DISSERTATION

EFFECTS OF LIPID-THERAPY INTENSIFICATION ON ENDOTHELIAL FUNCTION, CAROTID ARTERIES, LIPOPROTEIN PARTICLE SUBFRACTIONS, INFLAMMATION AND POST-PRANDIAL LIPEMIA

submitted by

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2022

Statutory Declaration and Disclosures

At the time of thesis registration at the Medical University of Graz with the initial working title “Effects of lipid-therapy intensification and post-prandial lipemia in clinical routine” in April 2017 and during the conduct of the thesis, I was an employee of Sanofi-Aventis Austria GmbH. Potential conflicts of interests were reported to the ethics committee as well as to Sanofi-Aventis. A declaration of confidentiality was signed regarding the work of this thesis with the Medical University of Graz as well as with Sanofi-Aventis to protect the legal interests of both institutions. An extended written agreement was signed with Sanofi-Aventis to ensure my independent academic work in my private time beyond my commitments for Sanofi-Aventis. I did not receive financial contributions from any institution regarding this thesis.

In course of formulation and specification of a medical scientific “state-of-the-art” hypothesis, a clinical trial protocol was drafted in close collaboration with the ethics committee and the regulatory authority of Austria (AGES). I was responsible for obtaining regulatory authority and ethics committee approvals including the adaptation of informed consent forms as well as for the communication with these authorities on an ongoing basis involving the preparation of clinical trial reports. The final approved study protocol with the short-name “ALIROCKS” had the title: “A pilot study investigating the effects of lipid-therapy intensification with alirocumab on endothelial function, carotid arteries, lipoprotein particle subfractions, inflammation and post-prandial lipemia in clinical routine”. My further accountabilities included the conduct of laboratory assessments, data documentation via case report and laboratory forms, the statistical analysis, interpretation of data, medical writing, and publication. All tasks were performed in alignment with my supervisor.

Clinical Trial Registration, Institutional Review Board, and Informed Consent Statement

The clinical trial protocol was reported to the European Medicines Agency (EMA), recorded under the EudraCT-number: 2018-000981-12 and was registered in the U.S. National Library of Medicine (clinicaltrials.gov; unique identifier: NCT03559309) before the trial was initiated. The Ethics Committee of the Medical University of Graz approved the study protocol with the code: 29-519 ex 16/17 on the 6 April 2018. The study and thus the thesis was conducted according to the Declaration of Helsinki and informed consent was therefore obtained from all participants included.

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Beside following the Guidelines for the Preparation of a Dissertation of the Medical University of Graz (Version, 28 June 2019), I also followed the guidelines for prevention of auto-plagiarism of the Charite Medical University of Berlin (Version, 16 August 2021). In this respect I want to clearly emphasize that parts of this thesis have been published in a peer-reviewed journal and were reproduced identically, I as author possess full copyrights (Metzner et al., 2022a, Metzner et al., 2022b) including a CC BY 4.0 license. In this respect I would also like to thank the Medical University of Graz for funding these publications as Open Access with CC BY licenses in MDPI Biomedicines. Beyond this copyright and plagiarism statement regarding attributions I also acknowledged the creators of the work directly at the tables and figures presented in this thesis including myself.

Publications and Presentations

- **Short-term Effects of Intensive LDL-Cholesterol Lowering with Alirocumab on Vascular Structure and Function**
  T. Metzner, D.R. Leitner, G. Dimsity, F. Gunzer, P. Opriessnig, K. Mellitzer, A. Beck, H. Sourij, T. Stojakovic, H. Deutschmann, W. März, U. Landmesser, M. Brodmann, G. Reishofer, H. Scharnagl, H. Toplak, G. Silbernagel; 89th European Atherosclerosis Society (EAS) Congress, 30th May to 02nd June **2021**. Abstract #206; E-Poster-Presentation.

  With special thanks to the European Atherosclerosis Society for the honor of a ***Young Investigator Fellowship.***

- **Short-Term Treatment with Alirocumab, Flow-Dependent Dilatation of the Brachial Artery and Use of Magnetic Resonance Diffusion Tensor Imaging to Evaluate Vascular Structure: An Exploratory Pilot Study**

  T. Metzner, D.R. Leitner, G. Dimsity, F. Gunzer, P. Opriessnig, K. Mellitzer, A. Beck, H. Sourij, T. Stojakovic, H. Deutschmann, W. März, U. Landmesser, M. Brodmann, G. Reishofer, H. Scharnagl, H. Toplak, and G. Silbernagel; Biomedicines **2022**, 10, 152; https://doi.org/10.3390/biomedicines10010152, published: 11 January 2022; Impact Factor 6.081 (2021 Journal Citation Reports®), Open Access with CC BY License.

- **Effects of Alirocumab on Triglyceride Metabolism: A Fat-Tolerance Test and Nuclear Magnetic Resonance Spectroscopy Study**

  T. Metzner, D.R. Leitner, K. Mellitzer, A. Beck, H. Sourij, T. Stojakovic, G. Reishofer, W. März, U. Landmesser, H. Scharnagl, H. Toplak, and G. Silbernagel; Biomedicines **2022**, 10, 193. https://doi.org/10.3390/biomedicines10010193, published: 17 January 2022; Impact Factor 6.081 (2021 Journal Citation Reports®), Open Access with CC BY License.

Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice”.

Graz, 14 March 2022 Thomas Metzner

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A big thank you!

Thomas Metzner

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Abbreviations and Definitions

ANGPTL-3	Angiopoietin-like-3
ANGPTL-4	Angiopoietin-like-4
ApoB	Apolipoprotein B
ApoCIII	Apolipoprotein CIII
ASCVD	Atherosclerotic Cardiovascular Disease
AUC	Area Under the Curve
CABG	Coronary Artery Bypass Graft
CHD	Coronary Heart Disease
C-IMT	Carotid-Intima Media Thickness
CM	Chylomicrone Particles
CMR	Cardiovascular Magnetic Resonance
CRP	C-Reactive Protein
COVID-19	Coronavirus Disease-2019
IL-6	Interleukin-6
FA	Fractional Anisotropy
FDD	Flow-Dependent Dilatation
FH	Familial Hypercholesterolemia
GPIHBP-1	Glycosylhosphatidylinositol-anchored High-density Lipoprotein-binding Protein-1
HDL	High Density Lipoprotein
Lp(a)	Lipoprotein-a Particles
LDL	Low Density Lipoprotein
MCP-1	Monocyte Chemoattractant Protein-1
MI	Myocardial Infarction
MRI	Magnetic Resonance Imaging
NMR	Nuclear Magnetic Resonance
PCI	Percutaneous Intervention
PCSK9	Proprotein Convertase Subtilisin/Kexin type 9
TG	Triglycerides
TIA	Transient Ischemic Attack
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low-Density Lipoprotein Particles

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Abstract in German (Zusammenfassung)

Ziele: Die Wirkung von Alirocumab auf die Gefäßfunktion und -struktur sind bisher kaum untersucht worden. Darüber hinaus gibt es keine zuverlässigen, nicht-invasiven Methoden zur Bewertung atherosklerotischer Veränderungen des Gefäßsystems. Die ALIROCKS Studie evaluierte in diesem Zusammenhang die Verwendung von Ultraschall-basierten Standardverfahren sowie einer neuen Magnetresonanz-basierten Methode.

Methoden: Es wurden 24 Patienten mit Indikation zur Behandlung mit PCSK9-Antikörpern rekrutiert, mit 2 geplanten Studienvisiten, die erste vor Beginn der Behandlung mit Alirocumab und die zweite nach 10 Wochen Behandlung. Zu den wichtigsten Endpunkten gehörten die Änderung der fraktionellen Anisotropie der Halsschlagader als Maß für die strukturelle Integrität der Gefäßwand, sowie die Ultraschall-gemessene Änderung der Fluss-abhängigen Dilatation der Brachialarterie als Maß für die Endothelfunktion.

Ergebnisse: Insgesamt 21 Teilnehmer hatten eine erste und zweite Studienvisite. Davon injizierten zwei Teilnehmer nur zweimal Alirocumab, 19 Patienten beendeten die Studie gemäß Protokoll. Ihre mittlere LDL-Cholesterinkonzentration wurde mit Alirocumab signifikant reduziert ($p < 0{,}001$). Die Partikelkonzentration vom Gesamt- sowie kleinem dichten LDL nahm ebenfalls signifikant ab ($p < 0{,}001$), während die HDL-Partikelkonzentration signifikant zunahm ($p = 0{,}001$). Die Fluss-abhängige Dilatation der Brachialarterie nahm nominal um 41% zu, aber dies war statistisch nicht signifikant. Die fraktionelle Anisotropie der Gefäßwand der Halsschlagader veränderte sich nicht. Die PCSK9 Plasmakonzentration stieg durch die Behandlung mit Alirocumab deutlich an ($p < 0{,}001$). Während das PCSK9 bei allen 19 adhärenten Patienten um mehr als das Dreifache anstieg, blieb sie bei den beiden Patienten mit nur zwei Injektionen unverändert.

Schlussfolgerung: Trotz einer nominalen Verbesserung der Fluss-abhängigen Dilatation waren keine signifikanten Effekte einer Kurzzeitbehandlung mit Alirocumab auf die Gefäßfunktion nachweisbar. Für den Einsatz der neuen Magnetresonanz-basierten Methodik in der klinischen Atherosklerose Forschung wären weiterführende Untersuchungen erforderlich. Die PCSK9 Plasmakonzentration könnte ein nützlicher Biomarker sein, um die Nicht-Adhärenz von einem Nicht-Ansprechen auf PCSK9-Antikörper zu unterscheiden.

Registrierung der klinischen Studie: URL: http://www.clinicaltrials.gov (NCT03559309).

Publiziert: (Metzner et al., 2022a, Metzner et al., 2022b).

Abstract in English

Aims: The effects of alirocumab on vascular structure and function have been sparsely evaluated. Besides, non-invasive reliable imaging techniques are needed to evaluate pathological changes caused by atherosclerosis. To address these issues, we conducted ultrasound-based and magnetic resonance-based imaging assessments.

Methods: All 24 recruited patients had an indication for PCSK9-antibody treatment. The treatment duration with alirocumab was 10 weeks. Outcome measures involved the change of fractional anisotropy of the carotid artery as a marker for vascular integrity, change of flow-dependent dilatation of the brachial artery as parameter for vascular function, change in the lipoprotein particle profile as well as the change of systemic inflammation or vascular biomarkers.

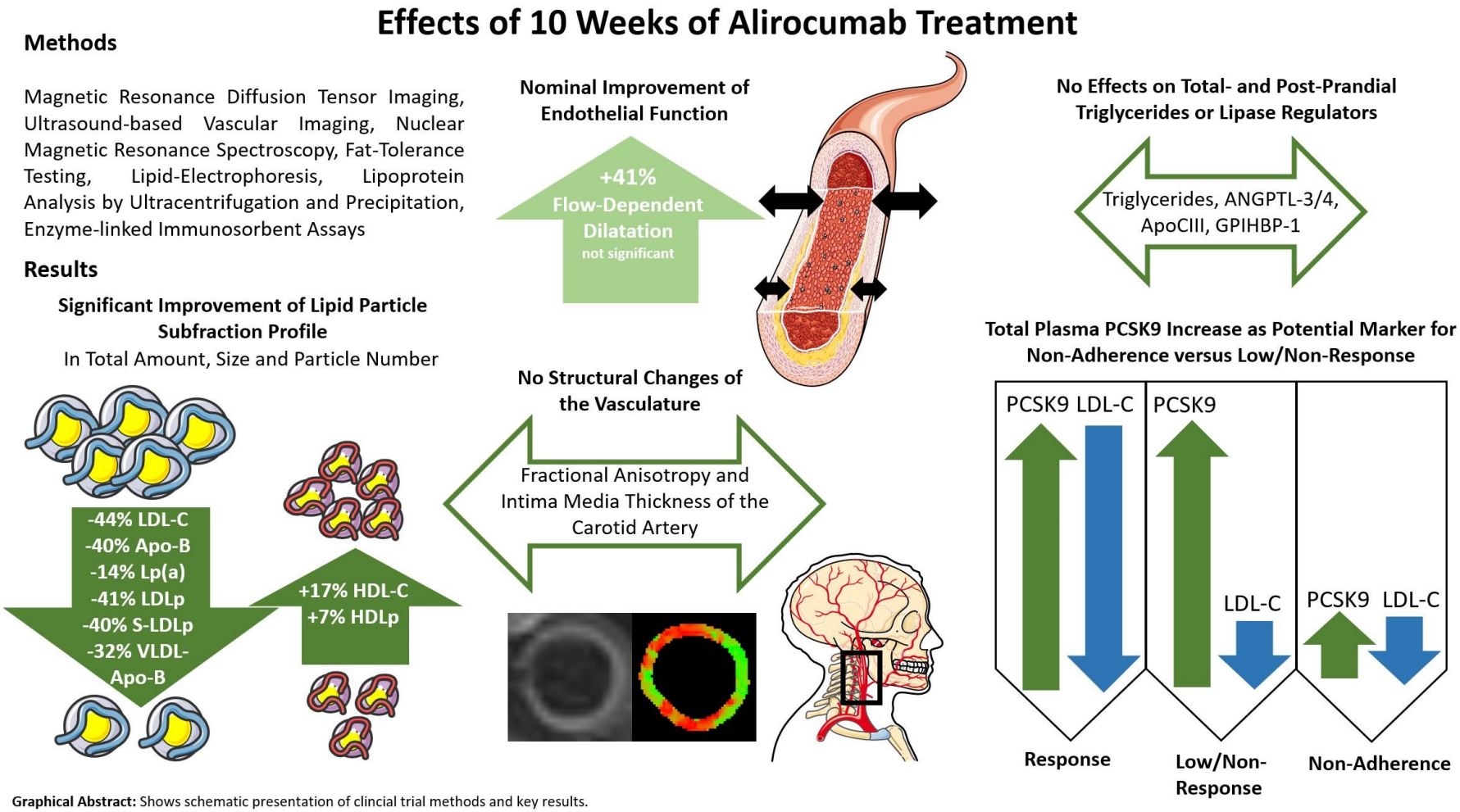
Results: Among all recruited patients, 21 trial participants had a baseline and a week 10 visit as planned. Two patients injected alirocumab only twice, thus 19 participants completed the trial per protocol. Their mean LDL-cholesterol concentration was significantly reduced by alirocumab ($p < 0.001$). The particle concentrations of total and small-dense LDL also significantly decreased ($p < 0.001$), whereas the HDL particle concentration significantly increased ($p = 0.001$). Flow-dependent dilatation nominally increased by 41% but this was not statistically significant. Fractional anisotropy of the carotid artery did not change. Total plasma PCSK9 increased more than 3-fold in all 19 adherent patients ($p < 0.001$) but did not change in those two non-adherent patients with two injections of alirocumab only. Changes of plasma biomarkers regarding systemic inflammation or vascular function were not detectable.

Conclusion: Statistically significant effects of alirocumab on vascular function or structure could not be shown in our exploratory pilot trial. More research efforts are needed to investigate the potential role of the novel magnetic resonance imaging technique in the clinical research setting. The assessment of total circulating PCSK9 concentration may be useful to distinguish non-adherent from non-responder patients.

Clinical Trial Registration: URL: http://www.clinicaltrials.gov. Unique identifier: NCT03559.

Published: (Metzner et al., 2022a, Metzner et al., 2022b).

Graphical Abstract



Graphical Abstract: Shows schematic presentation of clincial trial methods and key results.
Medical images were provided by Les Laboratoires Servier with permission for free use (smart.servier.com). The magnetic resonance images of the carotid artery were provided by our radiology team with permission for use.

1 INTRODUCTION

1.1 General Aspects of Atherosclerosis

Atherosclerosis can be considered as having the longest incubation periods among all human diseases, as its lesions are formed in humans over the course of years to decades, but despite this chronicity of the disease - the clinical consequences occur suddenly and often without warning. It is this long incubation period of the disease that creates difficult discussions of its main drivers, the beginning of “secondary” cardiovascular prevention and when advanced imaging techniques or medical treatment may be indicated. Meta-analysis and randomized clinical studies strengthened the direct link between the accumulation of LDL cholesterol (LDL-C) in blood vessels and inflammation (Catapano et al., 2017b, Mach et al., 2020).

Both processes have been studied widely separated over several decades. To date the reduction of LDL-C is key to improve cardiovascular outcome and therefore is the main target in all current international guidelines for the treatment of vascular risk patients. The lifelong progression of atherosclerosis mainly by lipid particle accumulation in the wall effects all blood vessels in general, but is getting serious, symptomatic, and life-threatening obviously when essential body parts are affected by the narrowing of the vessel lumen and might subsequently lead to a complete restriction in blood supply, alone or in combination with thrombus formation, plaque rupture and inflammation (Figure 1). These cardiovascular events, require immediate hospitalization when getting symptomatic, and affect most often the heart (coronary heart disease), the brain (cerebrovascular artery disease) or the legs (peripheral artery disease) leading to progressive tissue death if not treated accordingly. It is

well known that death from cardiovascular disease is the number one “killer” in industrialized countries nowadays, accountable for more than 4 million deaths in Europe each year (Libby, 2013, Mach et al., 2020).

It seems that plaque growth takes place extra luminal in the early phases of atherosclerosis, without significantly affecting the luminal area of the blood vessel for a very long period. This might lead to the impression that months or years prior to the myocardial infarction or other serious cardiovascular events, the situation is considered as “not clinically relevant” to be medically treated at all, or not with the required intensity to stop the progression of the disease (Figure 1). The common misperception that most acute coronary events arise from ruptured of mildly stenotic plaques arose from multiple studies that had measured the luminal narrowing months to years before myocardial infarction. Recently, postmortem pathological studies and angiographic studies within 3 months before myocardial infarction have all shown that culprit plaques in the acute phase of myocardial infarction are severely stenotic. It was also shown that plaque morphology might help to detect vulnerable plaques with an increased potential for adverse outcomes. For instance, when considering the plaque atheroma volume, necrotic core size, inflammation, thin fibrous caps, recurrent signs of plaque rupture and healing, intra plaque neovascularization or hemorrhage (Ahmadi et al., 2015, Ahmadi et al., 2019).

Acute coronary syndromes can be sub-classified in four causative mechanisms driving the atherosclerotic process:

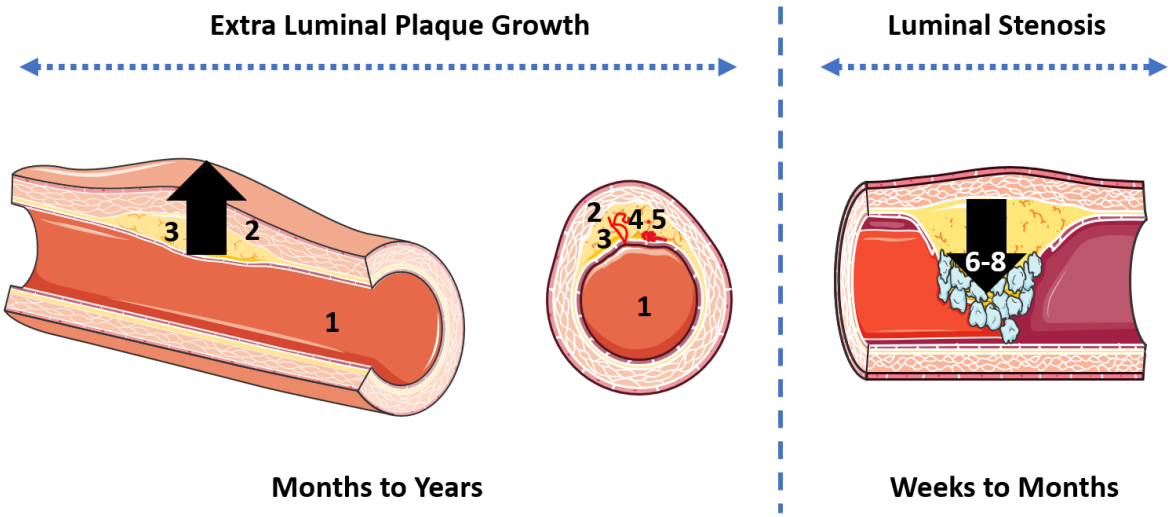
A. Plaque fissure or rupture with local and systemic inflammation accompanied with an increase in blood C-reactive protein (CRP).

B. Plaque rupture without systemic inflammation, thus no observed increases in C-reactive protein and large collections of intimal macrophages.

C. Plaque erosion exhibiting characteristics of white platelet-rich structures. No fissure, with neutrophil accumulation often provoking non-ST-segment-elevation myocardial infarction.

D. Epicardial or microvascular spasm without thrombus formation. The vasospasms can also cause acute coronary symptoms affecting epicardial arteries as well as coronary microcirculation.

Figure 1. Proposed mechanisms for plaque progression.



Legend: The plaque is expanding outwards at early stages, while the luminal constriction is quite stable despite ongoing plaque growth, someday the plaque may reach the limit of outward expansion and begins to rapidly grow intraluminal. Possible mechanisms for this rapid luminal stenosis at the final stage before a possible myocardial infarction are listed from 4-9.: *1. Artery lumen; 2. Fibrous plaque; 3. Necrotic core; 4. Intra plaque neovascularization; 5. Red blood cell leakage from intra-plaque vessels; 6. Intra-plaque hemorrhage; 7. Subclinical plaque rupture; 8. Thrombus formation.* This figure for illustrational purposes was created with images obtained from Les Laboratoires Servier with permission for free use (smart.servier.com) according to (Ahmadi et al., 2015, Ahmadi et al., 2019).

These sub-classifications of acute coronary syndromes driving the future of personalized medicine are likely to become increasingly important as new treatment approaches and clinical trials are targeting inflammatory pathways. Therefore, the differentiation between lipid-driven versus inflammation-driven atherosclerosis might be of clinical relevance, especially when considering the residual risk of a patient that is already on intense lipid-therapy expecting a shift in the nature of the disease (Crea and Libby, 2017).

