)</td>
<td>8.6 (7.8)</td>
<td>20.0 (9.2)</td>
<td>9.9 (6.8)</td>
<td>0.946</td>
</tr>
<tr>
<td>Campesterol: cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.658</td>
</tr>
<tr>
<td>Absolute, µmol/mmol</td>
<td>0.30 (0.19)</td>
<td>0.02 (0.09)</td>
<td>0.21 (0.25)</td>
<td>-0.15 (0.19)</td>
<td>0.562</td>
</tr>
<tr>
<td>%</td>
<td>16.4 (7.4)</td>
<td>8.0 (8.4)</td>
<td>10.0 (8.6)</td>
<td>11.5 (9.6)</td>
<td>0.719</td>
</tr>
<tr>
<td>Sitosterol: cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.658</td>
</tr>
<tr>
<td>Absolute, µmol/mmol</td>
<td>0.58 (0.25)</td>
<td>0.09 (0.16)</td>
<td>0.42 (0.35)</td>
<td>0.01 (0.20)</td>
<td>0.562</td>
</tr>
<tr>
<td>%</td>
<td>24.7 (7.9)</td>
<td>27.8 (16.5)</td>
<td>21.8 (11.6)</td>
<td>13.4 (6.3)</td>
<td>0.946</td>
</tr>
<tr>
<td>Campesterol: lathosterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.658</td>
</tr>
<tr>
<td>Absolute</td>
<td>-0.01 (0.18)</td>
<td>-0.09 (0.07)</td>
<td>-0.95 (0.54)</td>
<td>0.01 (0.10)</td>
<td>0.562</td>
</tr>
<tr>
<td>%</td>
<td>24.5 (8.3)</td>
<td>5.4 (8.0)</td>
<td>29.2 (36.3)</td>
<td>11.8 (7.6)</td>
<td>0.719</td>
</tr>
<tr>
<td>Sitosterol: lathosterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.562</td>
</tr>
<tr>
<td>Absolute</td>
<td>0.22 (0.33)</td>
<td>-0.12 (0.11)</td>
<td>-0.25 (0.43)</td>
<td>0.17 (0.18)</td>
<td>0.562</td>
</tr>
<tr>
<td>%</td>
<td>37.2 (10.7)</td>
<td>23.7 (14.0)</td>
<td>42.5 (41.7)</td>
<td>15.1 (6.0)</td>
<td>0.719</td>
</tr>
</tbody>
</table>

Legend: Values are means with standard error; * calculated with ANalysis Of VAriance; † numbers: 14/4/10/19
4 Discussion

4.1 Baseline concentrations of lipids and PCSK9

In our study in participants without statin or ezetimibe pretreatment, the baseline concentrations of total cholesterol and LDL-C were 7.27 mmol/l and 4.57 mmol/l, respectively. This reflects that the study participants suffered from hypercholesterolemia. Further in our study, the participants without statin or ezetimibe pretreatment had baseline plasma campesterol, sitosterol, and lathosterol concentrations of 16.3 µmol/l, 17.3 µmol/l and 18.9 µmol/l respectively.

In a Finnish study in patients with severe primary hypercholesterolemia prior to lovastatin therapy by Uusitupa et al., the plasma concentrations were 21.0 µmol/l for campesterol, 13.3 µmol/l for sitosterol, and 11.2 µmol/l for lathosterol. The baseline concentration of total cholesterol was 10.6 mmol/l and of LDL-C was 8.5 mmol/l (57). A Japanese study of patients with dyslipidemia by Hiramitsu et al. reported baseline concentrations of 7.0 µmol/l for campesterol, 4.3 µmol/l for sitosterol, and 5.7 µmol/l for lathosterol. Baseline lipids were 6.2 mmol/l for total cholesterol and for LDL-C 4.1 mmol/l (58). Taken together, non-cholesterol sterols appear to correlate in a positive way with total cholesterol and LDL-C levels.

4.2 Impact of pretreatment

Our investigation confirms that treatment with ezetimibe and/or statins is associated with lower plasma total cholesterol and LDL-C values. We saw the highest total cholesterol and LDL-C levels in patients without lipid-lowering pretreatment. Ezetimibe lowers total cholesterol and LDL-C concentrations by 18 - 20 % (25). Versus the ezetimibe treated group, the statin treated group had lower concentrations of total cholesterol and LDL-C. This is in line with the literature showing that statin monotherapy reduces LDL-C by 46 – 60 % (25). Of interest, several reports suggest that statins can reduce LDL-C more effectively in persons having elevated cholesterol synthesis than in persons with a high absorption (59). On the other hand, therapy with ezetimibe has been more effective in reducing LDL-C in people characterized by elevated cholesterol absorption and decreased in those characterized by high cholesterol synthesis (60). The group receiving ezetimibe and statins had the lowest amount of total cholesterol and LDL-C.