1.2 Lipoprotein Particles and Additional Risk Factors

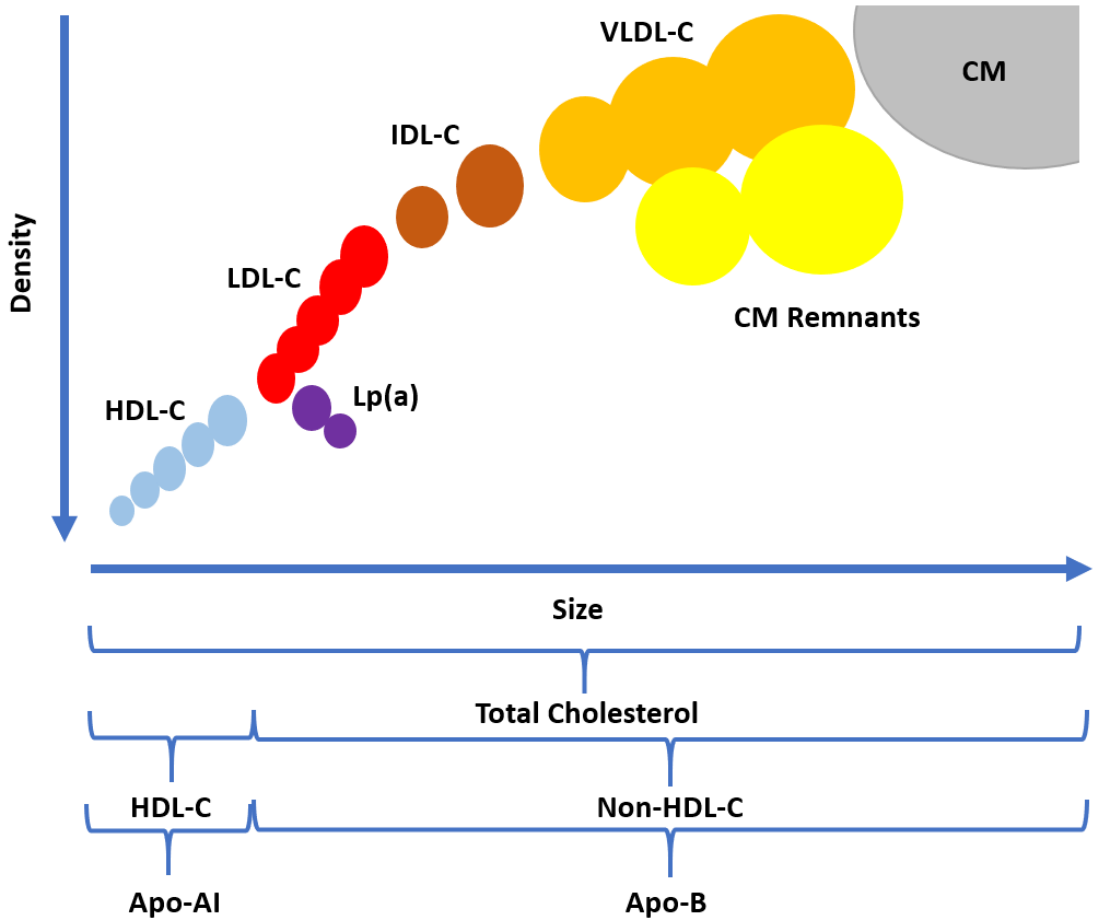
To date LDL-C is the best described and investigated causal biomarker for the development of atherosclerotic cardiovascular disease (ASCVD). The amount of this "vascular toxin" in the blood as well as the duration of exposure are the major determinants of individual cardiovascular risk. Therefore, patients with familial hypercholesterolemia (FH) carry a high cardiovascular risk due to early exposure of very high LDL-C concentrations which is caused by inherited mutations within the LDL-C regulatory pathway. On the other hand, reduction of this individual cardiovascular risk is the highest when LDL-C is reduced early and intensively. Physiologically this pathogenesis is conveyed by endothelial penetration of these particles and deposition in the subintimal space (Ference et al., 2018, Boren et al., 2020, Wiegman et al., 2015, Ference et al., 2020). It is also supposed that LDL particles increase pro-inflammatory cytokines such as interleukin-6 as well as C-reactive protein and reactive oxygen species. This vicious cycle is thought to promote endothelial dysfunction and accelerate the process of atherosclerosis and vice versa (Catapano et al., 2017b). It is believed that oxidized LDL particles have a central role in atherogenesis by recruiting monocyte-derived macrophages into the blood vessels, accumulating esterified cholesterol as well as foam cell formation of macrophages and modulating adherence between endothelial cells and monocytes (Mertens and Holvoet, 2001, Steinberg and Witztum, 2002, Itabe, 2003). The formation of oxidized LDL particles happens during conditions of increased oxidative stress. The postprandial oxidative stress is a subclass of nutritional oxidative stress resulting from sustained post-prandial hyperlipidemia for instance, which in turn is associated with a much higher risk for atherosclerosis (Sies et al., 2005, Homma et al., 2015).

However, patients still experience recurrent major cardiovascular events in spite of powerful lowering of LDL-C with statins and newer lipid-lowering medications (Sampson et al., 2012). A great variety of other cardiovascular risk factors determine the individual risk and are responsible for this "residual risk", when LDL-C is already treated below the recommended guideline targets (Boren et al., 2020, Mach et al., 2020). Well described additional cardiovascular risk factors can be impaired fasting glucose, type-2 diabetes, hypertriglyceridemia, low non-high-density lipoprotein cholesterol (non-HDL-C), large waist circumference, high apolipoprotein B (ApoB), small-dense LDL-C and elevated blood pressure (Figure 2). Therefore, further high-risk populations are often described as having a

mixed dyslipidemia, characterized by elevated triglycerides, high LDL-C, and low high-density lipoprotein cholesterol (HDL-C). Around half of these patients also have type-2 diabetes described as diabetic dyslipidemia, and the population itself is a subpopulation of patients with metabolic syndrome, thus patients defined as having four central components: Hypertension, abnormal glucose metabolism, dyslipidemia and obesity (Halcox and Misra, 2015). In this context, a lipid profile with a large amount of small-dense LDL particles, known to be highly prevalent in patients with diabetic dyslipidemia, has been described to be especially atherogenic as these particles can penetrate the endothelium more easily due to their reduced size (Meeusen, 2021, Hoogeveen et al., 2014, Krauss, 2004). In contrast, a large amount of small HDL particles has been shown to be protective for the vasculature as these particles convey the important mechanism of reverse cholesterol transport (Marz et al., 2017, Silbernagel et al., 2017).

Hypertriglyceridemia is the result of increased triglyceride-rich lipoprotein (TRL) particles (Chylomicrons, VLDL). Elevations of circulating TRLs are induced by their reduced clearance from the blood and / or their overproduction in the liver (Packard et al., 2020). These ApoB containing lipoprotein particles carry high amounts of cholesterol and can also enter the endothelium of the vasculature, especially as remnants after its triglycerides are hydrolysed by lipoprotein lipases (Nordestgaard, 2016, Ference et al., 2020). This accumulation process of cholesterol in the subintimal space may subsequently lead to the formation of atherosclerotic plaques in a time and dose dependent manner (Boren et al., 2020). A key modulator of the TRL metabolism is apolipoprotein CIII (ApoCIII), which inhibits the hepatic uptake of triglyceride-rich lipoproteins via inhibition of the lipoprotein lipase.

Figure 2. Plasma lipoprotein particles according to size and density, as well as their representative cholesterol and apolipoprotein markers.



Legend: CM: Chylomicrons, VLDL-C: Very low-density lipoprotein cholesterol, IDL-C: Intermediate-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, HDL-C: High-density lipoprotein cholesterol, Lp(a): Apolipoprotein a cholesterol. This illustrational figure was reproduced with minor adaptations from (Kjellmo et al., 2018) with permission (CC BY License).

Loss-of-function mutations in the ApoCIII gene lower plasma triglyceride levels and reduce the risk of coronary heart disease. It was shown that patients with type-2 diabetes mellitus with increased levels of plasma Apo-CIII have higher triglyceride levels and coronary artery calcifications (Qamar et al., 2015). Monoclonal antibodies against ApoCIII and human missense variants accelerate clearance of the protein and lower TRL levels (Khetarpal et al., 2017). Angiopoietin-like (ANGPTL) protein family members such as ANGPTL-3 and -4 have emerged as important regulators of plasma lipoproteins by inhibiting the lipoprotein lipase

enzyme. Compared to ANGPTL-4, the ANGPTL-3 protein is exclusively produced in the liver, thus may be considered as the true hepatokine and is mainly active post-prandial, while ANGPTL-4 is mostly active after fasting. Inactivation of ANGPTL-3 leads to reduced plasma triglycerides, free fatty acids and supresses the progression of atherosclerosis. Homozygous loss-of-function mutations in humans lead to a combined hypolipidemia with low plasma LDL-C, HDL-C, and triglyceride levels. Heterozygous carriers have a lower risk of coronary artery disease. Monoclonal antibodies and antisense oligonucleotides are already in development for these potential therapeutic targets (Kersten, 2017). Beside the TRL metabolism ANGPTL-4 might be also a key factor in the regulation of insulin resistance, insulin secretion and rheumatoid arthritis by driving chronic inflammation (Kim et al., 2017, Masuko, 2017). Like ANGPTL-3, the ANGPTL-4 protein is suggested to be a new therapeutic target for the treatment of cardiovascular disease, and both angiopoietin-like family members are evolving markers for chronic hepatitis and several types of carcinomas probably via their inflammatory stimulating attributes (Olshan and Rader, 2018, Chen et al., 2018, El-Shal et al., 2017, Hata et al., 2017).

The measurement of lipoprotein a particles (Lp(a)) is also important for further risk categorization and currently proposed as additional risk factor (Mach et al., 2020). Its evaluation is recommended in most dyslipidemia guidelines for patients with high cardiovascular risk and has a suggested cut-off value of 50 mg/dL (Figure 2). Above this, Lp(a) has been shown to be significantly associated with an increased cardiovascular risk (Nordestgaard et al., 2010, Catapano et al., 2016). Structurally, the particles can be considered as LDL-C with an additional lipoprotein on its surface - the Apolipoprotein a (Apo (a)), it is this protein that is believed to convey an additional pro-thrombotic effect by its structural similarity to plasminogen, thereby further driving the process of atherothrombotic progression. Importantly, it might not only be the amount of Lp(a) in the blood vessels but also a higher Apo-a kringle number of the protein that increase the risk for cardiovascular events. Thus, the hypothesis is that patients with higher kringle numbers have smaller Lp(a) particles, that can more easily invade the vessel endothelium, and therefore are believed to possess an even more atherogenic lipid profile. This kringle isoform size heterogeneity, as well as the total amount of Lp(a) particles in the blood stream, have been shown to be highly genetically determined (Borrelli et al., 2019, Nordestgaard et al., 2010, Nordestgaard and Langsted, 2016).

However, the exact measurement and/or interpretation of lipoprotein particles is especially important for patients with high triglycerides or high lipoprotein-a particles because these patients might have wrong calculations of concentrations by the Friedewald equation but this is most frequently used in clinical practice (Nordestgaard et al., 2016, Mach et al., 2020).

Friedewald equation:

LDL-C = Cholesterol - HDL-C - (TG/2.2) in mmol/l or

LDL-C = Cholesterol - HDL-C - (TG/5) in mg/dl

The equation assumes a constant triglyceride/cholesterol ratio in VLDL particles, that there are no chylomicrons present and includes cholesterol present in IDL as well as Lp(a) particles (Figure 2). It is known therefore that the equation significantly underestimates LDL-C compared to direct measurements when the triglyceride concentration is above 4,5 mmol/l or in the case of low LDL-C levels alone or accompanied by a triglyceride elevation (Scharnagl et al., 2001). This is especially important when PCSK9 inhibitors are considered as a therapeutic option which can result in extremely low LDL-C concentrations, together with the fact that LDL-C is still the most important target in cardiovascular risk management. The consideration of non-HDL-C levels or Apo-B levels might correct a possible misinterpretation in individual risk assessments, thus direct LDL-C measurements might not be essentially needed in the clinical routine when focusing on individual non-HDL-C and Apo-B targets in patients having increased triglycerides. Beside this, the calculation of Apo-B concentrations in clinical routine might be a useful risk modifier as it presents the total atherogenic particle number than focusing solely on the particle masses. Thus, patients with the same particle masses, for instance 70 mg/dL LDL-C might have different levels of Apo-B representing the particle numbers and are believed to convey different risks, therefore (Mach et al., 2020).

Finally, inflammation has been made accountable for being a key driver of residual cardiovascular risk and as initially stated in this chapter is thought to be associated with high LDL-C levels. Most recently, the CANTOS trial investigating the anti-IL1ß antibody canakinumab was the first randomized cardiovascular outcome trial that proofed causality of inflammation in the process of atherosclerosis (Ridker et al., 2017a). Systemic inflammation monitored by CRP changes seemed not to be affected by treatment of PCSK9-antibodies at a first glance (Momtazi-Borojeni et al., 2019). However, other in-vitro and in-vivo studies in

mice do suggest an involvement of the PCSK9 antigen in important inflammatory pathways driving atherosclerosis (Tang et al., 2017, Tang et al., 2019, Momtazi-Borojeni et al., 2019, Liu et al., 2019). Therefore, rapid improvement of endothelial dysfunction and inflammatory processes by PCSK9 antibodies might also contribute to its positive treatment effects on "hard endpoints" such as the reduction of major cardiovascular events and all-cause mortality. However, potential inflammatory factors driving the pathogenesis of atherosclerosis involve a great variety of different cytokines and its receptors of yet unclear pathways (Wu et al., 2017, Back and Hansson, 2015). The ATHEROMO-IVUS study investigated the relation of serum PCSK9 levels to coronary plaque inflammation in 581 patients by intravascular ultrasound virtual histology (IVUS-VH) imaging. It was shown that PCSK9 levels were linearly associated with the fraction and amount of necrotic core tissue, independently of serum LDL-C levels and statin use (Cheng et al., 2016). PCSK9 silencing experiments in apolipoprotein E knockout (ApoE KO) mice showed less aortic atherosclerotic plaques compared with the control group and had a decreased expression of vascular inflammation regulators, such as IL-1βa and MCP-1 for instance. In vitro overexpression of PCSK9 in macrophages increased the secretion of pro-inflammatory cytokines (Tang et al., 2017). To date a great variety of pre-clinical studies, support the thesis that PCSK9 down-regulation might attenuate plaque inflammation and atherosclerotic development independent of lipoprotein plasma levels. Apart from the liver, PCSK9 is also expressed by many other organs and there is evolving evidence that PCSK9 is expressed in human atherosclerotic plaques as well (Tang et al., 2019, Momtazi-Borojeni et al., 2019).

1.3 Oral Lipid-modifying Therapies

Treatment with statins and other oral lipid-modifying therapies are often insufficient to reach the current treatment targets for LDL cholesterol (Marz et al., 2016). Fenofibrates can be added to statins in case patients display high triglycerides and low HDL-C. Fibrates are fibric-acid derivates and are selective agonists of the peroxisome proliferator receptor-α (PPAR-α) and are well known to reduce triglyceride levels and increase HDL-C simultaneously. However, special caution needs to be taken in co-medication with statins, as there is evidence for an increase of muscle disorders. In contrast, applied as monotherapies, there remains only

a low risk of myopathy. Omega-3 polyunsaturated fatty acids (PUFAs) can be prescribed for patients with elevated triglycerides, and are known to have beneficial effects not only for reducing triglycerides, but also positively modify VLDL-C levels (Halcox and Misra, 2015).

Since publication of the positive cardiovascular outcome results of the IMPROVE-IT trial investigating ezetimibe + simvastatin versus simvastatin alone, the cholesterol absorption inhibitor became a feasible option beside statins to reduce elevated LDL-C. The molecule binds the Niemann-Pick C1-like 1 protein responsible for cholesterol uptake or resorption in the intestine (Cannon et al., 2015). The clinical usage of ezetimibe for LDL-C reduction and thus prevention of cardiovascular events varies greatly even between industrialized countries. This variety derives mainly through differing economic situations and considerations. High dose statins lead to an LDL-C reduction of around 50%. In contrast, addition of ezetimibe only produces LDL-C reductions of around 15-20%. Therefore, depending on the market situation and on a distance-to-target principle, ezetimibe is or is not an option between statins and PCSK9 antibodies, that induce an additional LDL-C reduction of around 60% (Sabatine, 2017, Waters and Boekholdt, 2017, Catapano et al., 2016).

Recently a cardiovascular outcome trial with icosapent ethyl reducing hypertriglyceridemia reported positive results regarding a composite primary endpoint of cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, coronary revascularization, or unstable angina. Icosapent ethyl is a highly stable eisosapentaenoic acid (EPA) ethyl ester, and besides lowering triglycerides, may have anti-inflammatory, anti-oxidative and plaque stabilizing effects. The trial was randomized, double-blind and placebo controlled and recruited around 8000 patients with a median follow-up of approx. 5 years. Eligible patients had fasting triglyceride levels of 150 to 499 mg/dl and LDL-C levels between 41 to 100 mg/dl and receiving stable statin doses for at least 4 weeks. The trial participants received 2g icosapent ethyl twice daily with food (4g/day) or placebo containing mineral oil to mimic the color and consistency. Composite primary endpoint events occurred in 17,2% of the patients in the verum and in 22,0% of the patients in the placebo group. Significant safety findings involved an increase of patients hospitalized for atrial fibrillation or flutter and serious bleeding events, but the absolute difference between the groups was 1% or below and altogether the event rates were very rare (Bhatt et al., 2019).

Another lipid-modifying substance that may become clinically relevant soon is bempedoic acid, an inhibitor of the ATP citrate lyase. Several phase-3 trials were published in the years of 2018 and 2019 evaluating the safety and efficacy in patient populations with or without statin intolerance, established ASCVD or equivalent risk factors such as familial hypercholesterolemia. The primary endpoint of the trials was LDL-C lowering, and all were randomized as well as placebo controlled. The LDL-C lowering effect of bempedoic acid was stronger in the statin intolerance patient population as monotherapy or in combination with ezetimibe, responsible for around 20-30% LDL-C reduction compared to placebo (Laufs et al., 2019, Ballantyne et al., 2018). In the trials that recruited patients on statins as monotherapy or oral-combinations with ezetimibe the addition of bempedoic acid led to an LDL-C reduction of around 15% compared to placebo (Ray et al., 2019a, Goldberg et al., 2019).

It is important to mention that the therapeutic evidence for reducing cardiovascular events is highest for statins compared to all other oral lipid-modifying therapies. Several meta-analyses evaluated their clinical net benefit by investigating various randomized cardiovascular outcome trials, while there is only one published for ezetimibe so far. The “Cholesterol Treatment Trialists Collaborators” published already in 2005, that in a prospective meta-analysis of data from around 90.000 patients in 14 randomized trials of statins, treatment can safely reduce the 5-year incidence of major cardiovascular events by 20% per each 40 mg/dl LDL-C reduction, irrespective of the initial lipid profile or other baseline characteristics. The absolute benefit related to an individual’s absolute risk and to the absolute reduction of LDL-C achieved (2005). Subsequent meta-analyses published by the collaboration group showed that especially diabetic individuals, but even people with low risk of vascular disease benefited greatly of a statin therapy (2012, 2008). Another important learning from the statin trials was, that a more intense therapy and thereby further LDL-C lowering led to a significant better result in reducing cardiovascular events. The analysis also confirmed previous investigations by suggesting that each 100 mg/dl reduction in LDL-C reduces cardiovascular risk of around 50% (2010).

In general, statins are very well tolerated and for the clinical trial populations described above, the net benefit in cardiovascular risk reduction outweighs potential side effects by far. The most frequent adverse effects are mild such as discolored urine, gastrointestinal upset and hepatotoxicity most often characterized by an increase of hepatic aminotransferases, but in

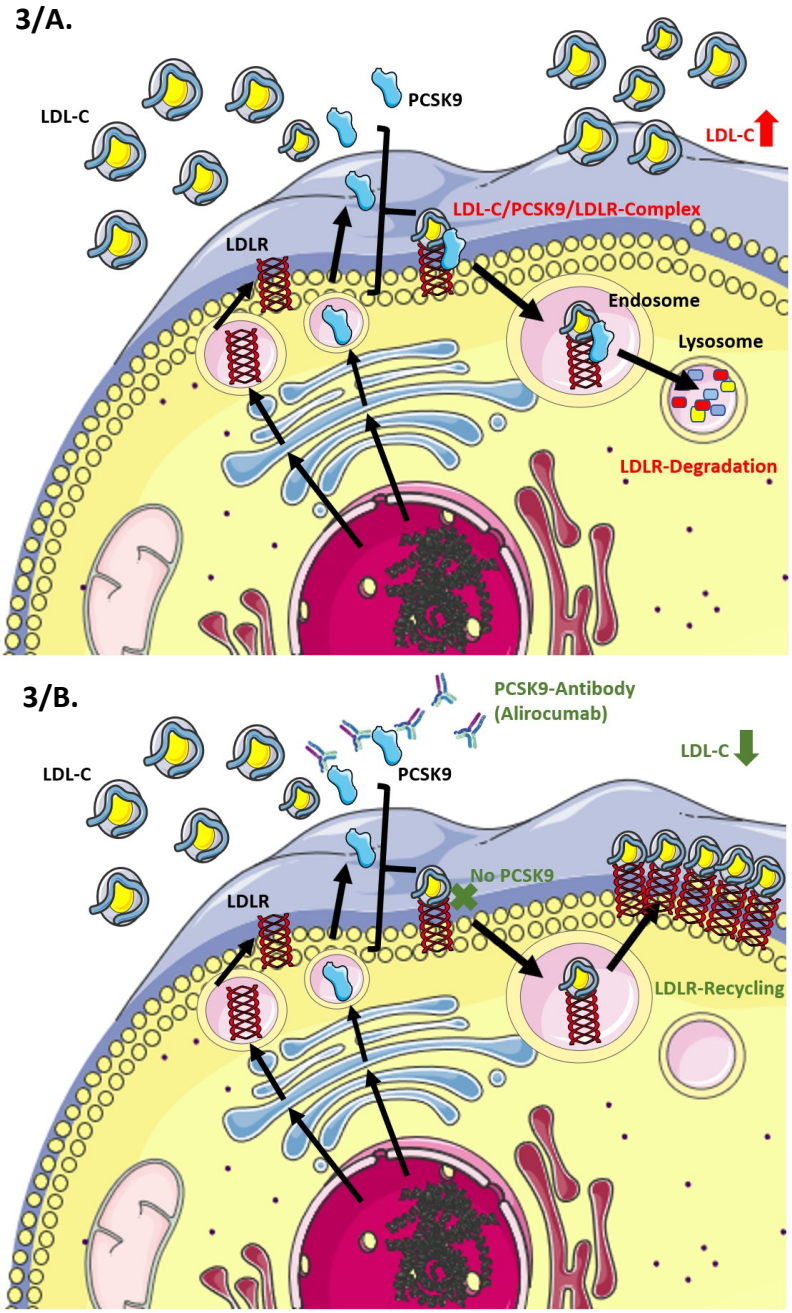
very rare cases might lead to hepatocellular injury and fulminant liver failure (Liu et al., 2010). In addition, it is known that myotoxicity such as myalgia or myopathy occurs in around 10% of statin treated patients and it may progress in very rare cases to rhabdomyolysis, characterized by myoglobinuria, massive muscle necrosis and acute renal failure (Williams and Feely, 2002). The potential side effects are known to be dose and class dependent. The rank order of myotoxicity, without considering the LDL-C lowering effect by dose, was described to be simvastatin > fluvastatiin > atorvastatin > lovastatin > pitavastatin >> rosuvastatin = pravastatin (Kobayashi et al., 2008). Drug-drug interactions might induce or intensify potential statin side effects, resulting from the inhibition of the CYP3A4 enzyme by changing the metabolism of statins and vis-a-versa (Gazzerro et al., 2012). Statin adherence is known to be a major barrier in LDL-C target achievement and might be a devastating consequence of misleading media information regarding its drug safety profile over the last decades. Low adherence is associated with increasing cardiovascular events and fatal outcomes. A recently published ESC position paper tries to argue for lipid-modifying combination therapies with lower dosages of statins and new lipid-lowering drugs to increase adherence and LDL-C target achievement (Drexel et al., 2020a).

1.4 PCSK9-Inhibition: From Theory to Practice

The involvement of the proprotein-convertase-subtilisin/kexin type 9 (PCSK9) gene in LDL circulation was first described around 20 years ago. Genetic analysis showed that PCSK9 mutations lead to autosomal dominant hypercholesterolemia characterized by isolated elevation of LDL particles that were already known to increase cardiovascular events and mortality. It turned out that loss-of-function or gain-of-function mutations may occur in the PCSK9 gene that can either extensively increase or decrease plasma LDL particles. High plasma PCSK9 protein levels were shown to decrease LDL receptors at hepatic cell surfaces by accelerated receptor degradation, thereby leading to increasing plasma LDL-C via less receptor-mediated particle internalization (Figure 3). It was known at this time that LDL-lowering mutations can take place in the Apo-B or LDL receptor gene causing familial hypercholesterolemia. With the involvement of PCSK9 in the LDL particle regulatory

mechanism another key mutation site was found (Abifadel et al., 2009b, Abifadel et al., 2009a, Varret et al., 1999, Abifadel et al., 2003).

Figure 3. PCSK9-mediated degradation of LDL-receptors and its inhibition by PCSK9 antibodies.



Legend: LDL-C: Low-density lipoprotein cholesterol particles, LDLR: Low-density lipoprotein cholesterol particle receptors, PCSK9: Proprotein-convertase-subtilisin/kexin type 9.

3/A. PCSK9-mediated degradation of the LDLR/LDL-C complex. The PCSK9 protein acts as glue leading to the degradation of the whole complex instead of LDL-receptor recycling to the surface.
3/B. Antibody mediated inhibition of PCSK9 in the plasma preventing the target antigen from binding LDL-receptors at the hepatic cell surface. Receptors can recycle to the surface thereby increasing the number of receptors, LDL-particle binding, and internalization resulting in a decrease of plasma LDL particles. This illustrational figure was reproduced with minor adaptations from (Rana et al., 2019) with permission (CC BY-NC). Images were obtained from smart.servier.com with permission for free use.

The PCSK9 gene locus and protein structure, as well as its life cycle were intensively studied during subsequent research. It was shown that LDL receptors recirculate to the hepatic cell surface around 100-times, after LDL particle internalization (Figure 3). This recycling process facilitates repeated binding and internalization of more LDL particle ligands at the cell surface. When PCSK9 is secreted by hepatic cells into the plasma, it binds to the external epidermal growth factor-like repeat A (EGF-A) domain of the LDL receptors. The whole LDL/LDLR/PCSK9 complex will be internalized and the presence of PCSK9 prevents the LDL-receptor to undergo conformational changes needed for its recycling. This leads to the lysosomal degradation of the whole protein complex instead of only recycling LDL particles. Taking this pathway together one can argue that the PCSK9 protein acts as glue for the complex, thereby preventing the LDL receptor recycling and leading to less functional receptors at the hepatic surface (Horton et al., 2009).

Further in-vivo and in-vitro studies strengthened the importance of this regulatory pathway and quickly lead to the first clinical anti-PCSK9 antibody trials in humans that showed promising results making the protein a viable new therapeutic option for lowering plasma LDL-C (Lambert et al., 2012, Rana et al., 2019). The success of those trials, subsequently lead to huge phase-3 clinical trial programs investigating the safety and efficacy of alirocumab, evolocumab and bococizumab in a broad variety of cardiovascular high-risk patient populations with or without statin intolerance (Gouni-Berthold et al., 2016, Reiss et al., 2018). The cardiovascular outcome trials of these three anti-PCSK9 antibodies will be described in the following chapter.

1.5 PCSK9-Antibodies: Cardiovascular Outcome Trials

Three companies, Amgen (evolcumab), Sanofi (alirocumab) and Pfizer (bococizumab) conducted their cardiovascular outcome trials nearly in parallel and reported positive study results between 2017 and 2018. Further important insights evolved in the years afterwards when pre-specified and post-hoc subgroup analyses, as well as meta-analyses were published. Altogether these three clinical trials: ODYSSEY OUTCOMES (Schwartz et al., 2018), FOURIER (Sabatine et al., 2017), SPIRE-1 & SPIRE-2 (Ridker et al., 2017b), recruited more than 70.000 patients of high to very-high cardiovascular risk with a median study duration of around 1-3 years. It's also important to mention that this dissertation and the ALIROCKS trial protocol was drafted in 2017 before the ODYSSEY OUTCOMES trial for alirocumab was published, but when the two other PCSK9 antibodies, evolocumab and bococizumab have already reported positive cardiovascular outcome (Ridker et al., 2017b, Sabatine et al., 2017). Bococizumab was finally not applied for market approval because of reporting anti-drug antibodies that lead to a reduction in efficacy regarding LDL-C lowering over time. This immune response against the PCSK9-antibody is believed to be originated by the mouse derived part of the antibody (humanized), in contrast to the two other approved fully human antibodies evolocumab and alirocumab (Ridker et al., 2017c). Nevertheless, the positive PCSK9 antibody outcome results together with the positive outcome trial of Ezetimibe: IMPROVE-IT (Cannon et al., 2015), further strengthened the importance of LDL-C lowering in the prevention of cardiovascular events (Ference et al., 2017).

Both EMA and FDA approved fully human PCSK9-antibodies alirocumab and evolocumab showed a significant and consistent 15% relative risk reduction of major cardiovascular events (primary endpoints). Key differences of trial designs involved: study duration, patient population, background lipid therapy and LDL-C lowering approach. While the ODYSSEY OUTCOMES trial recruited a homogenous patient population of post-acute coronary syndrome patients that were randomized shortly after their index ACS event (< 2.6 months), the FOURIER trial recruited patients with clinically evident ASCVD such as myocardial infarction, non-hemorrhagic stroke, or symptomatic peripheral artery disease, as well as additional characteristics that placed them at higher cardiovascular risk. Around 80% of the patient population recruited had a prior myocardial infarction, nearly 20% had a non-

hemorrhagic stroke and 13% had a peripheral artery disease. However, the median time from the most recent myocardial infarction or stroke was 3.4 years or 3.2 years respectively, thus representing significantly larger time periods from index events to the time of randomization compared to ODYSSEY OUTCOMES (Schwartz et al., 2018, Sabatine et al., 2017). Another important difference was how the LDL-C lowering was approached. The FOURIER trial had no LDL-C target value implemented in the protocol, so all patients received their study medication regardless of their achieved LDL-C level. This procedure might be considered as "the lower the better" or the "one size fits all" approach. The ODYSSEY OUTCOMES trial started with a dosage of 75 mg alirocumab for all patients, but with a clear target corridor of < 50 mg/dL and > 15 mg/dL LDL-C, so patients could be up-titrated or down-titrated when necessary. Approximately 75% of the patients achieved their target value of < 50 mg/dL with half of the dosage (75 mg), while only around 25% needed up-titration to 150 mg alirocumab, 8,8% percent of the patients had at least 2 consecutive LDL-C values below 15 mg/dL and were blinded switched to placebo. Altogether this target approach was criticized when the results were presented, because this down-titration or placebo switch of alirocumab probably meant that the outcome effects could have been even better than reported. However, one must keep in mind that the trial protocol was written at a time when trial analyses of glucose lowering agents as well as blood pressure lowering drugs reported negative outcomes when levels got too low (J-curves). Also, the safety profile of PCSK9-antibodies was not yet proven in powerful outcome trials. The maintenance of very low LDL-C levels (< 25 mg/dL) is still in discussion in clinical practice, especially over a long treatment period in "primary" cardiovascular prevention. However, as of today a J-curve for LDL-C is not known and the clinical benefit is considered more important than the possible risks, especially when very-high cardiovascular risk patients are concerned (Qamar and Libby, 2019, Michos and Martin, 2019).

An important pre-specified subpopulation analysis of the ODYSSEY OUTCOMES trial evaluating alirocumab considered patients with pre-diabetes and diabetes mellitus at baseline. Out of 18.924 patients recruited 5444 patients (28.8%) had diabetes, 8246 (43.6%) had prediabetes, and 5234 (27.7%) had normoglycemia, defined by patient medical history, baseline HbA1c or fasting serum glucose values (Ray et al., 2019b, Ray et al., 2019c). The reduction of lipid parameters with alirocumab was independent of the glucose-metabolic status, the LDL-C was lowered approx. 65% at month four in all three categories. The relative

risk reductions for the composite primary endpoint were similar with alirocumab across the glycemic categories, but a greater absolute risk reduction was shown for the diabetic subgroup compared to pre-diabetic and normoglycemic patients. Importantly the probability for interaction was significant, these results might not be surprising because diabetes mellitus is a well-defined cardiovascular risk factor leading to increased event rates. The diabetes subpopulation had a MACE (major adverse cardiovascular events) incidence of 16.4% in the clinical trial, compared to 9.2% of pre-diabetic or 8.5% of normoglycemic patients ($p < 0.0001$). Noteworthy, alirocumab did not increase the risk of new-onset diabetes and produced around twice the absolute reduction in cardiovascular events among patients with diabetes as in those without diabetes (Ray et al., 2019b).

1.6 Clinical Guidelines and Treatment Targets for Dyslipidemia

The European Society of Cardiology (ESC), together with the European Atherosclerosis Society (EAS), presented a new guideline for the management of dyslipidemias to reduce cardiovascular risk at the ESC congress in summer 2019. Following the results of PCSK9 antibody cardiovascular endpoint trails with evolocumab (FOURIER) and alirocumab (ODYSSEY OUTCOMES) as described above, lower LDL-C targets for very high-risk patients were expected, but the accurate definition was unclear at this time. The recruitment of the ALIROCKS study, conducted in course of this dissertation, started in summer 2018 approximately one year before presentation of this new guideline. As study participants recruited for this trial were all very-high risk, this chapter will focus especially on this patient population and implemented key changes to the previous guideline from 2016 (Catapano et al., 2016, Mach et al., 2020). The LDL-C treatment goals for very-high cardiovascular risk patients were reduced from < 70 mg/dL to < 55 mg/dL with at least 50% LDL-C reduction from baseline values, which is most likely not to be achieved with moderate intensity statins or low dose oral combination therapy. Therefore, for the very-high risk patient population the first-line treatment with high intensity statins, such as atorvastatin or rosuvastatin, became the preferred option. It is also important to mention that in secondary prevention this target value is listed with the highest possible recommendation class and evidence level (I/A). For patients not meeting one of the very-high risk criteria, the LDL-C treatment targets are < 70 mg/dL

(high-risk), 100 mg/dL (moderate-risk) and < 116 mg/dL for low-risk. Because most clinical evidence regarding the importance of LDL-C lowering evolved from cardiovascular outcome trials including participants with high or very-high risk populations, the recommendation class and evidence level is I/A for both risk categories, in contrast to IIa/A (moderate risk) and IIb/A for low-risk patients. According to the guideline, risk factors resulting in the very-high risk categorization were documentation of ASCVD in the patient's medical history (clinical or imaging), diabetes mellitus including disease duration and target organ damage, chronic kidney disease, familial hypercholesterolemia, and blood pressure. Target organ damage in course of diabetes mellitus was defined as microalbuminuria, retinopathy, or neuropathy.

Accurate definition of very-high cardiovascular risk according to the new ESC/EAS dyslipidemia guideline 2019:

- **Documented ASCVD.** Either clinical or unequivocal on imaging, includes previous ACS (MI or unstable angina), stable angina, coronary revascularization (PCI, CABG, and other arterial revascularization procedures), stroke and TIA, and peripheral arterial disease. Unequivocally documented ASCVD on imaging includes those findings that are known to be predictive of clinical events, such as significant plaque on coronary angiography or computed tomography scan (multi-vessel coronary disease with two major epicardial arteries having >50% stenosis), or on carotid ultrasound.
- **Diabetes mellitus** with target organ damage, or at least three major risk factors, or early onset of T1DM of long duration (>20 years).
- **Severe CKD** (eGFR <30 mL/min/1.73 m2).
- **SCORE ≥ 10%** for 10-year risk of fatal CVD.
- **Familial hypercholesterolemia** with ASCVD or with another major risk factor.

Important for the clinical routine and in respect to this dissertation, the guideline clearly mentioned that patients with a significant plaque on carotid ultrasound or a CT scan displaying two arteries with >50% stenosis need to be treated as very-high risk patients. Patients with inconclusive carotid ultrasound results or unclear medical history might be risk re-classified via coronary computed tomography angiography (CTA) that has one of the highest reliabilities among coronary artery disease assessments to rule-in or -out for coronary disease (Knuuti et al., 2020). During a guideline task force discussion at the ESC congress in Paris 2019 the SCOT-HEART trial and its possible implications on the guideline were

debated. The trial showed significantly less cardiovascular events in the coronary CTA group compared with the standard of care (no CTA) group without affecting the percutaneous interventions. It was shown that the coronary CTA group received intensified lipid modifying therapies and might have improved adherence because of the diagnostic assessment (Investigators et al., 2018).

Together with carotid or femoral plaque burden detection by ultrasound, the coronary artery calcium score (CAC ^{score}) has been demonstrated to be highly predictive of cardiovascular events, while the intima media thickness was inferior to those assessments. Therefore, the CAC was also mentioned as an important risk modifier when >100 Agatston when the patient is asymptomatic and yet without documentation of ASCVD. However, the CAC score is increased following the treatment with statins, therefore patients that are already receiving lipid modifying therapies should be interpreted with caution. Another, probably surprising - major change in comparison to the previous 2016 guideline was the more complex risk separation of patients with diabetes mellitus, that now ranged from moderate to very-high risk depending on disease duration, target organ damage or additional major risk factors. Exact new guideline definitions for diabetes mellitus (DM) cardiovascular risk assessments are:

- **Very-high risk**: DM with target organ damage, or at least three major risk factors, or early onset of Type-1 DM of long duration (>20 years).
- **High risk:** Patients with DM without target organ damage, with DM duration >_10 years or another additional risk factor.
- **Moderate risk:** Young patients (T1DM <35 years; T2DM <50 years) with DM duration <10 years, without other risk factors.

In contrast to the exact definition of target organ damage as described above, major risk factors were not accurately described in the corresponding references, thus are open for diverging interpretations. This interpretation bias for the definition of risk factors is not only important for patients with diabetes mellitus, but also for those patients diagnosed with FH that are only considered as very-high risk in case of established ASCVD or with at least one other major risk factor. Nevertheless, a big achievement was the involvement of FH diagnosis in the risk category overview that was not considered in this detail in the previous guideline of 2016. The heterozygous familial hypercholesterolemia is one of the most prevalent monogenic disorders known to date and is estimated to be 1/200 – 250 resulting in a total

number of 14 – 34 million worldwide and around 30 thousand in Austria, but so far only a minor fraction has been identified. Around 90% of those monogenic mutations have been found in the LDL-receptor gene, the other 10% affect mutations in the PCSK9 or Apo-B gene (Abifadel et al., 2009a, Abifadel et al., 2009b, Abifadel et al., 2003).

The diagnosis of familial hypercholesterolemia by clinical evaluation (score) is recommended (Class: I, Level: C) for patients with:

- coronary heart disease diagnosed < 55 years for men and < 60 years for women,
- people with relatives with premature fatal or non-fatal CVD,
- people with relatives who have tendon xanthomas,
- in people with severely elevated LDL-C (> 190 mg/dL),
- in children > 150 mg/dL,
- first degree relatives of familial hypercholesterolemia patients.

It is also recommended that FH patients should be confirmed, when possible, by DNA analysis. In case an index patient is diagnosed, a family cascade screening should be conducted. The prevalence of FH patients within the patient population getting potentially prescribed PCSK9-antibodies is very high and vice versa. The diagnosis of familial hypercholesterolemia is crucial for early therapeutic intervention and clinical outcome, as well as of special interest regarding this dissertation. Especially for the general practitioner but even for medical universities the clinical FH diagnosis by scores, genetic analysis and familial cascade screenings are extensively time consuming and costly and is therefore only done by specialized centers in Austria. The commonly used clinical criteria (score) for FH diagnosis are from the Dutch Lipid Clinic Network, but other criteria such as the Simon Broome register, or the WHO criteria might be used as well and are accepted. The genetic analysis is recommended but not necessary from a clinical perspective (Mach et al., 2020).

The clinical data and established hypothesis regarding Lp(a) as an important cardiovascular risk factor was noted already in a previous chapter. Because there have been no new clinical trial results in the meanwhile, prospectively investigating the causal relationship of the particles with the cardiovascular outcome of patients, the new guideline of 2019 still considers Lp(a) only as a risk factor important for individual assessments and LDL-C target determination but does not give any recommendations towards targeted medical intervention.

Specific recommendations for Lipoprotein-a particle assessment and interpretation in the new guideline for dyslipidemia are:

- Certain individuals declare themselves to be at high or very high CVD risk without needing risk scoring, this includes patients with extreme Lp(a) elevations.
- Lp(a) measurement should be considered at least once in each adult person's lifetime to identify those with very high inherited Lp(a) levels >180 mg/dL (>430 nmol/L) who may have a lifetime risk of ASCVD equivalent to the risk associated with heterozygous familial hypercholesterolaemia (IIa/C).
- Lp(a) should be considered in selected patients with a family history of premature CVD, and for reclassification in people who are borderline between moderate and high-risk (IIa/C).

Other lipid biomarkers with recommended treatment targets were defined as Non-HDL-C particles and Apo-B lipoproteins, mainly to correct for possible risk misinterpretations for patients with high triglycerides or smokers that might have a higher amount of atherogenic Non-HDL particles on one side or higher small dense LDL particle numbers on the other side, consequently leading to an increased cardiovascular risk without changing the total amount of LDL-C compared to patients with low triglycerides or non-smokers. Accurate guideline recommendations are as followed (Mach et al., 2020):

- Non-HDL-C secondary goals are <2.2, 2.6, and 3.4 mmol/L (<85, 100, and 130 mg/dL) for very-high-, high-, and moderate-risk people, respectively.
- Apo-B secondary goals are <65, 80, and 100 mg/dL for very-high-, high-, and moderate-risk people, respectively.
- No specific goal is recommended for triglycerides, but <1.7 mmol/L (<150 mg/dL) indicates lower risk and higher levels indicate a need to look for other risk factors.

1.7 Current Imaging Techniques of Atherosclerosis

Imaging techniques for atherosclerotic disease can help to further specify risk categorization of patients and may also increase their adherence to drug therapy and lifestyle changes (Investigators et al., 2018). Some diagnostic interventions have already shown to improve

cardiovascular outcomes. Novel diagnostic techniques might be especially important for patients with intermediate to high cardiovascular risk that are still in primary prevention, such as patients with diabetes, chronic kidney disease, high LDL-C values and/or a diagnosis of familial hypercholesterolemia, but still prior to myocardial infarction or stroke (Perez de Isla et al., 2018, Beller et al., 2018, Celeng et al., 2016, Investigators et al., 2018).

In 2019, the updated European clinical guidelines for chronic coronary syndrome and dyslipidemia focused on risk assessment, and main changes included new recommendations considering imaging and functional diagnosis techniques for the progression of atherosclerosis (Knuuti et al., 2020). The possible impact of CAC and coronary CTA was briefly summarized in the previous chapter. The new recommendations view coronary artery disease (CAD) as a serious disease although it may seem clinically silent from time to time. This dynamic characteristic of the disease leads to a variety of clinical presentations that can be categorized either as acute or chronic coronary syndrome. The cardiac risk during this dynamic process is highly dependent on the diagnosis of disease progression and subsequent interventional procedures. In respect to this dissertation major new recommendations can be summarized as followed, all are class I recommendations, thus are indicated (Knuuti et al., 2020):

- Coronary CTA or functional imaging (non-invasive) is initially recommended for diagnosis of CAD in symptomatic patients when obstructive CAD could not be excluded by clinical evaluation.
- The clinical probability of an established CAD should determine which initial non-invasive diagnostic test is chosen, further influential decision factors may be patient characteristics, team expertise, and availability of this diagnostic tests.
- In case coronary CTA indicates a CAD with unclear diagnostic or functional relevance further functional imaging evaluating cardiac ischemia is recommended.
- In patients with very-high probability of an acute cardiovascular event, such as patients with severe symptomatic or angina at low stress burden, invasive angiography is an alternative by guideline recommendations.

1.8 Magnetic Resonance Imaging, Flow-Dependent Dilatation, and Intima-Media Thickness

Novel imaging and functional techniques may further drive a personalized approach and assist in the decision of novel drug prescription, as well as providing intermediate endpoints for further treatment decisions (Schiele et al., 2017). Recently a new in-vivo cardiovascular magnetic resonance technique of 2D vessel wall diffusion anisotropy in carotid arteries was published in the Journal of Cardiovascular Magnetic Resonance by our group. The investigation was done in healthy subjects and the results showed that fractional anisotropy (FA) might be a sensitive parameter to grade vascular aging and the development or progression of atherosclerosis (Opriessnig et al., 2016). The principles of diffusion-weighted magnetic resonance imaging are based on the physics of molecular diffusion. A water molecule in a glass of water without any structural boundaries will diffuse completely random by thermal energy and is described in statistical terms by a displacement distribution. In a surrounding of complex structural restrictions, the diffusion of molecules will be limited and may be displayed as structural dependent diffusion probability.

The distribution is like unrestricted diffusion but narrower because there are structural barriers hindering molecular displacement and the expected displacement distance is reduced, therefore. In free diffusion the displacement is described as isotropic, while biological tissues are highly heterogeneous media consisting of various compartments and barriers of different diffusivities it is described as anisotropic. For instance, neuronal tissue is highly defined by a fibrillary structure of tightly packed and coherently aligned axons often organized in bundles. This leads to a greater molecular restriction in the perpendicular direction of the axon, compared to parallel diffusion with less structural restrictions. The distribution may be even more complicated if the underlying tissue contains fibers with various orientations. Diffusion must be linked to the signal intensity measured at magnetic resonance imaging, thus the motion of spins such as hydrogen protons of the water molecule, in the presence of a heterogeneous magnetic field lead to a decrease in signal intensity resulting in fundamental equations in diffusion imaging (Hagmann et al., 2006).

Prior investigations already showed the great potential of diffusion weighted cardiovascular magnetic resonance concerning the discrimination between healthy and diseased vessel tissue by evaluating the apparent diffusion coefficient along the arterial axis (Xie et al., 2014, Kim et al., 2009, Kim et al., 2011). Ex-vivo studies of porcine aortas have successfully visualized the fibrous structure of the vessel wall by investigating diffusion anisotropy of tensor imaging (Ghazanfari et al., 2012, Flamini et al., 2013). The recently published trial conducted by our group investigated high resolution diffusion tensor magnetic resonance imaging of 12 healthy males between 25 – 60 years. Vessel wall fractional anisotropy values ranged from 0.7 in the youngest participant to 0.56 for the oldest. Investigations by using linear regression analysis between fractional anisotropy values and age showed a significant correlation ($p < 0.01$) with an adjusted R^2 of 0.52 (Opriessnig et al., 2016). These results suggested that fractional anisotropy might be a very good marker that could be able to detect changes in the vascular structure with increasing age. Thereby, a decrease in fractional anisotropy values in course of advancing age might indicate pathological changes of tissue microstructural integrity and therefore might be a reliable marker for the progression of atherosclerosis. These trial results demonstrated for the first time the feasibility of measuring fractional anisotropy in human carotids in-vivo, in a non-destructive and non-invasive manner (Opriessnig et al., 2016). This novel magnetic resonance parameter is also thought to be a potential marker for alterations in the alignment of collagen fiber bundles in vessel walls of carotid arteries. (Opriessnig et al., 2016). The imaging method already demonstrated a high spatial resolution in visualizing collagen fiber bundles of the atherosclerotic fibrous cap in an ex-vivo approach (Opriessnig et al., 2018).

The vascular endothelium can be described as a functional organ playing an important role in the regulation of vasomotor tone, homeostasis, and immune response (Hafner et al., 2010). This inner lining of the arteries can be also defined as the first target in the pathogenesis of atherosclerosis (Gimbrone and Garcia-Cardena, 2016, Insull, 2009). A great variety of clinical trials showed an association of endothelial function and cardiovascular risk (Celermajer et al., 1992, Celermajer et al., 1994, Brevetti et al., 2003, Anderson et al., 1995, Hafner et al., 2014, Hafner et al., 2010). Statin therapies showed to improve endothelial function and another trial investigating endothelial function in 14 patients treated with evolocumab, suggested an improvement of endothelial function after 2 months of treatment with this PCSK9-antibody (Landmesser et al., 2005, Katsiki et al., 2018, Maulucci et al., 2018).

It was also shown that reduced endothelial function predicts the progression of carotid intima media thickness (C-IMT), another well described non-invasive marker of cardiovascular disease (Halcox et al., 2009). A meta-analysis investigating the outcome of several randomized clinical trials concluded that the C-IMT is a strong predictor of future vascular events (Lorenz et al., 2007). These findings were confirmed in a prospective outcome trial (SUITA) with the conclusion that the maximum IMT in the common carotid arteria (CCA) contributed significantly but modestly to the predictive power of calculating traditional risk factors. The trial provided the first demonstration that new progression of an incident carotid plaque is a cardiovascular disease risk (Kokubo et al., 2018). Another recently published systematic review investigating the relation between age and C-IMT showed a strong positive association between age and carotid IMT in the healthy population with a gradual and linear increase. Although patients with cardiovascular disease had higher C-IMT values compared with the population free of cardiovascular disease, a linear relation between age and C-IMT was also present in this population (van den Munckhof et al., 2018).

2 MATERIAL and METHODS

2.1 Rationale, Hypotheses and Trial Objectives

Elevated LDL-C is associated with increased atherosclerotic plaque volume (Virani et al., 2011). Lowering of LDL-C has been shown to significantly reduce the atherosclerotic plaque volume (Tsujita et al., 2015, Raber et al., 2015). Treatment with PCSK9 antibodies reduces plasma LDL-C by 50-60% (Schwartz et al., 2018, Sabatine et al., 2017, Leitner et al., 2020). Beside this, PCSK9 antibody treatment has also been shown to cause higher plaque stability analysed by intravascular ultrasound and optical coherence tomography (Nicholls et al., 2016, Ako et al., 2019, Yano et al., 2020, Sugizaki et al., 2020). However, optical coherence tomography and intravascular ultrasound are invasive procedures that may cause infection, allergy against contrast media, haematoma after arterial puncture, or catheter induced damage of the endothelium. In clinical practice ultrasound measurements of carotid intima-media thickness are frequently used as a non-invasive method to evaluate the atherosclerotic plaque status, but despite being safe for the patient the approach is considered imprecise (Johri et al., 2020, Bots et al., 2016). The ultrasound-based assessment of endothelial function by measuring flow-dependent dilatation of the brachial artery represents another well described and safe method to investigate pathological effects of atherosclerosis on the vasculature, but this method does not yield any structural information of the arterial wall (Hafner et al., 2014, Hafner et al., 2010).