In our investigation the ezetimibe group had the highest level of lathosterol and the lowest concentrations of campesterol and of sitosterol in comparison to the other groups. Further,
the lathosterol to cholesterol ratio has been highest and the plant sterol to cholesterol ratios have been lowest in the ezetimibe cohort in comparison to the other cohorts. This observation may reflect compensation of a lowered cholesterol absorption by increased cholesterol synthesis. This counterregulation was also reported by Hiramitsu et al., where a 45.3 % increase in lathosterol, a 46.6 % reduction in campesterol and a 27.2 % reduction in sitosterol was observed after 12 weeks of ezetimibe therapy.

Statin therapy has the opposite impact compared with ezetimibe with regard to cholesterol synthesis and absorption. This is in agreement with our observation that the statin group had the lowest lathosterol level as well as the lowest lathosterol to cholesterol ratio compared to the other groups. Moreover, campesterol and sitosterol as well as their ratios to cholesterol were highest in the statin treated group in comparison to the other groups. In an investigation of Uusitupa et al. the lathosterol to cholesterol ratio decreased by about 45 %, the campesterol to cholesterol ratio increased by 26 % and the sitosterol to cholesterol ratio increased by 40 % after treatment with lovastatin for 18 weeks. Van Himbergen et al. investigated the impact of 6 weeks of treatment with rosuvastatin on cholesterol metabolism and observed a 64 % reduction of the lathosterol to cholesterol ratio, a 52 % increase of the campesterol to cholesterol ratio, as well as a 67 % increase of the sitosterol to cholesterol ratio (61). Hence, the low lathosterol to cholesterol ratio and the elevated ratios of campesterol and sitosterol to cholesterol in the statin treated group in our study are in accordance with the literature.

A Chinese investigation by Qi et al. investigating the effects of statin treatment with regard to cholesterol absorption and synthesis markers in cardiovascular high risk patients reported an interesting finding. Cholesterol has been reduced much more under therapy with statins in people with elevated baseline lathosterol compared to people characterized by a lower baseline of lathosterol. This is comprehensible since statins act via the synthesis pathway (62).

In our study, the group with the combination therapy of ezetimibe and statins had low lathosterol concentrations and low lathosterol to cholesterol ratios, but both not as low as in the statin group. Further we observed in the group with combination therapy values of campesterol, sitosterol and of the ratios of plant sterols to cholesterol that lie between the groups that received either statins only or ezetimibe only.
According to our results, in the study by Hiramitsu et al. combination therapy with addition of ezetimibe to statins caused an increase of lathosterol and a reduction of campesterol and of sitosterol after 12 weeks. Another Japanese investigation by Sasaki et al. found that addition of ezetimibe to pravastatin went in parallel with an elevation of lathosterol and a decrease of campesterol and of sitosterol in patients with hypercholesterolemia (63). In agreement, Okada et al. found that adding ezetimibe to statins will increase the lathosterol to cholesterol ratio and decrease the campesterol to cholesterol ratio in people suffering from coronary artery disease. LDL-C was most strongly lowered in participants characterized by an elevated cholesterol absorption and low cholesterol synthesis. The lowest LDL-C reduction has been found in people characterized by low cholesterol absorption and high cholesterol synthesis (64).

In our study, there is a gradual increase of circulating PCSK9 with increased intensity of lipid lowering pretreatment. So, PCSK9 is lowest in the untreated group with a stepwise increase to the ezetimibe plus statin group. Previous studies have already reported that circulating PCSK9 goes up after treatment with statins (36, 65). In contrast to our results, no rise of circulating PCSK9 after treatment with ezetimibe has been described so far. There are also controversial reports regarding the impact of combined treatment with statins and ezetimibe on circulating PCSK9. Most investigations reported no additional increase of PCSK9 compared to monotherapy with statins (66, 67). In our study, indeed, an additional rise of circulating PCSK9 has been observed compared to statin treatment alone. One further study described an additional increase of circulating PCSK9 after the combination therapy, but not in response to monotherapy with ezetimibe (68).

The elevation of circulating PCSK9 after lipid-lowering therapy could be a counter-regulation mediated by the sterol element binding protein 2. This protein affects cholesterol biosynthesis, LDL-receptors and PCSK9 (69). It seems, that the more potent a lipid-lowering therapy is, the more pronounced is the corresponding increase of circulating PCSK9 (66, 67). In our study, there was a modest increase of circulating PCSK9 in the group receiving monotherapy with ezetimibe, which also has the lowest lipid lowering potency. But why are in most other studies no further increases of circulating PCSK9 detected in response to the combination treatment of statins and ezetimibe? In persons receiving statin treatment circulating PCSK9 may have already reached a plateau from which no further increase is possible, even if lipid-lowering therapy is further intensified (66). In addition, ezetimibe has only minor effects on LDL receptor activity and may
therefore not induce a significant counter-regulation via PCSK9, which is a regulator of the LDL receptor (70).