Therefore, non-invasive and reliable approaches are urgently needed to determine pathological changes of atherosclerosis (Vlachopoulos et al., 2015). Additionally, evolving evidence also suggests that triglyceride-rich lipoproteins, inflammation, small-dense LDL particles and Lp(a) play a major role in the management of cardiovascular disease and residual risk (Nordestgaard, 2016, Ference et al., 2020, Boren et al., 2020). The effects of novel PCSK9 antibodies on LDL-C and cardiovascular event reduction have been intensively studied throughout the last decade but there have hardly been any investigations regarding its effects on inflammation, lipoprotein particle subfractions or triglyceride metabolism (Schwartz et al., 2018, Sabatine et al., 2017).

Our pilot study was designed therefore to investigate the effects of short-term treatment with alirocumab, a fully human PCSK9 antibody, on fractional anisotropy of the carotid arteries using magnetic resonance diffusion tensor imaging and exploring its usability in patients with progressed atherosclerotic vascular disease as well as to assess for the first time longitudinal clinical data for this potential parameter for vascular integrity. Besides, we investigated the effects of alirocumab on brachial artery flow-dependent dilatation, carotid intima-media thickness, and possible effects on inflammatory and vascular biomarkers. Moreover, we evaluated its effects on lipoprotein lipase regulators. To address these questions, we conducted nuclear magnetic resonance spectroscopy, standardized and commercially available fat-tolerance tests, enzyme-linked immunosorbent assays and direct lipoprotein analysis using a combined ultracentrifugation precipitation method (Metzner et al., 2022a, Metzner et al., 2022b).

The formulated hypotheses in this context were:

- Fractional anisotropy may be increased in response to alirocumab.
- Lipid therapy intensification with alirocumab may improve flow-dependent, endothelium-dependent dilatation.
- Alirocumab may reduce intima media thickness.
- Alirocumab may improve the post-prandial lipemia profile.

- Alirocumab may induce a change of the lipoprotein subfraction distribution. More precisely, alirocumab is expected to reduce the number of LDL particles and to increase the number of HDL particles, especially of small ones.
- Alirocumab may reduce inflammation.

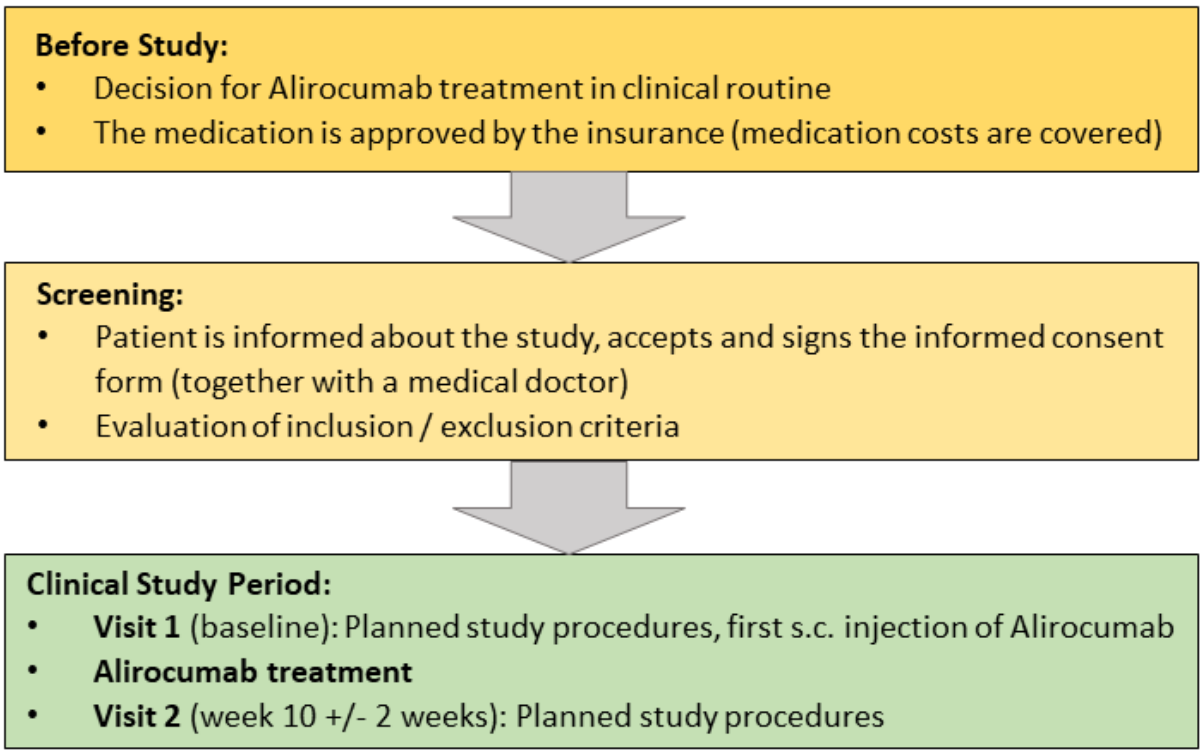
Exploratory study endpoints were defined as:

- Primary Endpoint:
    - Change of mean carotid vessel wall fractional anisotropy by alirocumab.
- Secondary Endpoints:
    - Change of flow-dependent dilatation in response to alirocumab
    - Change of intima media thickness in response to alirocumab.
    - Change of post-prandial lipemia in response to alirocumab.
    - Change of the lipoprotein subfractions in response to alirocumab
    - Changes of inflammatory parameters in response to alirocumab.

2.2 Study Design and Procedures

The ALIROCKS study (NCT03559309) is a prospective, single-centre, longitudinal pilot study with 24 patients scheduled for treatment with alirocumab (Figure 4). Treatment with alirocumab was initiated based on the current guidelines for the treatment of dyslipidaemia of the European Atherosclerosis Society and the European Society of Cardiology (Mach et al., 2020). Prescription of alirocumab and cost coverage by the insurance company was required prior to study inclusion. The patients were included into the study when they were scheduled for the first application of alirocumab in clinical routine and signed the trial specific informed consent forms. Drug administration took place via a subcutaneous pen injection once every two weeks or two injections every four weeks, for further details see “Summary of Product Characteristics (SmPC)” of Alirocumab (Praluent®, http://www.ema.europa.eu/).

Figure 4. Workflow overview



Diagnostic measurements defined per protocol were performed at baseline (BL, visit 1) the day at first alirocumab application and at week 10 (W10, visit 2) plus / minus 2 weeks in line with reimbursement obligations in course of clinical routine. In case the regulations for reimbursement changed during the study recruitment period, the recruitment of patients was automatically adapted to this new situation, thus it was defined prior per protocol that no amendment will be necessary due to any reimbursement changes. Study related diagnostic procedures were restricted to 2 ultrasound measurements of the brachial artery (flow-dependent dilatation / intima media thickness), and 2 magnetic resonance imaging investigations of the carotid arteries. The fat tolerance tests for both visits were optional and required 2 additional blood withdrawals at 2 and 4 hours after the high-fat test meal.

Diagnostic procedures at visit 1 included (after informed consent form has been signed):

- Documentation of demographics, medical history, and current pharmaceutical treatment
- Physical examination
- Fasting blood withdrawal
- Ultrasound measurement of the brachial artery
- Magnetic resonance imaging of the carotid arteries

- Optional: Fat-tolerance test

Diagnostic procedures at visit 2 were identical to the diagnostic procedures at visit 1 except for physical examination which has been waived. The ALIROCKS trial was approved by the Austrian Federal Office for Safety in Health Care (EudraCT: 2018-000981-12) and Ethics Committee of the Medical University of Graz (Protocol Nr.: 29-519 ex 16/17) and conducted according to the declaration of Helsinki, thus all patients gave written informed consent before their study inclusion.

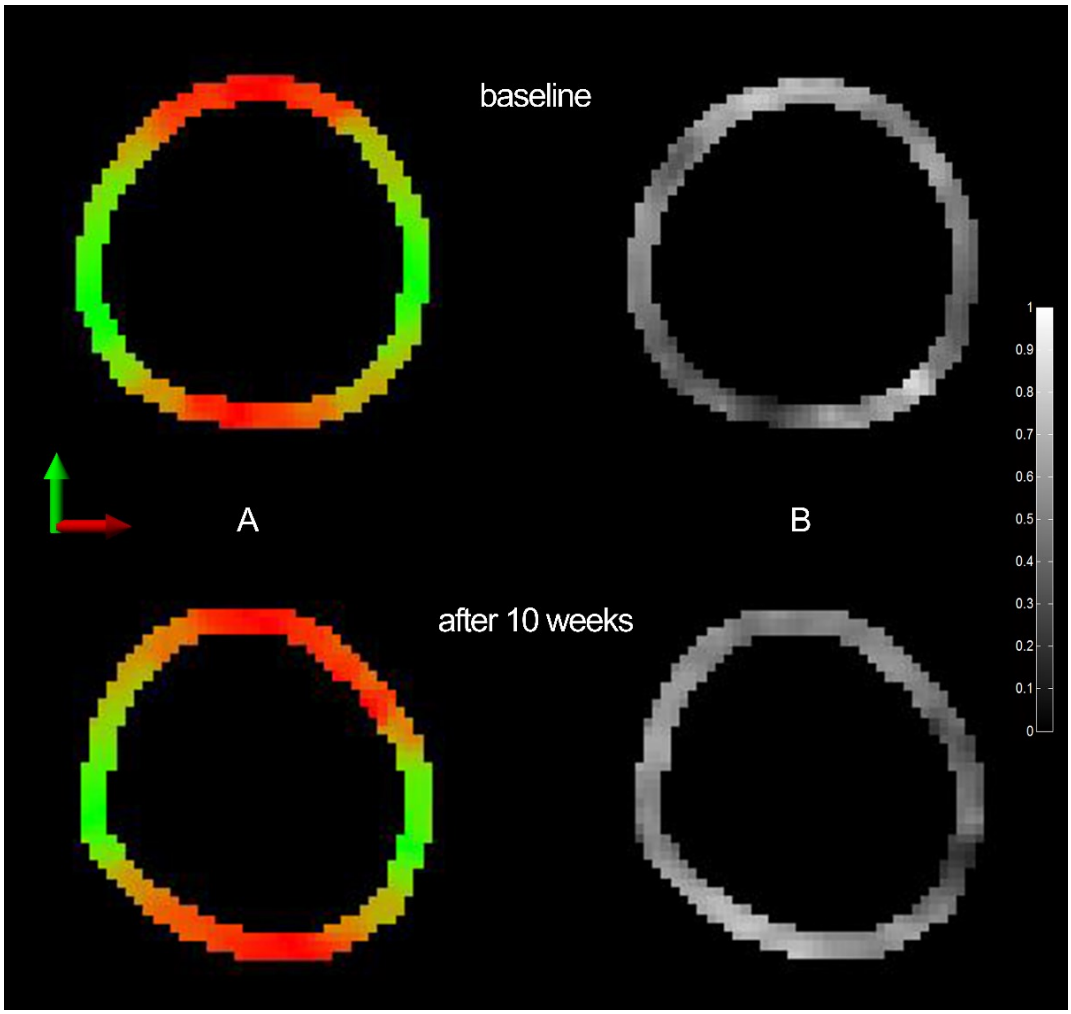
2.3 Patient Demographics and Medical History

The following parameters were documented for all eligible patients that signed the informed consent form. Some information was collected only once at BL, others were collected at both visits (not marked otherwise): Initials (BL), gender (BL), age and birthdate (BL), concomitant diseases and medications (no dosages), lipid-therapies (including dosage), medical history of cardiovascular events (summary of event and date). Patient specific data was collected by trained and experienced study investigators and coordinators at the Medical University of Graz. Documentation folders were provided as part of the study package electronically and / or as hard copy. Data management of patient related data was on a pseudo-anonymized basis using a pre-specified template with patient codes. Laboratory reports were not transferred into case report forms (CRFs), to limit the possibility of data errors. Laboratory results were copied and filed in the trial folders accordingly. The clinical trial protocol did not obligate the use of any specific scores or genetic tests to diagnose familial hypercholesterolemia, therefore it was determined depending on the preference of the treating physician and documented as such in the patient's medical record. Complete or partial statin intolerance was defined when trial participants did not receive high-intensity statins of ≥20 mg rosuvastatin or ≥40 mg atorvastatin. An example of a CRF is provided in the appendix of this dissertation. With special thanks to the community of the Global Health Network for providing this template for free with permission for adaptation to the needs of our clinical trial (Metzner et al., 2022a, Metzner et al., 2022b).

2.4 Magnetic Resonance Imaging of the Carotid Artery

The high-resolution diffusion tensor imaging was conducted by using a 2-dimensional diffusion acquisition approach. A readout-segmented Echo-Planar-Imaging (rs-EPI MRI) sequence was applied on a 3T whole-body magnetic resonance scanner (Prisma fit, Siemens Medical Solutions, Erlangen, Germany) with a 2 × 4 channel multifunctional coil (NORAS MRI products GmbH, Höchberg, Germany) and FOV = 189 × 189 mm2, matrix = 346 × 346, slice thickness = 10 mm, TR = 2RR intervals, trigger delay = 50ms to acquire images in the diastolic phase, TE = 93 ms, GRAPPA = 2, number of readout segments = 9, b-values = 0, 200, 400, 600 s/mm2, acquisition time = ~12 min. 18 gradient directions were defined on a hemicycle within a plane oriented perpendicular to the longitudinal axis of the carotid artery. The assessed parameter called fractional anisotropy can be described as a scalar value between zero and one that has no unit and is a measure of a diffusion process (Figure 5). In the hypothetical case that the value is one the diffusion is directed in one axis and restricted to all other directions, thus it can be imagined as diffusion in a straight line. On the other hand, a hypothetical fractional anisotropy value of zero describes diffusion as unrestricted in all directions or isotropic. If the structural integrity or alignment within the vessel wall is lost, this might reflect pathological changes by atherosclerosis and is considered to result in low fractional anisotropy values. (Opriessnig et al., 2016, Opriessnig et al., 2018). Per study protocol, the investigation of this novel structural marker was not performed in a blinded design, but patient numbers and time points of measurements were not reported to the person conducting the image analysis to reduce potential observer or interpretation bias (Metzner et al., 2022a, Metzner et al., 2022b).

Figure 5. High-resolution diffusion tensor imaging to assess fractional anisotropy.



Legend: The left column (A) shows colour coded vector images of the principal diffusion tensor direction with the corresponding grey-scale fractional anisotropy maps in the right column (B). The upper row shows the baseline measurement prior to the administration of alirocumab for a single participant (mean FA=0.500). The lower row shows the imaging result for a single participant after 10 weeks of intervention (mean FA=0.516). The fractional anisotropy parameter can be considered as scalar value between zero and one without unit. In the hypothetical case that the value is one the diffusion is directed in one axis and restricted to all other directions, thus can be imagined as diffusion in a straight line. On the other hand, a hypothetical fractional anisotropy value of zero describes diffusion as unrestricted in all directions or isotropic. In this illustrational example of a single patient the fractional anisotropy values did not change in response to alirocumab treatment meaning that no structural changes of carotid vessel wall could be detected. The image from this figure was provided by our radiology team with permission for use (Metzner et al., 2022a).

2.5 Flow-Dependent Dilatation and Intima Media Thickness

All flow dependent dilatation and intima media thickness assessments were conducted by one experienced person to restrict observer bias, and was performed at the Division of Angiology, Department of Internal Medicine, Medical University of Graz. A linear array transducer, 8–13 MHz (Sequoia 512, ACUSON Corp., Charleston Rd., Mountain View, CA, USA) was used to evaluate brachial artery vasodilatation. The artery was investigated in a longitudinal plane between 1 and 5 centimeters above the antecubital fossa by grey-scale imaging after resting 5 minutes in the supine position in a room with constant climate conditions. To ensure that all measurements were evaluated at the same segment of the artery the position of the ultrasound transducer was labelled at the skin with a marker. The image acquisition was performed in a one-centimeter-long segment for three times and the end-diastolic distances between the two intimal-interfaces were assessed using an electrocardiogram. The end-diastolic distance was defined as the start of the R-wave. The mean of those values was subsequently computed and used for all further statistical analysis. By applying a constant pressure of 50 mmHg the blood pressure cuff was inflated over a 5-minute period above the systolic blood pressure of the brachial artery. After the cuff release and a waiting time of 45 seconds the post-ischemic diameter of the brachial artery was assessed. The parameter of flow-dependent dilatation was calculated as the change of post-ischemic diameter from the baseline diameter in percentage. The intima-media thickness was assessed in a one-centimeter-long segment of the carotid arteria communis and again the mean of three measurements was used for further analysis. All these measurements were performed in a left (sinister) and right (dexter) position, subsequently the mean value of these two positions was statistically analyzed as carotid intima-media thickness (C-IMT), (Hafner et al., 2010, Metzner et al., 2022a, Metzner et al., 2022b).

2.6 Nuclear Magnetic Resonance Spectroscopy

To assess the lipoprotein particle subfraction profile we used:

- AXINON® lipoFIT® nuclear magnetic resonance assay (Numares, Regensburg, Germany).

- The Avance III HD nuclear magnetic resonance spectrometer (Bruker; Billerica, MA, USA), an Ascend 600 MHz magnet (Bruker).
- The TopSpin 3.2 (Bruker) and Axinon Suite 1.0.0.1 (Numares, Regensburg, Germany) software.

The following lipoprotein particle sizes were determined:

- Large VLDL particles (L-VLDLp): 50-240 nm.
- Large LDL particles (L-LDLp): 21.2-23 nm.
- Small LDL particles (S-LDLp): 18-21.2 nm.
- Large HDL particles (L-HDLp): 8.8-13 nm.
- Small HDL particles (S-HDLp): 7.3-8.8 nm.

Not provided by the assay are total- and small VLDL particles: AXINON® lipoFIT® nuclear magnetic resonance assay (Needham et al., 2019, Metzner et al., 2022a).

2.7 Laboratory Assessments

Lipoprotein-electrophoresis was performed to evaluate HDL-C and LDL-C while photometry was used to measure cholesterol and triglycerides. Immunoturbidimetry was conducted to assess C-reactive protein (CRP) and apolipoproteins (Apo). Electrochemiluminescence immunoassay was used to measure interleukin-6 (IL-6). Additional to the direct assessment method of lipoprotein particles by lipoprotein-electrophoresis we also performed a combined ultracentrifugation precipitation method (Wanner et al., 1991, Bachorik and Ross, 1995, Silbernagel et al., 2019a). Drawn blood samples were ultracentrifuged for 18 hours at a density of d = 1.0063 kg/L and at 30.000 rpm. Subsequently very low-density lipoprotein (VLDL) particle fractions were separated, and by applying phosphotungstic acid/MgCl2 the LDL fractions were precipitated. Enzymatic reagents were used from Diasys (Holzheim, Germany). Calibration standards were used from Roche Diagnostics (Mannheim, Germany).

Apolipoproteins (Apo), ApoB in VLDL and LDL, and Lp(a) were measured by using:

- Reagents from DiaSys (Holzheim, Germany)
- Standards from Siemens (Marburg, Germany; ApoAI, ApoB, ApoE), Kamiya Biomedical (Seattle, WA, USA; ApoAII, ApoCII, ApoCIII), and Diasys (Lp(a)).

The variations coefficients were below 5% (Hollstein et al., 2019, Metzner et al., 2022a, Metzner et al., 2022b).

Enzyme-linked immunosorbent assays performed in course of the trial were:

- Monocyte chemoattractant protein-1 (MCP-1): TECAN, Männedorf, Switzerland, ID: 30150435)
- Vascular endothelial growth factor (VEGF): IBL, Minneapolis, MN, USA, ID: JP27171)
- P-Selectin (CD62P): Bio-Techne (Minneapolis, MN, USA, ID: DPSE00).
- Angiopoietin-like-3 (ANGPTL-3): IBL (Minneapolis, MN, USA, ID: JP27750).
- Angiopoietin-like-4 (ANGPTL-4): IBL (Minneapolis, MN, USA, ID: JP27749).
- Glycosylhosphatidylinositol-anchored High-density Lipoprotein-binding Protein-1 (GPIHBP-1): TECAN (Männedorf, Switzerland, ID: 30131804).
- Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9): Bio-Techne (Minneapolis, MN, USA, ID: DPC900).

The PCSK9 enzyme-linked immunosorbent assay assessed total circulating PCSK9 independent of alirocumab binding. All blood samples were drawn during fasting conditions (Metzner et al., 2022a, Metzner et al., 2022b).

2.8 Determination of Post-Prandial Lipemia (Optional)

Post-prandial lipemia was assessed by fat-tolerance testing. Participants received the commercial Lipotest® meal which was specifically created for medical diagnostics and is characterized as "Food for specialized diagnostic determination of postprandial triglycerides". This standardized meal has 832 kcal total energy, with 75 g saturated fat, 25 g carbohydrates, 10 g protein, 2,1 g fiber and 0,15 g salt. This equates to 42% of the daily reference value according to the FDA (Food and Drug Administration) and European GDA (Guideline Daily

Amount). To date it is the best described meal to test post-prandial triglyceride tolerance worldwide and displays good precision and reproducibility. The 4 hours measurement after Lipotest® consumption captured the peak of postprandial triglyceride response in healthy subjects (Tentolouris et al., 2017, Metzner et al., 2022b).

The patients had to fast ≥10 hours before the test was performed (fasting condition). No additional meals or beverages were allowed until the blood withdrawal 4 hours after the meal. Even chewing gums, tea, soft drinks, and coffee were forbidden. Consumption of water was allowed during the time-period of the fat-tolerance test. Parameters of glucose and lipid metabolism were measured at hour 2 and 4 of the fat tolerance test. The area under the curve (AUC) was assessed before and after 10 weeks of Alirocumab therapy. The Lipotest® was provided in a sachet consisting of 115 g powder which was hydrated with 150 ml of water. The powder was added to the water into an appropriate cup and mixed until properly dissolved. When placed in the fridge (2-8 C°) overnight it became a tasty mousse (chocolate flavor). Nevertheless, because of the fat-rich character of the meal, the patients were advised to consume the meal at a good pace (< 30 minutes).

The ingredients of the fat-tolerance meal are:

- Vegetable oils fully hydrogenated (palm and coconut oil).
- Glucose syrup.
- Milk proteins.
- Sugar
- Lactic and acetic acid esters of monoglycerides and diglycerides of fatty acids acting as emulsifiers.
- Cocoa powder with 20-22% fat content and defatted cocoa powder with 10-12% fat content.
- Flavorings.

May contain traces of cereal containing gluten, soy, and products thereof (Lipotest®, Product specifications: N.O.M. Distribution Number: 13664/21-2-12, 75198/23-10-12, D. GENOMERES Advanced Medical Research, Athens, Greece). The fat-tolerance test was performed by the Department of Internal Medicine, Division of Endocrinology and Diabetology, Medical University of Graz (Metzner et al., 2022b).

2.9 Patient Visits (Overview)

The intima media thickness and endothelial function assessments were evaluated in fasting state, in the morning at the Division of Angiology. Afterwards, patients were sent to the Division of Diabetology and Endocrinology to complete their baseline or week 10 anamnesis visit and if applicable, their optional fat-tolerance test (Table 1). For the cardiovascular magnetic resonance measurements, exclusive visits were scheduled with the patients as it was considered scientifically important that vessel function and structural assessments took place before or separately from the fat-tolerance test, blood withdrawals and drug administration to prevent potential cross-reactions that may influence the outcome of those evaluations.

Table 1. Schedule of trial visits, procedures, and parameters to be documented.

Baseline	**Week 10** (plus / minus 2 weeks)
Check documentation of informed consent form	
Documentation of essential information of the medical history: Initials, gender, age and date of birth, concomitant diseases, and medications (no dosages necessary), lipid-therapies (including dosage), medical history of vascular events (summary of event and date), physical examination.	Documentation of essential information of the medical history: Any changes in concomitant diseases or medications (no dosages necessary), lipid-therapies (including dosage), medical history of vascular events (summary of event and date), lipid-parameters: LDL-C, TG and Lp(a).
Fasting blood sampling as part of clinical routine (t0).	Fasting blood sampling as part of clinical routine (t0).
Cardiovascular magnetic resonance of 2D vessel wall diffusion anisotropy in carotid vessels.	Cardiovascular magnetic resonance of 2D vessel wall diffusion anisotropy in carotid vessels.
Endothelial function and intima media thickness measurements	Endothelial function and intima media thickness
OPTIONAL: Standardized meal (fat tolerance test).	OPTIONAL: Standardized meal (fat tolerance test)
OPTIONAL: Post-prandial blood sampling, 2	OPTIONAL: Post-prandial blood

and 4 hours after fasting blood sampling (t2 and t4). In case the test requires a separate visit t0 blood sampling must be done as well.	sampling, 2 and 4 hours after fasting blood sampling (t2 and t4). In case the test requires a separate visit t0 blood sampling must be done as well.

Legend: The cardiovascular magnetic resonance or the optional fat-tolerance has required an additional patient visit for logistical reasons.

2.10 Inclusion and Exclusion Criteria

Patients scheduled for Alirocumab treatment in clinical routine were potential study participants. The decision for drug prescription was made by the physician in charge (Endocrinologist, Department of Internal Medicine, Division of Endocrinology and Diabetology) in collaboration with the responsible insurance company in clinical routine before study start. Patients were only included if cost coverage by the specific health insurance was ensured. Prescription guidelines from the insurance (Table 2) were adapted on a yearly basis and published at http://m.eko2go.at and http://www.hauptverband.at. The original national reimbursement guideline (German language) and its progress in course of the recruitment phase are enclosed to this dissertation (Appendix). Importantly, there were no major medical changes regarding the reimbursement text and in the end, it was always a decision between the doctor in charge and the chief physician of the responsible insurance company. The new ESC/EAS guideline for dyslipidemia published at the European Society of Cardiology (ESC) congress in August 2019 and especially the new LDL-C targets (< 55 mg/dL, very-high risk) may have influenced this reimbursement und recruitment decision (Mach et al., 2020).

The ALIROCKS trial did not influence the treatment or treatment decision with alirocumab as already stated, nevertheless, to minimize the probability of an existent pregnancy at screening and to fulfill national legal requirements (BGBl. I Nr. 35/2004 §30), a pregnancy test has been provided at screening for women of childbearing age beside the informed consent process (Figure 6). Women that were no longer of childbearing age required appropriate documentation in their medical history at screening. Considering the specific study design

(e.g., diagnostic interventions of low risk) and the quite short duration of 10 weeks, only one pregnancy test at screening was provided. Regarding the risk management of a potential pregnancy, it is important to quote that to date there was and still is no data from the use of alirocumab in pregnant women. Animal studies do not indicate harmful effects with respect to maintenance of pregnancy or embryo-fetal development. In clinical routine pregnancy tests are generally not conducted before or during the treatment with alirocumab.

Table 2. National health insurance reimbursement guideline for Praluent® (Alirocumab).

Primary Hypercholesterolemia in secondary prevention after an acute atherosclerotic, ischemic cardiovascular event for patients with diagnostically confirmed coronary heart disease and / or peripheral occlusive disease and / or arteria cerebral occlusive disease: - If an additional LDL-C lowering is medically indicated because of a very high cardiovascular risk AND - If a professional nutritional advisory takes place, arterial blood pressure is controlled, HbA1c is < 8% and smoking abstinence will be pursued AND -If over a minimum period of 3 month, a maximal tolerated dose of intensified LDL-C therapy with atorvastatin or rosuvastatin, each in combination with Ezetimibe (or Ezetimibe with / or without colesevelam in patients with statin intolerance) an LDL-C below 100 mg/dL cannot be achieved, or if these treatments are contraindicated. A statin intolerance is considered confirmed, if therapies with several statins – at least with atorvastatin, respectively rosuvastatin – resulted in myopathies and an increase of creatinine-kinase of at least 5x ULN (upper limit normal) or if statin treatment leaded to a severe hepatopathy. Diagnosis, family anamnesis and primary drug prescription must be conducted by a specialized center by a medical specialist for internal medicine and endocrinology in addition. The treatment with alirocumab can only be prolonged if LDL-C will be controlled 2-3 months after therapy initiation by laboratory controls and the value has decreased at least 40% from initial baseline value with this intensivied lipid-lowering therapy, respectively an LDL-C below 70 mg/dL was achieved.

Figure 6. Inclusion and exclusion criteria.

Inclusion Criteria:

1. Patient scheduled for treatment with Alirocumab in clinical routine (after approval of cost coverage by insurance company)
2. No previous treatment with PCSK9 antibodies
3. Signed informed consent form

Exclusion Criteria:

1. Age of < 18 years
2. Pregnancy (pregnancy test at screening visit)
3. Breast-feeding
4. Impossibility to perform magnetic resonance imaging of the carotid artery (claustrophobia, carotid stent)

2.11 Statistical Analysis

It was planned per protocol to recruit 24 patients (n = 24). Statistical analysis of this explorative pilot study was planned to be descriptive. Changes from baseline to week 10 were evaluated with the paired samples t-test and/or related samples Wilcoxon signed rank test in case non-normal distribution was determined by Shapiro-Wilk. The baseline characteristics are specified as means (standard deviations) in cases of continuous variables and as numbers (percentages) in cases of categorical variables. Regarding fat-tolerance testing, the area under the curve (AUC) analysis was assessed by a trapezoid approximation: ($AUC^{(T0-4)} = AUC^{(T0-2)} + AUC^{(T2-4)}$; $AUC^{(T0-2)} = (Value^{(T0)} + Value^{(T2)}) / 2*(Time^{(T2)} - Time^{(T0)})$, $AUC^{(T2-4)} = (Value^{(T2)} + Value^{(T4)}) / 2*(Time^{(T4)} - Time^{(T2)})$. It was considered statistically significant when p-values were reported ≤ 0.05. For all statistical analyses the SPSS 26.0 software was used (SPSS Inc., Chicago, IL, US), (Metzner et al., 2022a, Metzner et al., 2022b).

2.12 Data Quality and Monitoring

For key study outcome measures such as carotid vessel wall fractional anisotropy and for flow-dependent dilatation, source data verification (SDV) was conducted by an independent, external clinical research organization (MM Clinical Services & Consulting GmbH, Hart bei Graz, Austria). In addition, all informed consent forms (ICFs) were checked and monitored by this company (Metzner et al., 2022a).

2.13 Safety Reporting

Any serious adverse events (SAEs) were planned per protocol to be reported to the sponsor within 24 hours from time of notice to fulfill local regulations and local law. The SAE reporter was anybody involved in the clinical trial, thus authorized in the protocol approval form, and got informed and/or got aware of this serious adverse event. The SAE reporter filled the SAE reporting form provided for this study and reported the specific SAE to the sponsor per Mail (Scan PDF) to klinische-studien@medunigraz.at, Klinische Studien – Administration, Medizinische Universität Graz, Forschungsmanagement, Auenbruggerplatz 2/4. Stock, A-8036 Graz, Phone.: +43 (0)316 385 74094. Non-serious adverse events or deviations of laboratory parameters were not collected in course of this clinical trial, as drug or medical device safety was not in the focus of this clinical study. Other prospective, and from a statistical point of view – more powerful, clinical trials were already published and were currently ongoing at the time of trial protocol writing and study initiation. Definition of an adverse event: An adverse event was defined as any unfavorable and unintended sign, symptom, or disease temporally associated with the use of a medicinal product or diagnostic intervention, whether considered related to the medical product or intervention.

The decision whether expedited reporting was appropriate in other situations, such as important medical events that may have not been immediately life-threatening or resulted in death or hospitalization but could have jeopardized the patient or may have required intervention to prevent one of the other outcomes listed in the definition above were based on medical and scientific judgement. These events were generally also considered serious. Examples of such events were intensive treatment in an emergency room or at home for

allergic bronchospasm; blood dyscrasias or convulsions that did not result in hospitalization; or development of drug dependency or drug abuse. The term "life-threatening" in the definition of "serious" referred to an event in which the patient was at risk of death at the time of the event; it did not refer to an event which hypothetically might have caused death if it were more severe.

A serious adverse event (experience) or reaction was any untoward medical occurrence that:

• resulted in death,

• was life-threatening,

• required an inpatient hospitalization or prolongation of existing hospitalization,

• resulted in persistent or significant disability/incapacity, or

• in a congenital anomaly/birth defect.

(Adapted from ICH Topic E2A. Clinical Safety Data Management: Definitions and Standards for Expedited Reporting, June 1995, EMEA reproduction 2006).

2.14 Actual Trial Milestones and Regulatory Affairs

The dissertation topic providing a research frame was defined in 2017 April, subsequently first protocol drafts were written and discussed with supervisors and potential collaborators. In parallel, the protocol was also discussed with the ethics committee (EC) of the medical university of Graz, as well as with Austrian regulatory authorities represented by the Austrian Agency for Health & Food Safety (“AGES”) in mandate of the Federal Office for Safety in Health Care (“BASG”). The regulatory authority concluded that the protocol meets the criteria for a clinical trial of a substance class (ATC Code) and may result in new efficacy data (“Arzneimittelstudie”), although the protocol did not intervene with the drug prescription beyond clinical routine. The first protocol version agreed among all investigators, informed patient forms (ICFs) and all other essential documents were submitted to the ethics committee and regulatory authority. Final approval of the clinical trial, after minor adaptations of

protocol and ICFs, was received on the 06 April 2018 (see Appendix). Study reports to the ethics committee and subsequent approvals took place on a yearly basis.

Table 3. Clinical trial milestones (actual).

Finalized Protocol (1st Protocol Version): 13-JUN-2017
First EC Submission: 14-JUN-2017
EC Approval (3rd Protocol Version): 06-APR-2018
Study and Site Initiation: 20-JUN-2018
Recruitment Start: 20-JUN-2018
First Patient Visit: 21-SEP-2018
Recruitment End: 30-JAN-2020
Last Patient Visit: 05-FEB-2020
End of Study: 03-APR-2020 (Early termination due to COVID-19)

The recruitment was shortly delayed by a reimbursement halt in summer 2019 due to price negotiations with Sanofi-Aventis Austria. All 24 patients could be enrolled according to study protocol, with the last patient recruited in January 2020. When local authorities communicated the COVID-19 pandemic it was decided to cancel all scheduled trial visits until further information (risk/benefit assessment). The last study visit took place therefore on the 05. February 2020. The prolongation of the trial was “on-hold”, awaiting new recommendations and was not terminated immediately. On 10 April 2020 it was finally decided to prematurely terminate the clinical trial, after governmental officials explained their COVID-19 countermeasure plans, and it became obvious that we could not conduct the trial visits within or near the protocol plan (Table 3).

3 RESULTS

3.1 Trial Population

3.1.1 Definition of Study Populations, Treatment Duration and Dosage

All 24 trial participants were recruited according to the study protocol from mid-2018 to early 2020 (Table 3 and 4). The majority, about 80% of these patients received the lower dosage namely 75 mg of alirocumab every two weeks, while only around 20% administered 150 mg alirocumab every two weeks. Two patients were non-adherent to alirocumab treatment, administered the study medication only twice, at baseline and at week 2. According to the responsible investigators the reasons were described as communication and drug access hurdles. Minor deviations of concomitant oral lipid-lowering therapies were reported for four patients. Among these, one stopped ezetimibe, two patients stopped their red yeast rice product, and another one reduced his oral combination therapy to 20 mg atorvastatin monotherapy from initially 80 mg atorvastatin plus 10 mg ezetimibe. Implementation of countermeasures due to the COVID-19 pandemic restricted all hospital visits to essential interventions exclusively, therefore the ALIROCKS study had to be terminated before the last three participants had completed their final week 10 visits. Thus, 19 patients completed the trial without major protocol violations (Table 3 and 4). For these participants mean treatment duration with alirocumab was exactly 10.0 (SD ± 0.7) weeks (Metzner et al., 2022a, Metzner et al., 2022b).

Table 4. Dosage of alirocumab and trial populations.

Definition	Population (N)	Alirocumab 75 mg (%)	Alirocumab 150 mg (%)
Recruitment	24	19 (79.2)	5 (20.8)
Completion	19	16 (84.2)	3 (15.8)

Legend: Trial completion population (n=19) includes patients attending their baseline plus week 10 visit and injected alirocumab as planned. Three patients could not conduct their scheduled week 10 visit due to COVID-19 countermeasures. Two patients injected alirocumab only twice at baseline and at week 2 but not beyond (Metzner et al., 2022a, Metzner et al., 2022b).

3.1.2 Safety of Trial Population

Only one patient fulfilled the criteria for a serious adverse event (SAE). The patient (ID-10) was hospitalized due to a sports injury caused by a cross-country skiing accident. The study intervention was not discontinued due to the event. The supervising trial investigator marked this event as “clearly not related to the intervention” and signed of the SAE form accordingly.

3.2 Characteristics of Trial Participants

The ALIROCKS trial completion population consisted of elderly males and females that were balanced in the cohort, all had coronary artery disease (Table 5). Seven patients had poly-vascular disease with an additional diagnosis of cerebral and/or peripheral artery disease. Most of the patients had already received bypass surgery or coronary intervention (“secondary prevention”). The remaining three patients without surgery or prior coronary intervention (“primary prevention”) had progressed atherosclerotic disease documented as poly-vascular artery disease including a coronary stenosis of 50% which was confirmed by a cardiac computed tomography. Type-2 diabetes mellitus was documented in the medical records of four patients and familial hypercholesterolemia for two patients. The minority of patients received high-intensity statins at the time of their baseline visit, therefore sixteen patients were considered to have complete or partial statin intolerance. On the other hand, the majority

namely thirteen trial participants received ezetimibe as mono- or as combination therapy (Metzner et al., 2022a, Metzner et al., 2022b).

Table 5. Characteristics of study population.

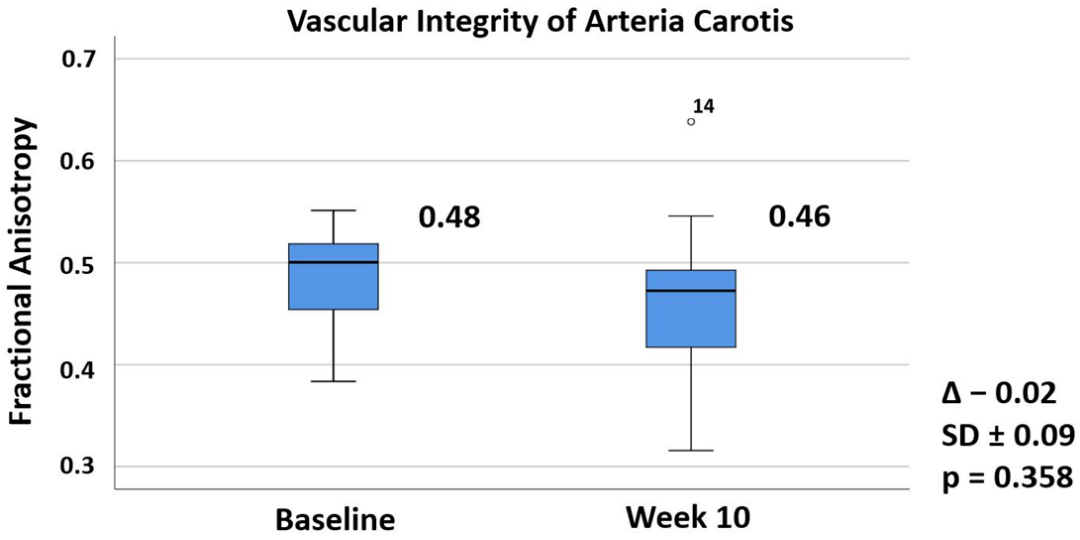
Characteristic	All Recruited (N=24)	Trial Completion (N=19)
Age - yr	66 (9)	66 (9)
Female sex - n (%)	9 (37.5)	9 (47.4)
Male sex - n (%)	15 (62.5)	10 (52.6)
Smoker[a] - n (%)	12 (50)	9 (47.4)
Current Smoker[b] - n (%)	5 (20.8)	4 (21.1)
Concomitant Diseases - n (%)		
Cardiovascular Disease	24 (100)	19 (100)
a. Coronary Heart Disease	23 (95.8)	19 (100)
Coronary Intervention or Surgery	19 (79.2)	16 (84.2)
Documentation of Coronary Stenosis[c]	4 (16.7)	3 (15.8)
b. Peripheral Artery Disease	3 (12.5)	3 (15.8)
c. Cerebral Artery Disease	8 (33.3)	6 (31.6)
Chronic Kidney Disease	5 (20.8)	4 (21.1)
Familial Hypercholesterolaemia[d]	4 (16.7)	2 (10.5)
Adiposity	4 (16.7)	4 (21.1)
Type-2 Diabetes Mellitus	4 (16.7)	4 (21.1)
Type-1 Diabetes Mellitus	0 (0)	0 (0)
Hypertension	19 (79.2)	15 (78.9)
Number of prior Cardiovascular Events[e] - n (%)		
Three	2 (8.3)	2 (10.5)
Two	6 (25)	5 (26.3)
One	12 (50)	9 (47.4)
Zero	4 (16.7)	3 (15.8)
Concomitant Lipid Medication – n (%)		
High-Intensity Statins[f]	5 (20.8)	3 (15.8)
Statins	7 (29.2)	5 (26.3)
Ezetimibe	15 (62.5)	13 (68.4)
Dietary Supplements[g]	6 (25)	5 (26.3)
Statin Intolerance[h]	19 (79.2)	16 (84.2)

Legend: Characteristics are shown by numbers (percentages) or means (standard deviations). [a]Former or current smoker. [b]Current smoker or no stop date documented. [c]Confirmation by cardiac computed tomography but without documentation of prior cardiovascular event ("primary prevention"). [d]Medical records entry. [e]Cardiovascular events such as myocardial infarctions, or percutaneous interventions, prior strokes/transient ischemic attacks, stents, balloons, coronary artery bypass grafts. [f]Therapy with ≥ 20 mg of rosuvastatin or ≥ 40 mg of atorvastatin. [g]Red yeast rice products (monacolin K). [h]All participants not taking high-intensity statins (Metzner et al., 2022a, Metzner et al., 2022b).

3.3 Evaluation of Vascular Function and Structure

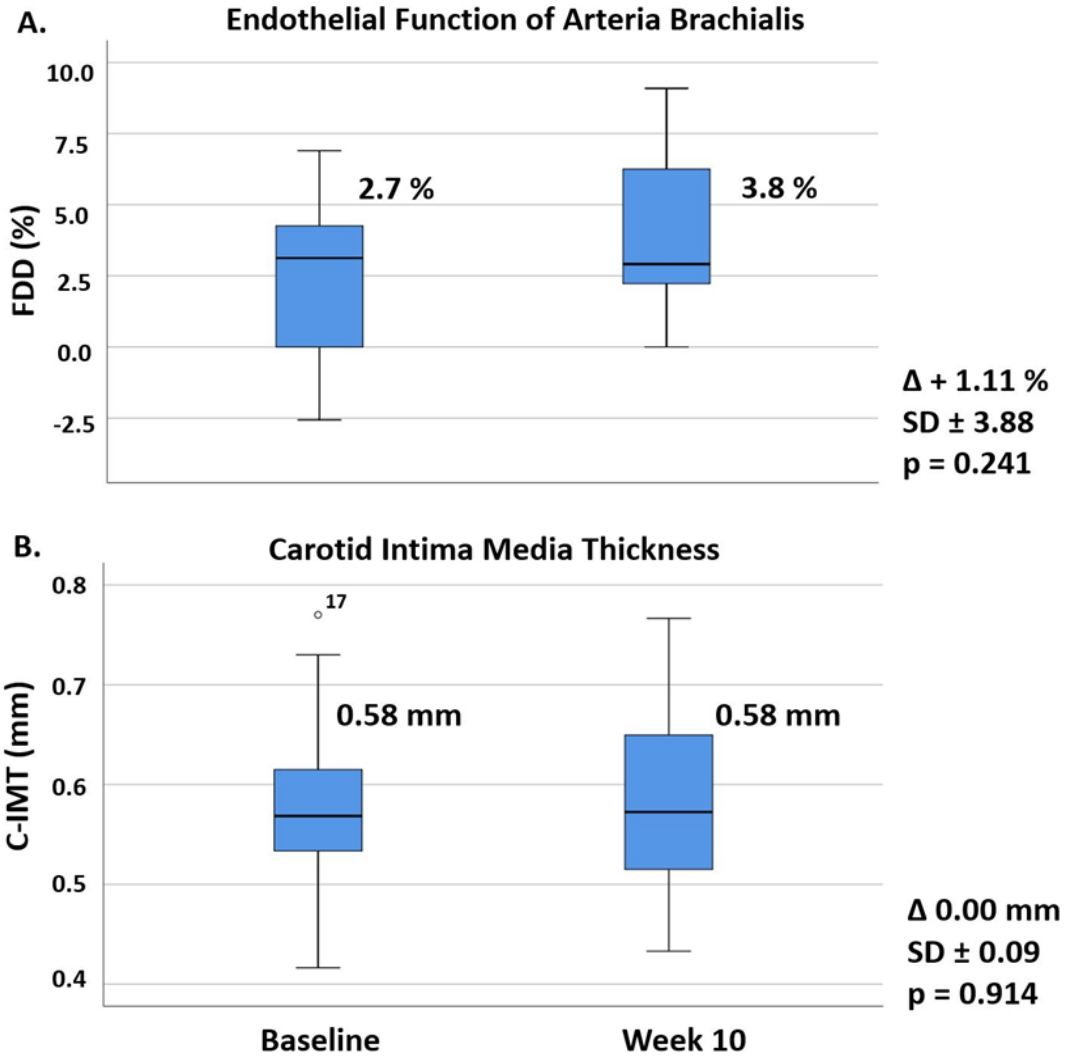
Novel magnetic resonance imaging and standard ultrasound-based evaluation of structural changes of the carotid vasculature did not reveal any changes by alirocumab treatment (Figures 7 and 8). We did also not observe any significant changes of flow-dependent dilatation of the arteria brachialis (p = 0.241) as marker for endothelial function, despite a numerical increase of 41% from baseline (Figure 8). Further correlation analysis (Figure 9) showed an inverse trend between the change of flow-dependent dilatation and LDL-C (Metzner et al., 2022a).

Figure 7. Effects of alirocumab on a novel magnetic resonance-based vascular parameter.



Legend: Assessment of vascular integrity by evaluating fractional anisotropy of the carotid vessel wall. The numerical data shows mean values at baseline and week 10 of alirocumab treatment with mean change, standard deviation, and p-value, while boxplots graphically represent medians and interquartiles. The small circle with the number 14 at the right upper side of the figure identifies an outlier measurement of patient number 14 at week 10. Outliers are defined as values between 1.5x and 3x interquartile ranges from the end of a box. Fractional anisotropy is a scalar value without a unit between one and zero. Lower values of fractional anisotropy are considered to reflect pathological changes of the carotid vessel wall structure. Trial-completion analysis of patients with two valid assessments for statistical analysis (n = 13). Paired t-test with a two-sided p-value (Metzner et al., 2022a).