4.3 Correlations

In the entire cohort we observed a significant inverse correlation between the lathosterol to cholesterol ratio and the plant sterol to cholesterol ratios. This inverse correlation was also seen in the two studies by Silbernagel et al. This observation confirms the inverse association of cholesterol absorption and cholesterol synthesis (56, 71). Furthermore, the campesterol and sitosterol to cholesterol ratios show strong and positive correlations, which explains their similar roles as absorption markers.

4.4 Effects of PCSK9-antibodies

In contrast to the therapy with statins or ezetimibe, the effects of PCSK9-antibody therapy on cholesterol metabolism have barely been studied and not completely clarified so far.

In our study treatment with PCSK9-antibodies caused a decrease of total cholesterol of about 39 % as well as a decrease in LDL-C of 52 %. Furthermore, treatment with PCSK9-antibodies caused a significant decrease of lathosterol of 30 % as well as significant reductions of campesterol of 31 % and sitosterol of 27 %. The lathosterol and campesterol to cholesterol ratios were not altered by treatment with PCSK9-antibodies. However, the sitosterol to cholesterol ratio raised in a modest manner.

We can compare our findings with the findings of three previous reports. Peach et al. investigated the effect of evelocumab on cholesterol metabolism in a post-hoc analysis in a cohort of 133 participants suffering from hypercholesterolemia. For lathosterol a significant reduction was observed after 2 weeks, but no significant change in the lathosterol to cholesterol ratio. Also, significant reductions of campesterol and sitosterol were observed. After 2 weeks the ratio of sitosterol to cholesterol significantly increased in response to therapy with 420 mg of evolocumab but not after 140 mg sc. The campesterol to cholesterol showed no significant alteration (72).

Watts et al. investigated the impact of evolocumab on lipoprotein metabolism in 89 normolipidaemic participants. A 35 % lathosterol reduction and a 30 % campesterol reduction were seen after 8 weeks of therapy with evolocumab. The lathosterol to cholesterol ratio as well as the campesterol to cholesterol ratio did not significantly change (37).
Kawashiri et al. studied the difference between a bi-weekly treatment with evolocumab instead of apheresis in 10 patients with familial hypercholesterolemia. No significant alterations could be observed with regard to the lathosterol to cholesterol ratio as well as the plant sterol to cholesterol ratios (73).

Although only minor changes in the ratios of the synthesis and absorption markers to cholesterol were observed, there was a marked reduction of blood lipids. In the investigation by Peach et al. an LDL-C decrease of 39 – 51 % was observed. In the study by Watts et al. there was a 59 % decrease in LDL-C. A 63 % LDL-C decrease was seen in the investigation of Kawashiri et al.

Our study described a moderate, but significant elevation of HDL-C of nearly 14 % after therapy with PCSK9-antibodies. For total triglycerides, a moderate, but also significant reduction of 17 % was detected.

Our findings of only moderate effects of PCSK9-antibodies on HDL-C and triglycerides are congruent with the literature. Watts et al. reported modest alterations of triglycerides and a HDL-C significant elevation of 15 %. Reyes-Soffer et al. conducted a placebo controlled study with alirocumab in 18 healthy participants to investigate the effects of PSCK9-antibodies on lipoprotein metabolism. They could not detect significant alterations of HDL-C and triglycerides in response to alirocumab (74).

To sum up, there is a significant decrease in total and LDL-C which is associated with a significant reduction of the non-cholesterol sterol concentrations. However, there are no major changes in the balance between cholesterol synthesis and absorption.

Possibly we have a trend for a slight increase of lipid absorption after PCSK9-antibody therapy. As mentioned, in the study by Peach et al. therapy with 420 mg of evolocumab caused a significant rise of the sitosterol to cholesterol ratio. We also observed a modest increase of the sitosterol to cholesterol ratio. The elevated ratio of sitosterol to cholesterol could indicate a compensatory counter-regulation mechanism in response to low total cholesterol concentrations. However, cholesterol synthesis seems to be unaffected.

These results indicate that PCSK9-antibody therapy has no or little effects on lipid synthesis and absorption but rather causes cholesterol reduction through increased lipid catabolism via hepatic uptake of LDL.