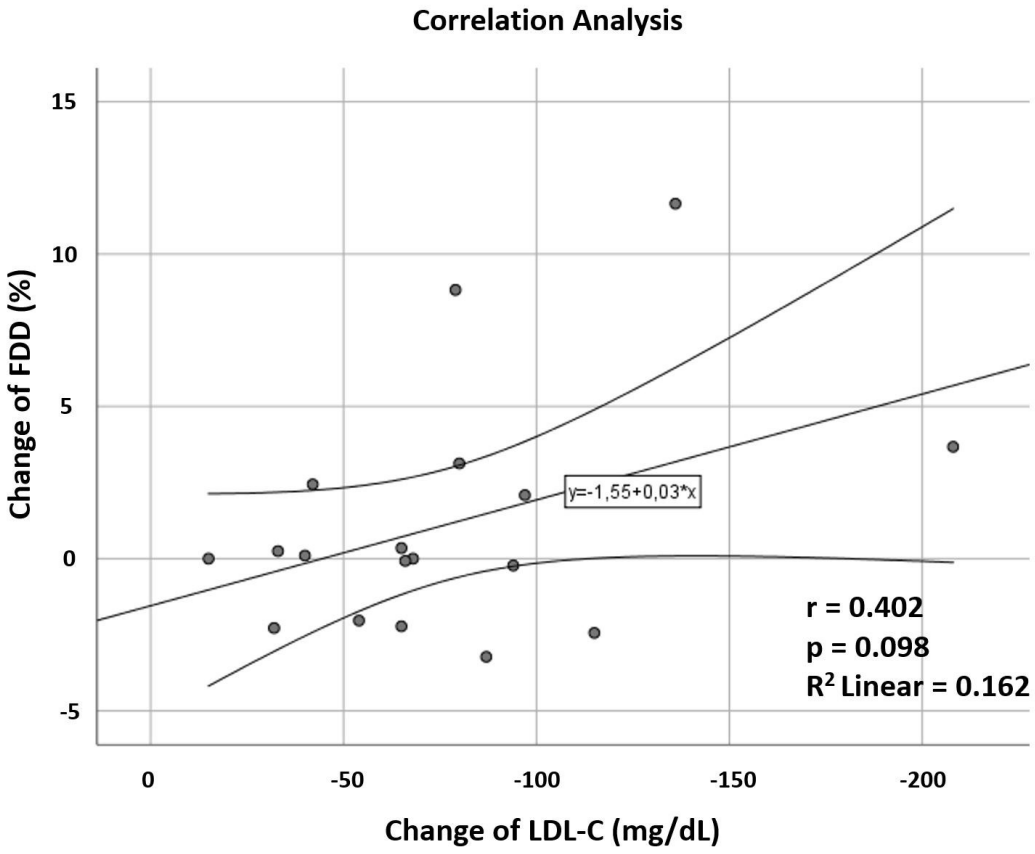
Figure 8. Effects of alirocumab on established ultrasound-based vascular parameters.



Legend: The numerical data shows mean values at baseline and after 10 weeks of alirocumab treatment with mean change, standard deviation, and p-value, while boxplots graphically represent medians and interquartiles. The small circle with the number 17 at the left upper side of the figure identifies an outlier measurement of patient number 17 at baseline. Outliers are defined as values between 1.5x and 3x interquartile ranges from the end of a box. Trial-completion analysis with two valid assessments for statistical analysis (n = 18); Paired t-test with a two-sided p-value. (**A**) Flow-dependent dilatation (FDD) of the arteria brachialis. (**B**) Carotid intima-media thickness (C-IMT) of the vessel wall (Metzner et al., 2022a).

Considering the magnetic resonance imaging regarding fractional anisotropy we obtained only for 13 participants two valid measurements (baseline and week 10) for statistical analysis. The high drop-out was accounted by one patient's anatomy, thus a short neck with adiposity, two had spontaneous claustrophobia during the investigation, one outlier measure had to be excluded from analysis, and the remaining two patients did not attend their planned magnetic resonance visits. For the ultrasound-based investigation of flow-dependent dilatation and carotid intima-media thickness we obtained valid measurements for 18 patients, one participant missed one of his scheduled trial visits (Metzner et al., 2022a).

Figure 9. Correlation of alirocumab treatment effect on flow-dependent dilatation of the arteria brachialis and LDL-C lowering.

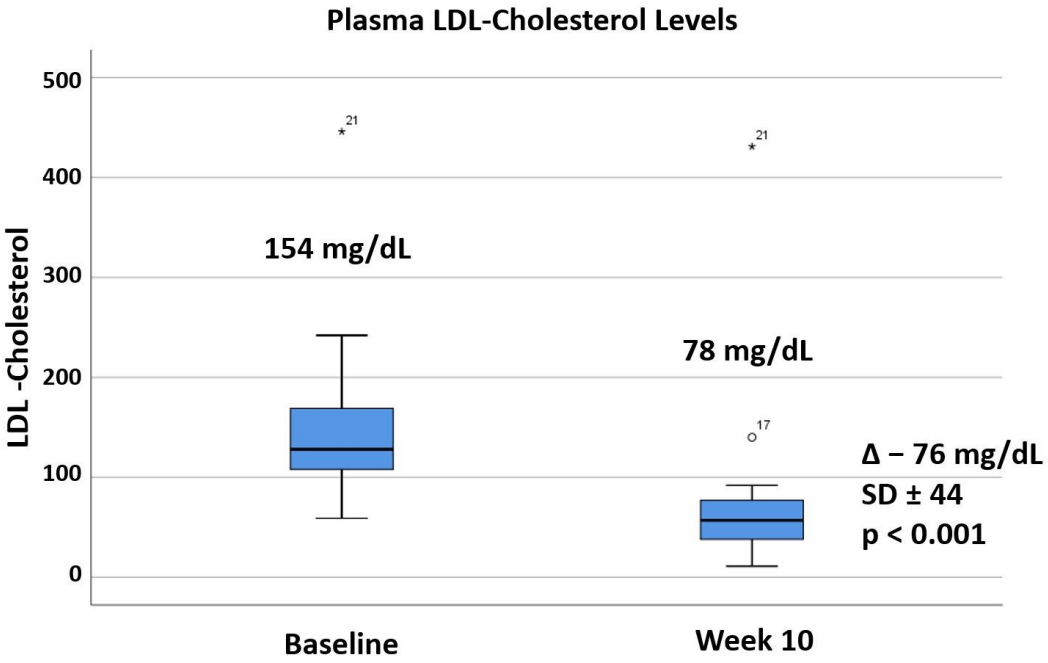


Legend: Figure shows trial-completion analysis (n = 19), one patient missed his scheduled ultrasound imaging visit. Pearson bivariate correlation coefficient (r). FDD: Flow-dependent dilatation. R^2 Linear: R-squared is a goodness-of-fit measure for linear regression models (Metzner et al., 2022a).

3.4 Lipids, Vascular Biomarkers, and Inflammation

Plasma LDL-C was significantly changed by an absolute reduction of 76 mg/dL in response to alirocumab treatment, this equates a relative reduction of 49% from baseline ($p < 0.001$, Figure 10 and Table 6). Further significant reductions of cardiovascular biomarkers were observed for Apo-B (39%), cholesterol (32%) and Lp(a) by 16%. Familial hypercholesterolemia was recorded for two patients, one showed baseline LDL-C of >400 mg/dL. Lp(a) measurements of >50 mg/dL at baseline was observed for eight patients, in this cohort Lp(a) was significantly reduced from mean 94 mg/dL to 81 mg/dL ($p = 0.018$), which equates an absolute Lp(a) reduction of 14 (12) mg/dL (Metzner et al., 2022a).

Figure 10. Plasma LDL-C levels at baseline and after 10 weeks of alirocumab treatment.



Legend: Figure shows trial-completion analysis (n = 19) by lipid-electrophoresis; Paired t-test with two-sided p-value; SD = Standard Deviation. The small circle in the right part of the figure identifies an outlier measurement, while asterisks identify extreme values. Outliers are defined as values between 1.5x and 3x interquartile ranges from the end of a box and extreme values are defined as >3x interquartile ranges (Metzner et al., 2022a).

Table 6. Change of lipids, vascular biomarkers, and inflammation in response to alirocumab.

Parameters	Baseline	±SD	Week 10	±SD	Change (mg/dL)	±SD	Change (%)	p-Value
Standard Lipids [a]								
LDL-C (mg/dL)	154	85	78	91	−76	44	−49	<0.001
Apo-B (mg/dL)	124	56	76	65	−48	28	−39	<0.001
Cholesterol (mg/dL)	244	104	166	102	−78	41	−32	<0.001
Lp(a) (mg/dL)	51	43	43	39	−8	11	−16	0.007
Triglycerides (mg/dL)	148	87	132	65	−17	73	−11	0.332
HDL-C (mg/dL)	50	13	59	16	+9	15	+18	0.018
Systemic Inflammation [b]								
CRP (mg/L)	2.7	3.5	2.6	3.1	−0.1	1.7	−4	0.902
IL-6 (pg/mL)	3.1	1.7	2.7	1.4	−0.5	0.9	−13	0.030
MCP-1 (pg/mL)	617	219	623	188	+6.2	99	+1	0.789
Vascular Biomarkers [c]								
VEGF (pg/dL)	6.2	6.1	7.5	7.8	+1.3	5.7	+21	0.409
P-Selectin/CD62P (ng/mL)	35.4	8.0	35.5	10.2	+0.1	7.4	+0.3	0.963

Legend: The table lists baseline and week 10 mean values and its changes of the trial completion population (n = 19), statistical analysis was a paired t-test reporting two-sided p-value.

[a]Lipid-electrophoresis was performed to assess LDL-C and HDL-C; Photometry was used to analyse triglycerides and total cholesterol; Immunoturbidimetry was performed to evaluate lipoproteins. [b]Immunoturbidimetry was also used to assess CRP; Electrochemiluminescence immunoassay was performed to measure IL-6; Enzyme-linked immunosorbent assay was conducted to assess MCP-1. [c]Enzyme-linked immunosorbent assays were also used to measure VEGF and P-Selectin/CD62P. Regarding the VEGF assessment only fourteen patients had valid measurements for statistical analysis, thus the VEGF antigen was below the limit of detection for five participants. (Metzner et al., 2022a).

Biomarkers for systemic inflammation (MCP-1 and CRP) were not affected by alirocumab treatment (Table 6). However, we could observe a minor change of IL-6 that was statistically significant. Plasma VEGF and P-selectin/CD62P as vascular biomarkers were also not changed by alirocumab (Metzner et al., 2022a).

3.5 Lipoproteins and Lipoprotein Particle Subfractions

The evaluation by ß-quantification using a combined method of ultracentrifugation and precipitation also showed an absolute LDL-C reduction of 69 mg/dL and relative reduction of -44% by alirocumab treatment (Table 7). In consistence with the lipid-electrophoresis method we observed a significant improvement of HDL-C (+17%). Despite a numerical reduction of 34% considering VLDL-C, the change was not statistically significant (Metzner et al., 2022b).

Table 7. Effects of alirocumab on lipids, lipoprotein particle subfractions, and lipoproteins.

Parameters	Baseline	Week 10	Absolute Change (mg/dL)	±SD	Relative Change (%)	p-value
Cholesterol (mg/dL)	236	162	−74	42	−31	<0.001
Triglycerides (mg/dL)	144	130	−15	69	−10	0.362
Lp(a) (mg/dL)	47	41	−7	8	−14	0.002
ApoAI (mg/dL)	154	164	+10	13	+7	0.002
ApoAII (mg/dL)	34	35	+1	4	+2	0.384
ApoB (mg/dL)	110	66	−44	23	−40	<0.001
ApoCII (mg/dL)	6	5	−1	1	−10	0.069
ApoCIII (mg/dL)	14	14	−1	3	−5	0.411
ApoE (mg/dL)	12	9	−3	3	−21	0.001
VLDL-C (mg/dL)	35	23	−12	41	−34	0.219
VLDL Triglycerides (mg/dL)	96	91	−5	67	−5	0.772

VLDL ApoB (mg/dL)	15	10	−5	10	−32	0.046
LDL-C (mg/dL)	158	89	−69	63	−44	<0.001
LDL Triglycerides (mg/dL)	36	24	−11	7	−32	<0.001
LDL ApoB (mg/dL)	95	56	−39	27	−41	<0.001
HDL-C (mg/dL)	42	49	+7	7	+17	<0.001
HDL Triglycerides (mg/dL)	13	14	+1	2	+9	0.055

Legend: Shows lipoprotein analysis results by ultracentrifugation and precipitation method. Trial completion population (n = 19). SD: Standard deviation; Mean values; Paired-samples t-test with two-sided p-value (Metzner et al., 2022b).

3.6 Lipoprotein Particle Numbers and Lipase Regulators

We observed a significant increase of the VLDL-triglycerides/VLDL-ApoB ratio by alirocumab. This ratio is thought to correlate with increasing VLDL size thus representing a potential marker for a less atherogenic profile (Hollstein et al., 2019). The VLDL size was evaluated by nuclear magnetic resonance and in consistence showed a modest but significant change of VLDL size. However, the large VLDL particle concentration seemed not to be affected by alirocumab treatment and there was no effect of alirocumab on lipoprotein lipase regulating proteins (Table 8 and Figure 11), (Metzner et al., 2022b).

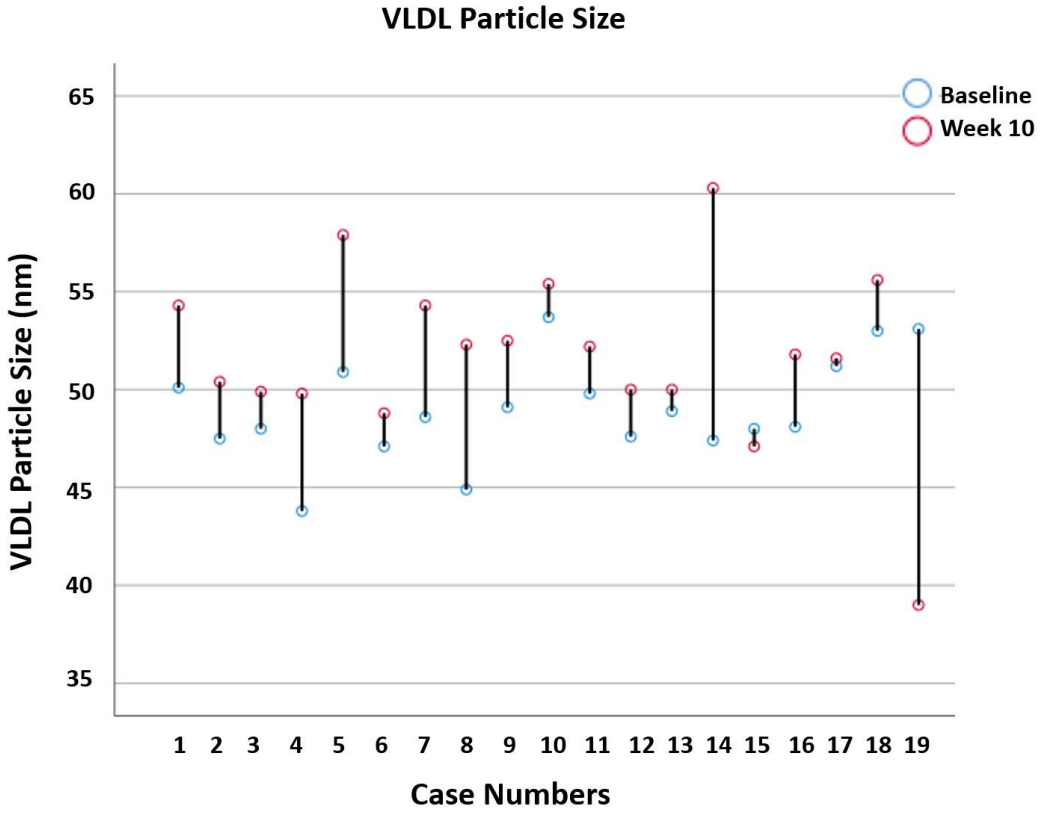
Table 8. Lipoprotein particle numbers, particle ratios, and lipoprotein lipase regulators.

Parameters	Baseline	Week 10	Absolute Change	±SD	Relative Change (%)	p-Value
Lipoprotein Particle Number [a]						
L-VLDLp (nmol/L)	6.5	6.9	+0.4	5.6	+6	0.752
LDLp (nmol/L)	1573	930	−643	317	−41	<0.001
L-LDLp (nmol/L)	832	498	−335	454	−40	0.005
S-LDLp (nmol/L)	741	448	−293	301	−40	<0.001

HDLp (nmol/L)	32391	34585	+2194	2294	+7	0.001
L-HDLp (nmol/L)	4758	6083	+1325	1382	+28	0.001
S-HDLp (nmol/L)	28436	28940	−593	2122	+2	0.252
Lipoprotein Particle Size [b]						
VLDL size (nm)	49	52	+3	5	+6	0.031
LDL size (nm)	21	21	0	0.5	0	0.159
HDL size (nm)	8.8	9.0	+0.2	0.2	+2.3	<0.001
Ratios of Lipoprotein Particles [c]						
Triglycerides/ApoB	1.3	2.2	+0.9	0.8	+69	<0.001
VLDL Triglycerides/ApoB	0.9	1.6	+0.7	0.8	+78	0.001
VLDL Triglycerides/VLDL-ApoB	6.9	9.7	+2.8	2.8	+41	<0.001
Cholesterol/ApoB	2.2	2.6	+0.5	0.3	+18	<0.001
Lipoprotein Lipase Regulators [d]						
ANGPTL-3 (ng/mL)	66.5	66.3	−0.2	4.3	−0.3	0.835
ANGPTL-4 (pg/mL)	138.3	140.6	−2.2	43.1	+1.7	0.825
GPIHBP-1 (pg/mL)	894.3	913.7	+19.4	118.3	+2.2	0.484

Legend: The table lists baseline and week 10 mean values and its changes of the trial completion population (n = 19), statistical analysis was a paired t-test reporting two-sided p-value. SD: Standard deviation. [a,b] Lipoprotein particle number concentration and particle size were assessed by nuclear magnetic resonance spectroscopy. L = large; S = small lipoprotein particles. Total and small VLDL particle concentrations are not measured by the Numares method. [c] Ratios of lipoprotein subfractions were calculated from lipoprotein analysis using the combined ultracentrifugation precipitation method. [d] Lipase regulators were calculated by enzyme-linked immunosorbent assays (Metzner et al., 2022b).

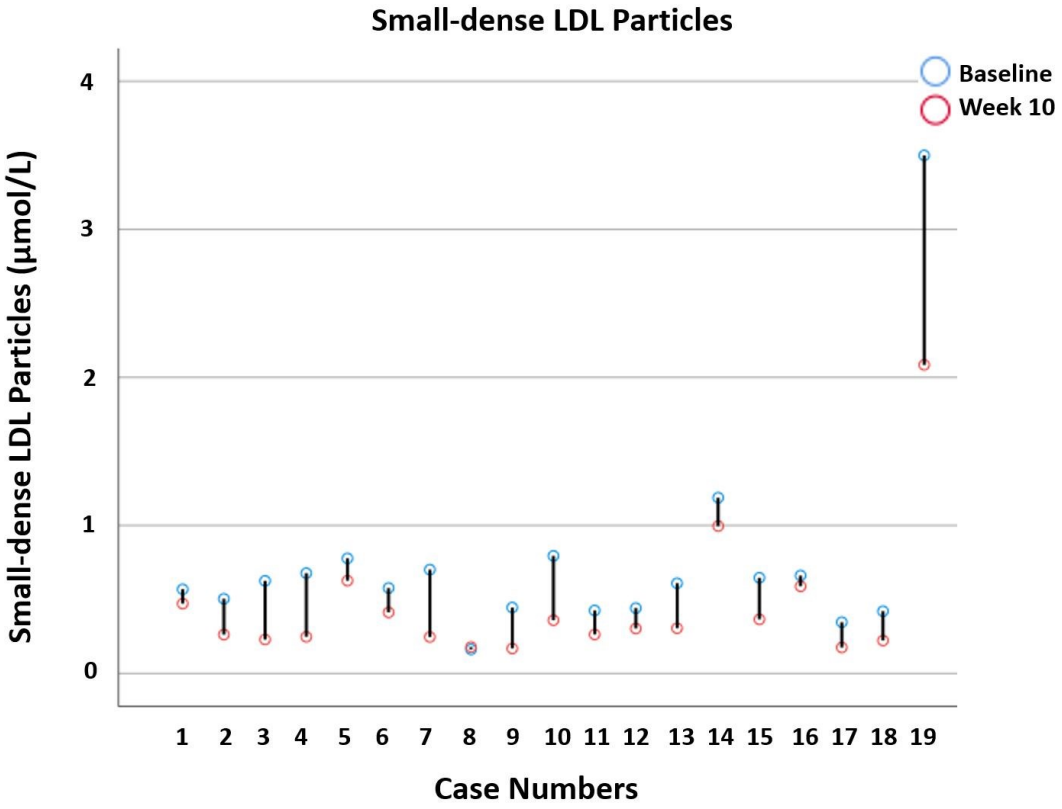
Figure 11. Distribution of VLDL particle size and changes by alirocumab treatment.



Legend: Shows nuclear magnetic resonance results of individual VLDL size and changes in response to alirocumab from baseline to week 10. Trial-completion population (n = 19). Case number 19 was a patient with mixed familial hyperlipidaemia and with baseline LDL-C of > 300 mg/dL, baseline small-dense LDL particles of 3500 nmol/L and with baseline triglyceride-rich large VLDL particles of 24 nmol/L (Metzner et al., 2022b).

Total LDL particle concentration was significantly decreased, while total HDL particles were significantly increased. The reduction of both large- and small LDL particles were accountable for the reduction of total LDL particle concentration, while the increase of total HDL particle concentration seemed to be driven by large HDL particles exclusively (Table 8 and Figure 12). Correlation analysis showed a positive association of baseline triglyceride-rich large VLDL particles and small dense LDL particles with statistical significance ($r = 0.883$, $p < 0.001$). On the other hand, large and small baseline LDL particles were not related ($r = 0.021$, $p = 0.932$), (Metzner et al., 2022b).

Figure 12. Distribution of small-dense LDL particles and changes by alirocumab treatment.



Legend: Shows nuclear magnetic resonance results of individual small-dense LDL particles and changes in response to alirocumab from baseline to week 10. Trial-completion population (n = 19). Case number 19 was a patient with mixed familial hyperlipidaemia and with baseline LDL-C of > 300 mg/dL, baseline small-dense LDL particles of 3500 nmol/L and with baseline triglyceride-rich large VLDL particles of 24 nmol/L (Metzner et al., 2022b).

3.7 Investigation of Post-Prandial Lipemia

The optional fat-tolerance testing was conducted by fourteen patients among all nineteen patients completing the ALIROCKS trial. The commercial Lipotest® meal significantly increased triglycerides at both trial visits ($p < 0.001$, Figure 13). The relative increase of triglycerides by the fat-tolerance meal was about 25% from 0 hours (t0) to 4 hours (t4), (Metzner et al., 2022b).

Figure 13. Fat-tolerance test results at baseline and after 10 weeks of treatment with alirocumab.

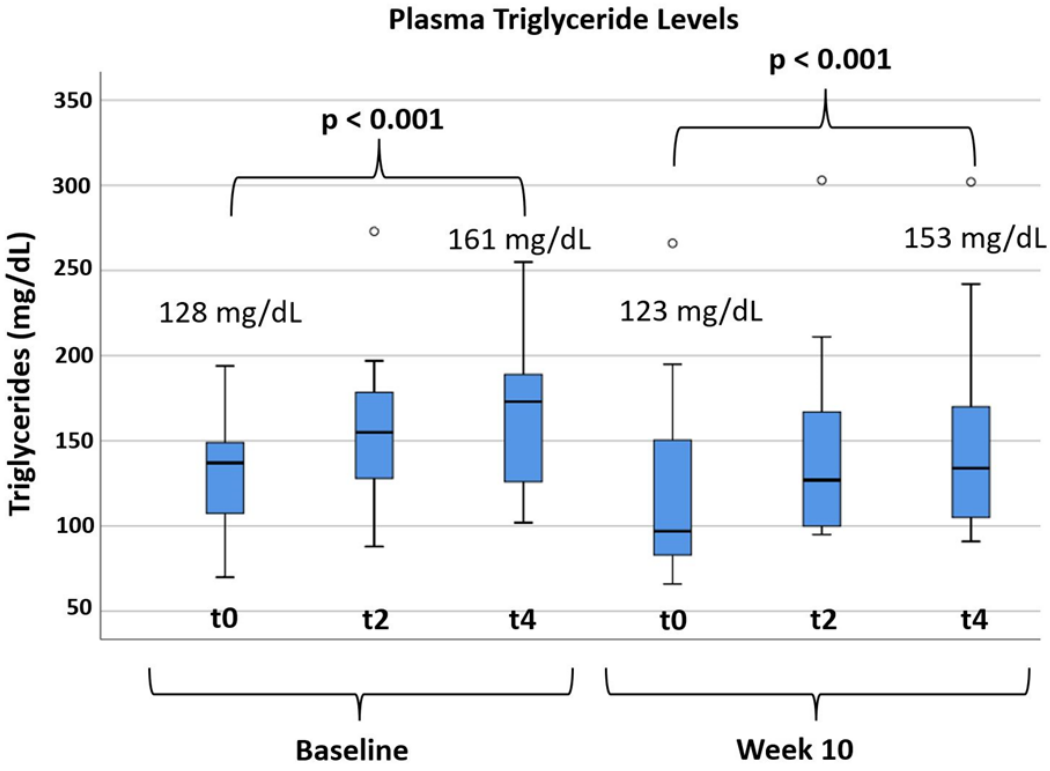
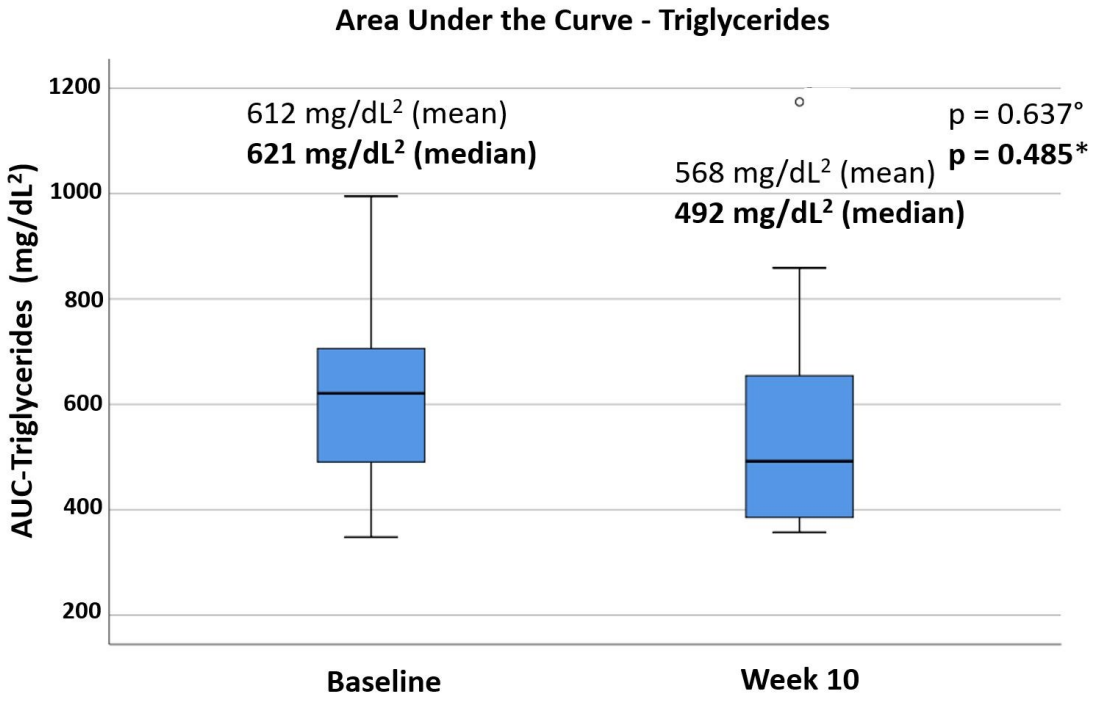


Figure 1. Fat-tolerance test results with Lipotest® meal at baseline and after 10 weeks of treatment with alirocumab. Legend: Triglyceride plasma levels at baseline and after 10 weeks of alirocumab treatment, before Lipotest® meal consumption (t0), at 2 h (t2), and 4 h (t4). Among the trial completion population, 14 patients participated in the per-protocol optional fat-tolerance assessment (n = 14). The small circles at the upper part of the figure identify outlier measurements. Outliers are defined as values between 1.5x and 3x interquartile ranges from the end of a box. Paired-samples t-test with two-sided p-value was performed (Metzner et al., 2022b).

We did not observe significant changes of post-prandial triglycerides by alirocumab, either by comparison of t0-t4 values (p = 0.446) or by comparison of areas-under-the-curves (AUC) before and after the treatment (p = 0.485, Figures 13 and 14). HDL-C, non-HDL-C and total cholesterol did not change in response to the fat-tolerance test or alirocumab treatment (Metzner et al., 2022b).

Figure 14. Area under the curve of triglycerides during fat-tolerance testing (t0-4 hours) at baseline visit and after 10 weeks of alirocumab.

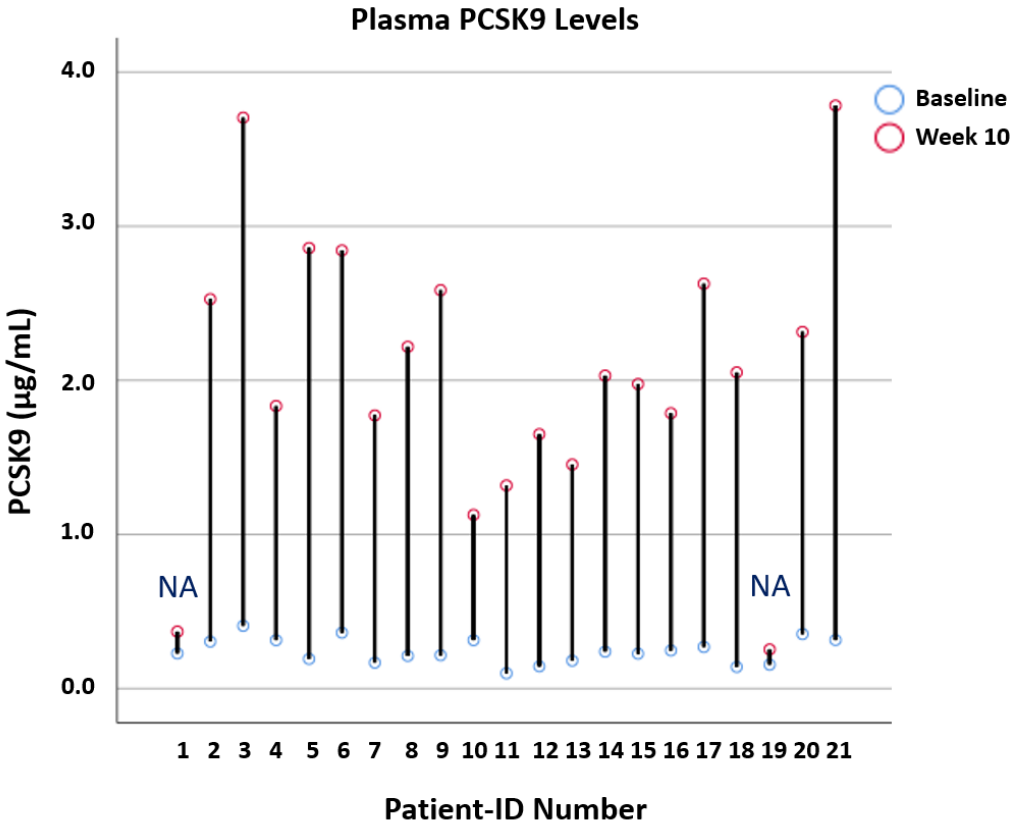


Legend: Among the trial-completion population, eleven patients had completed all measurements for AUC calculation (n = 11). Paired samples t-test with two-sided p-value. The small circle at the right upper part of the figure identifies an outlier measurement. Outliers are defined as values between 1.5x and 3x interquartile ranges from the end of a box. °Paired samples t-test; *Related samples Wilcoxon signed rank test. Calculations according to the trapezoid model (Metzner et al., 2022b).

3.8 Effects of Alirocumab on Circulating PCSK9

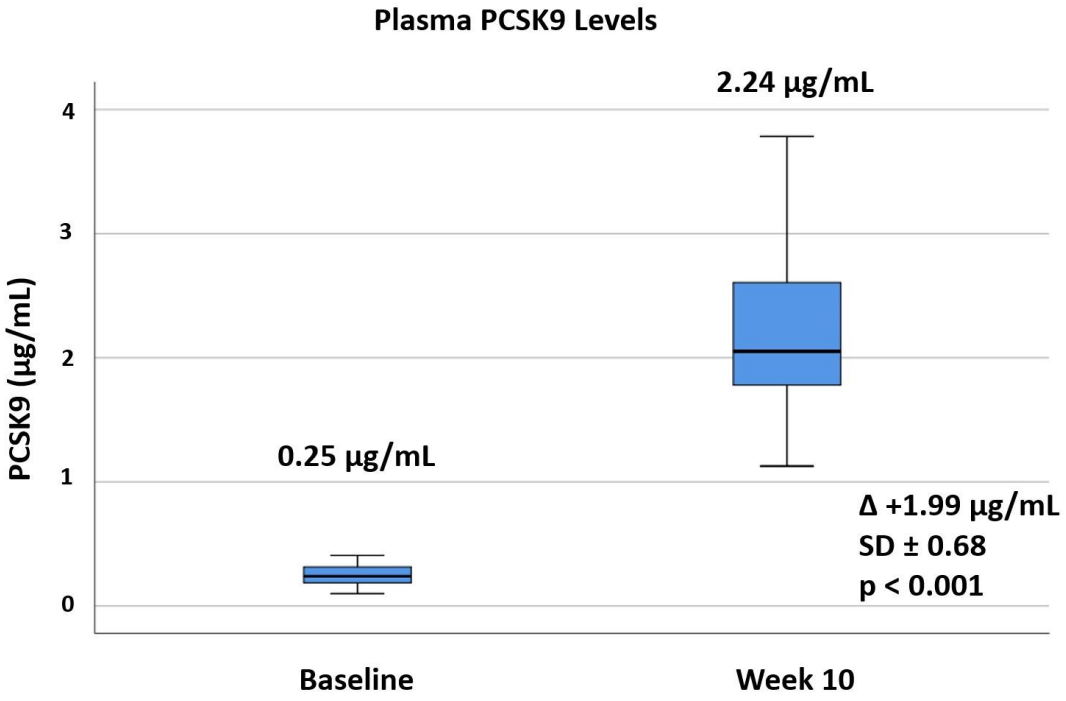
Plasma PCSK9 was increased >3-fold by alirocumab in all participants reporting treatment adherence (Figures 15, 16 and Table 9). The total PCSK9 concentration increased from 0.25 to 2.24 µg/mL in the trial completion population (n = 19), which resulted in an absolute change of +1.99 µg/mL (SD ± 0.68; $p < 0.001$) and equates to a nearly 10-fold increase from baseline (Metzner et al., 2022b).

Figure 15. Plasma PCSK9 increase per patient in response to alirocumab treatment.



Legend: The figure includes the 19 adherent patients and 2 non-adherent (NA) patients that did not receive alirocumab beyond week 2 (n = 21). The used enzyme-linked immunosorbent assay measured total plasma PCSK9 levels, and thus did not differentiate between bound and unbound PCSK9 (Metzner et al., 2022b).

Figure 16. Plasma PCSK9 levels at baseline and after 10 weeks of alirocumab treatment.



Legend: Figure shows trial-completion analysis (n = 19); Paired t-test with two-sided p-value; SD: Standard deviation; (Metzner et al., 2022b).

We did not observe an LDL-C reduction or PCSK9 increase (>3-fold) in the two non-adherent patients which administered only two alirocumab injections each at baseline and week 10, their total PCSK9 concentrations remained <0.4 µg/mL. In contrast, for all nineteen adherent patients measured plasma PCSK9 levels were between 1.1 and 3.8 µg/mL at week 10 of alirocumab treatment. Among those nineteen participants reporting treatment adherence, two patients can be classified as low/non-responders because they did not show the expected decrease in plasma LDL-C but displayed a 10- to 12-fold PCSK9 increase (Metzner et al., 2022b).

Table 9. LDL-C and PCSK9 plasma levels per patient.

Method	Lipoprotein-Electrophoresis			Lipoprotein Analysis (Combined)			ELISA		
Patient-ID	LDL-C Baseline	LDL-C Week 10	LDL-C Change (%)	LDL-C Baseline	LDL-C Week 10	LDL-C Change (%)	PCSK9 Baseline	PCSK9 Week 10	X-Fold Increase
1	147	155	+5	145	176	+21	0.23	0.37	<2
2	90	48	-47	119	74	-38	0.31	2.53	8
3	128	41	-68	142	53	-63	0.41	3.70	9
4	81	27	-67	85	46	-46	0.31	1.83	6
5	242	34	-86	278	63	-77	0.19	2.86	15
6	161	67	-58	168	58	-65	0.36	2.84	8
7	154	78	-49	160	94	-41	0.17	1.77	11
8	193	57	-70	197	61	-69	0.21	2.22	10
9	125	60	-52	148	87	-41	0.22	2.59	12
10	59	27	-54	86	49	-43	0.31	1.13	4
11	126	46	-63	121	47	-61	0.10	1.32	13
12	90	11	-88	99	52	-47	0.14	1.65	11
13	116	76	-34	119	78	-34	0.18	1.45	8
14	160	92	-43	181	114	-37	0.24	2.03	8
15	206	91	-56	215	82	-62	0.23	1.98	9
16	165	68	-59	160	68	-58	0.25	1.79	7
17	173	140	-19	172	134	-22	0.27	2.63	10
18	100	35	-65	110	43	-61	0.14	2.05	15
19	79	80	+1	122	94	-23	0.16	0.25	<2
20	117	51	-56	132	64	-52	0.35	2.31	7
21	446	431	-3	319	433	+36	0.32	3.78	12

Legend: The table shows mean LDL-C (mg/dL) and mean PCSK9 (µg/mL) values per patient with week 10 assessments (n = 21), as well as the relative impact of alirocumab treatment on these two parameters. Patients with the number 1 and 19 were non-adherent, thus did not inject alirocumab beyond week 2. Among participants with self-reported adherence, patient number 17 and 21 reflect low/non-response but show high PCSK9 increase. Green: Lipoprotein-Electrophoresis; Blue: Lipoprotein analysis using ultracentrifugation precipitation (ß-quantification); Purple: Enzyme-linked immunosorbent assay (ELISA), (Metzner et al., 2022b).

4 DISCUSSION

4.1 Vascular Structure and Function

We evaluated a novel magnetic resonance imaging method assessing the structural integrity of the carotid artery vessel wall. However, we obtained valid fractional anisotropy values for statistical analysis of only a subgroup of our clinical trial population, thereby limiting the scientific and clinical applicability of this technique. The reasons for this shortcoming were spontaneous claustrophobia experienced by patients during the assessments, the long duration of the imaging procedure, the patient's anatomy such as adiposity / short neck, as well as invalid outlier measures. Fractional anisotropy as a potential structural marker of the carotid artery did not change in response to alirocumab. Despite about 50% LDL-C reduction by alirocumab, the low sample size of the trial together with the short duration of only 10 weeks of treatment did not let expect significant structural changes. Because of the novelty of this imaging method there are also no established cut-off values enabling to differentiate pathological from normal anisotropy measures. In course of a previous trial our group evaluated the reproducibility of this novel imaging parameter in a population of healthy men by calculating the variation coefficients. These were shown to range from 2.5% to 5.4% in

four consecutive assessments of four trial participants. This investigation in a healthy population also reported an inverse association of increasing age and fractional anisotropy. These subjects had fractional anisotropy measures between approximately 0.70 at 27 years to 0.56 at 57 years of age (Opriessnig et al., 2016). In consistence with the hypothesis of advanced atherosclerosis and lower fractional anisotropy measures with increasing age, our ALIROCKS trial population had a mean baseline fractional anisotropy value of 0.48 and a mean age of 66 years. However, correlation analysis of LDL-C and fractional anisotropy did not reveal a significant association in our small trial population with progressed atherosclerotic disease (Metzner et al., 2022a).

Reliable and non-invasive methods to assess the progression of atherosclerosis are needed for improved treatment guidance and patient safety. In atherosclerosis research the usage of invasive procedures like optical coherence tomography showed that lipid cores of plaques can be reduced, and fibrous caps of atherosclerotic lesions can be stabilized by PCSK9-antibody treatment. A retrospective investigation of 18 patients with acute coronary syndrome using optical coherence tomography showed that 12 weeks of evolocumab increased fibrous-cap thickness while decreasing the lipid-rich plaque proportion (Yano et al., 2020). In this context, another clinical study also showed that alirocumab with a treatment duration of 36 weeks increased fibrous cap thickness and decreased the lipid core of plaques in 12 patients with coronary artery disease (Sugizaki et al., 2020). Lately a press release announced the positive results of a placebo-controlled double-blinded phase 3 study evaluating the effects of evolocumab on fibrous cap thickness using optical coherence tomography. The trial randomized 161 patients with myocardial infarction either to statin plus placebo or statin plus evolocumab. These study results showed that the PCSK9 antibody evolocumab can significantly increase fibrous cap thickness over an observation period of 52 weeks (Nicholls et al., 2021). In consistency, the better established invasive intravascular-ultrasound method in atherosclerosis research previously showed in the GLAGOV trial that evolocumab can significantly lower coronary atheroma volume. This placebo-controlled, randomized trial enrolled 968 patients with coronary heart disease and the study duration was 76 weeks (Nicholls et al., 2016). However, further sub-analysis of this trial investigating possible effects of evolocumab on plaque composition by virtual histology did not report significant findings (Nicholls et al., 2018). The randomized ODYSSEY J-IVUS study evaluated the effects of alirocumab on atheroma volume using intravascular-ultrasound and enrolled 206

patients after acute coronary syndrome. In concordance with the results of the GLAGOV trial this study showed a numerical decrease of total plaque atheroma volume with a much shorter treatment duration of 36 weeks only (Ako et al., 2019, Metzner et al., 2022a).

In our ALIROCKS trial we also used well described and established ultrasound-based approaches to investigate potential effects of alirocumab on the vasculature, beyond the novel magnetic resonance imaging technique. Firstly, structural changes of carotid arteries were analyzed by evaluating the intima-media thickness. In our study population with advanced atherosclerotic disease and age, baseline intima-media thickness ranged between 0.42 to 0.77 mm with a mean value of 0.58 mm. Interestingly, these results would not meet most recent recommendations by the American Society of Echocardiography for significant cardiovascular disease definition in primary prevention which suggests an intima-media thickness of ≥1.5 mm (Johri et al., 2020). Of importance, we did not screen for larger plaques or atherosclerotic target lesions in the carotid arteries in course of our study, but our findings underline the need for an improvement of cardiovascular imaging methods in the clinical setting, especially for patients with hypercholesterolemia in primary prevention (Randrianarisoa et al., 2015). In consistency with our observations, recommendations from the European Society of Cardiology and European Atherosclerosis Society explicitly state that the evaluation of carotid intima-media thickness is considered inferior to coronary computed tomography and / or coronary artery calcium scoring for cardiovascular risk assessment in primary prevention (Mach et al., 2020). The low sample size, the low baseline intima-media thickness without selection of target lesions and the short treatment duration of our clinical study did not let expect significant effects of alirocumab on the intima-media thickness. A recently published observational trial investigating the effects of evolocumab on intima-media thickness of carotid arteries showed a significant improvement, but treatment duration was one year and involved 229 subjects with concomitant statin treatment (Hirai et al., 2020). Additional LDL-C lowering by PCSK9-antibodies in patients on stable lipoprotein apheresis has been also shown to reduce intima-media thickness. Although only 14 patients with heterozygous familial hypercholesterolemia were analyzed in this retrospective investigation the results showed significant changes, but compared to our trial these subjects had a much higher mean LDL-C value at baseline (197 mg/dL), higher Lp(a) value (380 nmol/L) and were observed for 3.5 years (Metzner et al., 2022a, Sbrana et al., 2020).

Moreover, the ultrasound-based evaluation of endothelial function by assessment of flow-dependent dilatation in course of our trial did also not show significant effects of alirocumab treatment, although we detected a numerical improvement of +41%. An Italian study analyzed the effects of evolocumab on flow-dependent dilatation in 14 participants over a treatment period of 2 months and reported a significant improvement (Maulucci et al., 2018). Of interest, the effect size and treatment duration were comparable with our ALIROCKS trial, but mean baseline LDL-C and baseline flow-dependent dilatation was remarkably higher in this Italian investigation. These differences might be explained due to observer bias and /or differential patient characteristics The Italian trial analyzed patients following myocardial infarction without achieving their guideline recommended LDL-C goal although treated with ezetimibe and high-intensity statins. Besides, another trial evaluating the effects of PCSK9-antibodies in 25 participants with familial hypercholesterolemia also reported an improvement of endothelial function over a treatment period of 3 months (Di Minno et al., 2020). At baseline, their mean LDL-C was 201 mg/dL and their mean Lp(a) was 69 mg/dL. Summing up, differing trial results regarding the evaluation of endothelial function in response to PCSK9-antibodies may be due to diverging characteristics of patient populations especially when considering known cardiovascular risk factors and / or methodological restrictions of this ultrasound-based technique (Hijmering et al., 2001, Metzner et al., 2022a).

4.2 Lipid and Triglyceride Metabolism

The LDL-C lowering effect by alirocumab treatment was about 50% in consistence with previous clinical trials of higher sample size (Catapano et al., 2017a). The values varied depending on the respective method of lipid-analysis. This variation was expected and is not only known between calculated LDL-C by Friedewald formula and newer direct quantification methods such as lipid-electrophoresis or ß-quantification but has been also observed between direct lipid assessments (Miller et al., 2010, Chung et al., 2009, Scharnagl et al., 2001). The extent of measurement variation depends on the methodological bias and on the respective patient population of a specific lipoprotein particle profile. Therefore, especially in patients with hypertriglyceridemia, with a history of high Lp(a) or diabetic dyslipidaemia, direct, consistent and complete lipoprotein particle profiles should be

conducted for the right interpretation and to guide optimal medical therapies (Scharnagl et al., 2001, Mach et al., 2020, Miller et al., 2010). Of note, the patient characteristics of our clinical trial were heterogenous in this respect and consistent with a recently published "real-world" non-interventional alirocumab trial in clinical routine reporting similar LDL-C lowering effects by alirocumab treatment (Leitner et al., 2020). Although a significant reduction of LDL-C could be achieved by alirocumab treatment in our trial, mean week 10 values were still above the current guideline recommendations of < 55 mg/dL LDL-C (Mach et al., 2020). Only 47% (n = 9 of 19) of all patients completing the trial reached this value. The reason for this low target achievement in our PCSK9 population of clinical routine is the high prevalence of statin intolerance. Of all 19 patients, only 5 tolerated any concomitant statin and only 3 received high intensity statin therapy, defined as ≥ 40 mg of atorvastatin or ≥ 20 mg of rosuvastatin. Importantly, all 5 patients tolerating statins achieved the LDL-C target of < 55 mg/dL. These results clearly demonstrate the need for implementation of further non-statin oral lipid-therapy combinations into clinical practice, such as bemepedoic acid + ezetimibe poly pills. The bempedoic acid inhibits cholesterol synthesis in hepatic cells like statins but is administered as prodrug and is only active in hepatic cells. Therefore, this substance combination might be a good alternative for patients complaining about statin associated myopathy and might also improve the high prevalence of non-adherence to statins (Drexel et al., 2020b, Metzner et al., 2022b).

One of the key findings of our clinical study was that alirocumab treatment had nearly no effect on triglycerides and triglyceride-rich lipoproteins in our "real-world" PCSK9-antibody population. We did not detect any alirocumab treatment effects on post-prandial lipemia which was evaluated by standardized fat-tolerance testing. In consistence with these results key lipoprotein lipase modulators like ANGPTL-3, ANGPTL-4, GPIHBP-1, Apo-CII and ApoCIII were also not affected by alirocumab. Altogether this strongly suggests that PCSK9 regulation does not substantially affect triglyceride metabolism in an unselected PCSK9-antibody population. There may be a numerical trend towards a reduction of fasting plasma triglycerides of 10% without reaching statistical significance. However, this effect size is consistent with a more powerful, pooled analysis of ten alirocumab studies involving 4983 patients which showed a modest but significant decrease in triglycerides of around 10-15% (Henry et al., 2018). Of note, this investigation of several pooled alirocumab studies did also suggest that this triglyceride reduction effect was irrespective of metabolic syndrome, but

patients with diabetes mellitus were not included. As expected, the ODYSSEY DM-INSULIN trial showed that PCSK9-antibody effects on triglycerides are increased in a more selected patient population of mixed dyslipidaemia with insulin-dependent type-2 diabetes mellitus (Ray et al., 2019c). In consistence, a much smaller but randomized and placebo-controlled clinical study investigating 12 participants of type-2 diabetes with insulin dependence showed similar treatment effects of alirocumab regarding post-prandial as well as fasting triglycerides (Burggraaf et al., 2020). Evolocumab also showed comparable changes regarding post-prandial lipemia in a study of non-randomized design evaluating its effects in 15 patients with type-2 diabetes mellitus (Taskinen et al., 2020, Metzner et al., 2022b).