If PCSK9-antibodies have no or little effect on lipid synthesis and absorption, why are the absolute values of the synthesis and absorption markers due to PCSK9-antibody therapy reduced? It could be due to concomitant uptake of cholesterol and non-cholesterol sterols
by the liver. A similar extent of cholesterol and non-cholesterol sterol uptake by the liver may result in only minor changes of the non-cholesterol sterols to cholesterol ratios.

Treatment with PCSK9-antibodies causes a massive increase in circulating PCSK9 of 760%. Kawashiri et al. also reported that PCSK9-antibody therapy resulted in a rapid increase of circulating PCSK9 by over 700%. This increase is due to the fact that our PCSK9 assay also detects PCSK9 bound to the antibody (73). An assay that only detects free PCSK9 was not available for the present analyses.

4.5 Effects of PCSK9-antibodies according to pretreatment

In contrast to the results of Peach et al., Watts et al., and Kawashiri et al., our investigation also provides information on how lipid lowering pretreatment affects therapy with PCSK9-antibodies. Again, we have to differentiate the four groups of pretreatment with ezetimibe or statins or ezetimibe plus statins or without previous therapy. The measurements revealed the four pretreatment cohorts have similar changes of circulating PCSK9, major lipids, non-cholesterol sterols, and the various ratios of non-cholesterol sterols in response to treatment with PCSK9-antibodies. This is compatible with the assumption that the different pretreatments have no impact on the effect of PCSK9-antibodies. This result also confirms our conclusion that PCSK9-antibodies have no influence on lipid synthesis or absorption and thus complement each other very well with cholesterol absorption or synthesis inhibitors.

4.6 Limitations of the study

First, our study has no placebo control group. However, all participants included into the study had the indication for PCSK9 antibody treatment. Therefore, it would have not been ethically acceptable to treat half of the participants with placebo. Furthermore, we did not record any dietary habits of the participants. Finally, the duration of the treatment period was 4 – 8 weeks only. For evaluating the long-term impact of PCSK9-antibodies on cholesterol metabolism additional studies with a longer treatment duration would be required.

4.7 Outlook

Our study confirmed the high potential of PCSK9-antibodies to reduce total cholesterol and LDL-C. In addition, we could detect that this potential is independent of lipid-lowering pretreatment.
It seems that PCSK9-antibodies are very well tolerated and hardly cause side effects. PCSK9-antibodies represent a good treatment strategy for patients with increased total cholesterol and LDL-C, especially in persons suffering from familial hypercholesterolemia. Therefore, it would be pivotal to identify affected persons as early as possible to prevent long-term effects of elevated cholesterol levels. This requires efficient screening both on the population level and cascade screening. It is the aim of the Austrian Atherosclerosis Society to establish a national registry of persons with familial hypercholesterolemia in order to screen potential affected family members as early as possible.

Treatment with PCSK9-antibodies is very expensive. Therefore, it is important to use PCSK9-antibodies very efficiently and with a clear indication, which can be ensured by an optimal patient screening.

It would also be useful to investigate the impact of PCSK9-antibodies on lipoprotein subclasses and their lipid content. So it is known for example, that PCSK9-antibodies lower atherogenic Lp(a) plasma concentrations.

After the 4 to 8 weeks of therapy with PCSK9-antibodies LDL-C concentrations were significantly reduced. It is also known that cardiovascular events may be reduced by PCSK9-antibodies. However, survival curves do not diverge before 1 year. Hence it will be of interest, whether atherosclerotic plaques may be reduced within a shorter period of time.

4.8 Conclusion

There is a gradual increase in circulating PCSK9 with increased intensity of lipid lowering pretreatment with ezetimibe or statins or both. This seems to be a counter-regulation against the pretreatment, so that PCSK9-antibodies have a strong effect in lowering cholesterol as an add-on therapy by blocking this counter-regulation-mechanism.

Furthermore, we found a significant reduction of total cholesterol and LDL-C concomitant to a significant decrease of the non-cholesterol sterol levels, but without significant alterations in the equilibrium of cholesterol synthesis and absorption. A similar extent of cholesterol and non-cholesterol sterol uptake by the liver may result in only minor changes of the non-cholesterol sterols to cholesterol ratios. So we might conclude, that PCSK9-antibodies have no strong effect on the balance between cholesterol absorption or synthesis.
The different pretreatments have no impact on the lipid-lowering effects of PCSK9-antibodies. This result confirms our conclusion that PCSK9-antibodies have strong potency in reducing LDL-C in people without pretreatment and in people with statin and/or ezetimibe therapy.
5 References


43. www.kvwl.de/arzt/verordnung/arzneimittel/info/invo/inhibitoren_lipidsenkung_pcs k9_invo.pdf


45. www-1pschyrembel-1de-1pschyrembel.han.medunigraz.at/Sterole/K0LL1/doc/


This refers to figures 1, 2 and tables 5, 6, 7, 8.