Another key outcome measure of our ALIROCKS study was to evaluate changes of lipoprotein particle subfractions in response to alirocumab. Correlation analysis of baseline triglyceride-rich large VLDL particles and baseline small-dense LDL particles revealed a significant positive relationship. This association has been well described in patients with diabetic dyslipidaemia as both particle subfractions are especially elevated in this disease characteristic (Krauss, 2004). Most important we showed that alirocumab treatment significantly reduced not only total LDL particles, but also small-dense LDL particles that are considered to convey additional atherosclerotic risk because of their smaller size and increased ability to invade the blood vessel wall. On the other hand, HDL particles were significantly elevated by alirocumab suggesting an improved reverse-cholesterol transport. These findings of our clinical trial are in consistence with the results of a post-hoc investigation using nuclear magnetic resonance spectroscopy involving 29 alirocumab patients (Koren et al., 2015). In course of our lipoprotein particle subfraction analysis we identified one patient in our trial population (patient ID: 21, case number: 19) with seemingly no response to alirocumab regarding LDL-C but having high absolute small-dense LDL particle concentration and experiencing a relative reduction of about 40% by alirocumab at week 10. This resulted in an absolute reduction of 1500 nmol/L in small-dene LDL particles and might convey a treatment benefit irrespective of LDL-C. This underlines the importance of a direct and complete lipoprotein particle assessment beyond LDL-C calculation by the Friedewald formula as already explained. Non-HDL and ApoB assessments help to improve target guidance in patients with hypertriglyceridemia or diabetic dyslipidaemia but would probably not have identified this positive effect on small-dense LDL particles by alirocumab that can be especially important when proof of efficacy needs to be documented for insurance

reimbursement of this medication (Mach et al., 2020, Metzner et al., 2022b). In consistence with a preceding trial of our team members investigating 350 patients treated with PCSK9-antibodies, our ALIROCKS trial results also suggested a VLDL size increase by alirocumab calculated by a VLDL-triglycerides/VLDL-ApoB ratio. In line with this finding, alirocumab treatment was also associated with a decrease of VLDL-associated apolipoproteins indicating an increased clearance of smaller, more atherogenic VLDL remnants from the plasma (Hollstein et al., 2019). This effect on VLDL size increase by PCSK9-antibodies was affirmed by our nuclear magnetic resonance spectroscopy investigation. Of note, we also found a statistically significant reduction of Lp(a) by alirocumab treatment. In a prespecified sub-analysis of the ODYSSEY OUTCOMES trial evaluating the effects of alirocumab on the reduction of major cardiovascular events, the Lp(a) lowering effect was suggested to additionally decrease the event rate irrespective of LDL-C reduction (Bittner et al., 2020). However, the causality of Lp(a) reduction with major cardiovascular event reduction must be proven by a randomized, placebo-controlled cardiovascular outcome study which is still ongoing, and no results are published yet (Tsimikas et al., 2021). Eventually, all these "pleiotropic" alirocumab effects on the lipoprotein subfraction profile, such as the reduction of small-dense LDL particles, increase of VLDL size and decrease of Lp(a), are promising to add cardiovascular benefit beyond LDL-C lowering (Metzner et al., 2022b).

4.3 Inflammation and Vascular Biomarkers

Further outcome measures of our clinical study investigated possible changes of systemic vascular- and inflammatory biomarkers by alirocumab treatment. While several, mainly pre-clinical investigations supported the hypothesis that high PCSK9 levels increase inflammation in the atherosclerotic pathogenesis, clinical trials did not suggest an effect of PCSK9-antibodies on systemic inflammation (Momtazi-Borojeni et al., 2019, Trankle et al., 2019). In consistency with these studies, we could not show any alirocumab treatment effects either on plasma MCP-1 or on CRP. The minor decrease of IL-6 was considered to be neglectable (Momtazi-Borojeni et al., 2019). Of note, our trial population was not preselected regarding their inflammation status, thus mean inflammatory plasma markers such as CRP or IL-6 were not elevated at baseline. Another clinical trial investigating the change of local vessel wall

inflammation in response to alirocumab using using positron-emission computed tomography reported significant positive effects, while systemic biomarkers for inflammation were also not affected by the treatment (Hoogeveen et al., 2019). Interestingly, we could not show a link between LDL-C lowering and change of systemic vascular inflammation as suggested by a recent review (Catapano et al., 2017b). We could also not observe any alirocumab treatment effects on vascular biomarkers such as plasma VEGF or P-selectin, although a promising pre-clinical trial investigating evolocumab showed stimulating angiogenic effects by elevations of VEGF levels in an in-vitro setting (Safaeian et al., 2019). In consistency with another trial evaluating effects of PCSK9-antibodies on vascular biomarkers, we did not find changes of circulating P-selectin by short-term treatment (Barale et al., 2020). Of interest, this comparable study conducted by an Italian group had included another measurement time point for plasma P-selectin after a treatment period of 12-month. With this longer follow-up they were able to report a statistically significant decrease of this biomarker, thereby proposing a time-dependent effect (Barale et al., 2020, Metzner et al., 2022a).

Another important finding of our trial was that circulating PCSK9 could be a potential biomarker for treatment adherence of patients receiving a PCSK9-antibody in clinical practice. Of note, the enzyme-linked immunosorbent assay performed for this analysis did not distinguish between antibody-bound PCSK9 and unbound PCSK9 but measured the total PCSK9 plasma concentration. In consistency with another trial recently published by our group, mean PCSK9 levels increased about the 10-fold from baseline in response to alirocumab treatment (Silbernagel et al., 2019b). While those two patients with self-reported non-adherence, having administered alirocumab only twice, showed neither a decrease of LDL-C nor an increase of PCSK9, all other patients with reported adherence had a >3-fold increase of plasma PCSK9 levels. Therefore, exclusively two patients of our trial population can be categorized as real non- or low-responder, thus having a >3-fold increase of plasma PCSK9 but an LDL-C lowering effect of below 25%. To sum this up, assessing total PCSK9 levels in PCSK9-patients with seemingly low LDL-C lowering effects may be a useful strategy to differentiate non-response from non-adherence in the clinical setting (Table 12). Reliable evaluation of treatment adherence may be especially eminent in times of limited health-care budgets and expensive medications. Besides, it’s also important to keep in mind that non-adherence has a high prevalence in patients receiving life-long lipid-lowering therapies and is accountable for major cardiovascular events, but non-adherence is reversible

and needs to be handled completely different to non-respondence (Drexel et al., 2020a, Metzner et al., 2022b).

Table 10. Proposed evaluation for PCSK9 antibody adherence.

	Responder	**Non-Responder**	**Non-Adherent**
LDL-C ↓	**Yes**	**No**	**No**
PCSK9 ↑ (x3)	**N.A.**	**Yes**	**No**

Legend: Proposed evaluation strategy for testing low- to non-responder versus non-adherence. Total circulating PCSK9 level may differentiate between non-responder and non-adherent. Suggested cut-off value is a >3-fold PCSK9 level increase from baseline. ↑ = Increase, ↓ = Decrease; N.A.: Not Applicable (Metzner et al., 2022b).

4.4 Strengths and Limitations

The major strength of our trial is the usage of a completely novel magnetic resonance imaging method to investigate structural changes of the vasculature in patients with cardiovascular disease for the first time. Moreover, the parallel study design in combination with high-quality and better-established ultrasound-based imaging is unique. These ultrasound assessments were also conducted by only one methodological expert to exclude inter-observer variability. It is a strength that both data sets, obtained from magnetic resonance (fractional anisotropy) and from ultrasound evaluations (flow-dependent dilatation), had been monitored by an external clinical research organization. To reflect the "real world" of clinical practice we enrolled PCSK9-antibody candidates with great diversity within the area of cardiovascular disease such as localization of atherosclerotic lesions, concomitant diseases, lipid levels and medication. This trial design minimizes patient selection bias but simultaneously increases heterogeneity of the study population which needs to be considered when interpreting our results. We conducted a profound and accurate analysis of metabolic and lipidomic characteristics using nuclear magnetic resonance spectroscopy, lipid-electrophoresis, combined lipoprotein analysis (ß-quantification) and fat-tolerance testing. The used Lipotest®

meal is the best described fat-tolerance test in the literature to date and due to its high standardization promises great reproducibility compared to non-commercial solutions (Tentolouris et al., 2017). Finally, this is the first clinical study evaluating changes of lipoprotein particle subfractions of alirocumab treatment using nuclear magnetic resonance spectroscopy in a clinical routine setting. It is a limitation that we had no control group, but all patients were guideline recommended candidates for PCSK9-antibodies, it was therefore rejected as unethical. The magnetic resonance imaging technique was not yet evaluated in a comparative trial of a healthy versus atherosclerotic vasculature, thus there are currently no established reference measures for fractional anisotropy. Our exploratory study design with rather low sample size is also a weakness. However, trials with even smaller or similar sample size and shorter treatment duration reported improvements of vascular function by lipid lowering therapies (Landmesser et al., 2005, Yoshida et al., 2010, Amudha et al., 2008, Karatzis et al., 2005, Maulucci et al., 2018, Di Minno et al., 2020). Confirmatory trials with larger sample size and longer treatment durations are certainly encouraged (Metzner et al., 2022a, Metzner et al., 2022b).

4.5 Implications for the Future and Conclusion

In summary, we could not detect significant changes of vascular structure or function in response to short-term alirocumab treatment. It will require further research efforts to evaluate our magnetic resonance imaging method and the potential of fractional anisotropy as marker for atherosclerosis. We could show that alirocumab does hardly affect post-prandial lipemia or triglyceride metabolism in an unselected PCSK9-antibody population of clinical routine. Importantly, we illustrated that total plasma PCSK9 assessments for patients with seemingly no or low LDL-C lowering effect by PCSK9-antibodies may help to differentiate real non-responders from non-adherent patients. Further trials are needed to challenge or confirm our findings (Metzner et al., 2022a, Metzner et al., 2022b).

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6 APPENDIX

6.1 Ethics Committee Approval

Ethikkommission

Medizinische Universität Graz

Auenbruggerplatz 2, A-8036 Graz
ethikkommission@medunigraz.at
Tel.: +43 / 316 / 385-13928, Fax: -14348

VOTUM

gültig bis 06.04.2019

EK-Nummer:	29-519 ex 16/17 **EudraCT Nr.:** 2018-000981-12
Studientitel:	A PILOT STUDY INVESTIGATING THE EFFECTS OF LIPID-THERAPY INTENSIFICATION WITH ALIROCUMAB ON ENDOTHELIAL FUNCTION, CAROTID ARTERIES, LIPOPROTEIN PARTICLE SUBFRACTIONS, INFLAMMATION AND POST-PRANDIAL LIPEMIA IN CLINICAL ROUTINE
Prüfer:	Ass.-Prof.Dr.med.univ.et scien Günther Silbernagel Klinische Abteilung für Angiologie, Universitätsklinik für Innere Medizin, Mediz
Sponsor:	Med.Uni Graz, Univ.Klinik für Innere Medizin
Ansprechpartner:	Ass.-Prof.Dr. Günther Silbernagel, 8036 Graz, Auenbruggerplatz 15
CRO:	-
Antragsteller:	Med. Uni. Graz
Ansprechpartner:	Mag.rer.nat. Thomas Metzner

Die o.a. Studie wurde von der Ethikkommission erstmals in der Sitzung 10-16/17 am 10.07.2017 behandelt.
Die Ethikkommission ist zu folgendem Schluss gekommen:

Es besteht kein Einwand gegen die Durchführung der Studie in der vorliegenden Form.

Stimmberechtigte bzw. anwesende Mitglieder bei der Behandlung waren: Siehe beiliegende Liste vom 10.07.2017.

Kommissionsmitglieder, die für diesen Tagesordnungspunkt als befangen anzusehen waren und daher gemäß Geschäftsordnung an der Entscheidungsfindung und Abstimmung nicht teilgenommen haben: Univ.Prof.Dr.Hermann Toplak

Zur Beurteilung vorliegende Dokumente:

Dokumente eingegangen am 19.06.2017, begutachtet in der Sitzung 10-16/17 am 10.07.2017

✓	Cover Letter Anschreiben_EK_Metzner_Dissertant 1	18.06.2017
✓	Antragsformular ECS	19.06.2017
	Originalprotokoll Clinical Trial Protocol_ALIROCKS_Version 1_13JUN2017 1	13.06.2017
	Informed Consent Form Patienteneininformation_ALIROCKS_Version 1_10JUN2017 1	10.06.2017
✓	Conflict of Interest Erklärung CoI Erklärung Prüfer_Silbernagel 1	17.06.2017
✓	Conflict of Interest Erklärung CoI Erklärung Antragsteller_Metzner 1	17.06.2017
✓	CV CV Silbernagel	16.06.2017
✓	CV CV Toplak	13.06.2017
✓	CV CV Scharnagl	13.06.2017
✓	CV CV Reishofer	24.05.2017
✓	CV CV Metzner	14.06.2017
✓	CV CV Mellitzer	13.06.2017
✓	CV CV Leitner	12.06.2017
✓	CV CV Beck	13.06.2017

Medizinische Universität Graz, Auenbruggerplatz 2, A-8036 Graz. www.medunigraz.at
Rechtsform: Juristische Person öffentlichen Rechts gem. Universitätsgesetz 2002. Information: Mitteilungsblatt der Universität und www.medunigraz.at. DVR-Nr. 210 9494, UID: ATU 575 111 79. Bankverbindung: Bank Austria Creditanstalt BLZ 12000 Konto-Nr. 500 948 400 04, Raiffeisen Landesbank Steiermark BLZ 38000 Konto-Nr. 49510.

✓ Sonstiges: EK Antrag_ALIROCKS Studie_14JUN2017 1	14.06.2017
Dokumente eingegangen am 12.09.2017 (in der nächsten Begutachtung mitbegutachtet)	
✓ Originalprotokoll 2	12.09.2017
Informed Consent Form 2	12.09.2017
✓ SmPC/Fachinformation Praluent	23.09.2015
✓ Sonstiges: E-Mail inkl. Stellungnahme zur Bearbeitungsmitteilung	12.09.2017
✓ Letter of Authorization	05.09.2017
Dokumente eingegangen am 14.09.2017 (in der nächsten Begutachtung mitbegutachtet)	
✓ Sonstiges: Stellungnahme Prüfer	14.09.2017
Dokumente eingegangen am 02.10.2017, begutachtet im 'expedited Review' am 24.10.2017	
✓ Sonstiges: Arbeitsaufteilung undatiert	
✓ Sonstiges: E-Mail Stellungnahme zur Bearbeitungsmitteilung	02.10.2017
Dokumente eingegangen am 27.10.2017 (in der nächsten Begutachtung mitbegutachtet)	
✓ Antragsformular ECS Unterschriftenseiten	09.10.2017
Dokumente eingegangen am 08.03.2018 (in der nächsten Begutachtung mitbegutachtet)	
✓ Originalprotokoll 3	05.03.2018
✓ Informed Consent Form 3	05.03.2018
✓ Informed Consent Form MRT Untersuchung 1	05.03.2018
✓ CV Mitarbeiter Sourij	21.11.2017
✓ CV Mitarbeiter Opriessnig	20.02.2018
✓ CV Mitarbeiter Deutschmann	27.02.2018
✓ CV Mitarbeiter Dimsity	18.10.2017
✓ CV Mitarbeiter Brodmann	20.09.2017
✓ EudraCT Formular (CT1)	07.03.2018
✓ Sonstiges: Übersicht der Blutabnahmen undatiert	
✓ Sonstiges: Stellungnahme zur Bearbeitungsmitteilung 1	05.03.2018
Dokumente eingegangen am 20.03.2018, begutachtet im 'expedited Review' am 06.04.2018	
✓ Versicherungsbestätigung Wiener Städtische 08-N811.957	20.03.2018

Die Ethikkommission geht – rechtlich unverbindlich – davon aus, dass es sich um eine klinische Prüfung nach AMG handelt und macht darauf aufmerksam, dass vor Beginn der Prüfung ein ordnungsgemäßer Antrag auf Genehmigung an das Bundesamt für Sicherheit im Gesundheitswesen zu stellen ist.

Es handelt sich um eine Studie im Rahmen einer Dissertation.

Das Votum der Ethikkommission berührt in keiner Weise die alleinige Verantwortung der Prüferin / des Prüfers / der Prüfer für die ordnungsgemäße Durchführung der Studie unter Einhaltung aller einschlägiger gesetzlicher Bestimmungen und Richtlinien.

Weiters machen wir darauf aufmerksam, dass der Kommission unverzüglich zu melden sind:

- Abweichungen vom Protokoll aus Sicherheitsgründen oder Protokolländerungen

- Änderungen, die das Risiko der Teilnehmer/-innen erhöhen oder die Durchführung der Studie wesentlich beeinflussen

- Mutmaßliche unerwartete schwerwiegende Nebenwirkungen - SUSARs (AMG-Studien ab 1.5.2004) oder schwerwiegende unerwünschte Ereignisse - SAEs (andere Studien)

- Jegliche Information über sonstige Umstände, die die Sicherheit der Teilnehmer/-innen oder die Durchführung der Studie beeinträchtigen können

Begründung: Es handelt sich um eine relevante Fragestellung, die mit geeigneter Methodik beantwortet werden soll. Die vom Antragsteller vorgenommene Bewertung des Nutzen/Risiko-Verhältnisses ist plausibel.

Dieses Votum gilt für ein Jahr ab dem Datum der Ausstellung. Bei längerer Studiendauer ist rechtzeitig vor Ablauf der Gültigkeit des Votums ein Zwischenbericht vorzulegen (Berichtsformular), um eine etwaige

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Verlängerung zu erlangen.

Graz, 06. April 2018

Univ.Prof.DI Dr.Josef Haas
Vorsitzender

Univ.Prof.Dr.Hermann Toplak
Stv. Vorsitzender

Achtung: Bitte bei allen das Projekt betreffende Schreiben oder telefonischen Anfragen die EK-Nummer angeben!

6.2 Regulatory Authority Approval

Bundesamt für Sicherheit im Gesundheitswesen

BASG/AGES
Institute Surveillance
Traisengasse 5, 1200 Vienna

Med. Univ. Graz, Univ.-Klinik f. Innere Medizin,
Abt. f. Angiologie
Priska Hirschmann
Auenbruggerplatz 15
8036 Graz
Österreich

date: 29.03.2018
department: clinical trials - CLTR
phone: +43(0)5 0555 36663
e-mail: clinicaltrials@ages.at
reference: 10765717

Betreff / *RE:* Vollständigkeitsbestätigung, *Confirmation of Formal Completeness*

EudraCT Nr.: 2018-000981-12

Sehr geehrte(r) Antragsteller(in)!

Das Bundesamt für Sicherheit im Gesundheitswesen bestätigt hiermit die formale Vollständigkeit des oben genannten Genehmigungsantrages zur Durchführung einer klinischen Prüfung gemäß § 40 Arzneimittelgesetz (AMG) in der geltenden Fassung mit **27.03.2018**. Die formale Vollständigkeit schließt inhaltliche Nachforderungen nicht aus.

Ihr Antrag gilt als genehmigt, wenn innerhalb von 35 Kalendertagen kein Einwand seitens der Behörde erhoben wird. Die Frist beginnt am Stichtag der Vollständigkeit.

Die klinische Arzneimittelprüfung kann nur begonnen werden, wenn seitens des Bundesamtes keine Einwände erhoben wurden sowie ein positives Votum der zuständigen Ethikkommission vorliegt.

Bei Kombinationsstudien (Studien gemäß Arzneimittelgesetz und Medizinproduktegesetz) sind neben dem Genehmigungsverfahren nach AMG auch die Vorraussetzungen für die Durchführung einer klinischen Prüfung nach MPG zu beachten. Dieses Dokument gilt nicht als Bestätigung der ordnungsgemäßen Einreichung einer Klinischen Prüfung nach Medizinproduktegesetz.

Dear Applicant!

The Austrian Competent Authority herewith confirms the formal completeness of the submitted Clinical Trial Application according to § 40 Austrian Medicinal Products Act, as amended, per **27.03.2018**. *Formal completeness does not preclude later requests for content.*

Your application will be considered approved, if no objections are raised within 35 calender days, starting with the date of formal completeness.

The Clinical Trial can only be initiated if no objections have been raised by the Authority and the concerned Ethics Committee has reached a positive opinion regarding the trial.

Combination studies (investigation of medicinal products and medical devices in the same trial) need to fulfill the requirements of both the Austrian Medicinal Products Act and the Austrian Medical Devices Act.

Bundesamt für Sicherheit
im Gesundheitswesen

BASG/AGES
Institute Surveillance
Traisengasse 5, 1200 Vienna

This document should not be treated as confirmation of formal completeness according to the Austrian Medical Devices Act.

Für das Bundesamt:

On behalf of the Competent Authority:

Katharina Moser

Moser Katharina
am 29.3.2018

BUNDESAMT FÜR SICHERHEIT IM GESUNDHEITSWESEN @ AMTSSIGNATUR	Dieses Dokument wurde amtssigniert. Informationen zur Prüfung der elektronischen Signatur und des Ausdrucks finden Sie unter http://www.basg.gv.at/amtssignatur. Bundesamt für Sicherheit im Gesundheitswesen Traisengasse 5, 1200 Wien	
	Signaturwert	TDstfi/czIbsmu0TGhtaw5DfiWIk glfpbldAggs0fbwu/5Av5rBisc/Dc ÄDnGBicmilecpcDvPhscg zfS0dTgPlGwsloGSa0ps1ruwolzBA aAde/f5seWTfG2WDs2BAilD5nb TkaISrml2gkihpzvrA5drevr rnGi51kP2cmhasBaf0Gfw/td

6.3 Health Insurance Prescription Guideline for Alirocumab (Original)

Praluent® (Alirocumab), Regeltext für den Gelben Bereich / RE1

Änderungen des Regeltextes wurden farblich markiert.

Ab 01. Juli 2016:

Bei primärer Hypercholesterinämie zur Sekundärprävention nach einem akuten atherosklerotisch bedingten, ischämischen kardiovaskulären Ereignis bei PatientInnen mit diagnostisch gesicherter koronarer Herzkrankheit und/oder peripherer arterieller Verschlusskrankheit und/oder zerebraler arterieller Verschlusskrankheit

- wenn aufgrund des sehr hohen kardiovaskulären Risikos (größer gleich 10 % gemäß ESC-Leitlinie) eine zusätzliche Senkung von LDL-C medizinisch erforderlich ist,
und
- wenn eine professionelle Ernährungsberatung erfolgt, der arterielle Blutdruck kontrolliert und der Blutzucker auf ein HbA1c kleiner 7.5 % eingestellt ist sowie eine Tabakrauchabstinenz angestrebt wird,
und
-wenn über mindestens 3 Monate mit der maximal verträglichen Dosierung einer intensivierten LDL-
C senkenden Therapie mit Atorvastatin bzw. Rosuvastatin, jeweils in Kombination mit Ezetimib (oder Ezetimib mit oder ohne Colesevelam bei Statinunverträglichkeit) ein LDL-Wert von kleiner als 100 mg/dl nicht erreicht werden kann, oder wenn diese Behandlungen kontraindiziert sind.

Eine Unverträglichkeit gegenüber Statinen gilt jedenfalls als belegt, wenn Therapieversuche mit mehreren Statinen - jedenfalls auch Atorvastatin und Rosuvastatin - zu Myalgien und einem Anstieg der Kreatinin-Kinase auf mindestens das Fünffache des oberen Normwertes führten oder wenn durch ein Statin eine schwere Hepatopathie aufgetreten ist.
Diagnose und Erstverordnung durch ein spezialisiertes Zentrum von einem Facharzt/eine Fachärztin für Innere Medizin mit dem Additivfach Endokrinologie und Stoffwechselerkrankungen.
Die Liste der für die Erstverordnung in Frage kommenden Einrichtungen wird vom Hauptverband
erstellt und unter [www.hauptverband.at] publiziert.

Regelmäßige Kontrollen durch entsprechende Fachabteilung bzw. Zentrum bzw. durch einen/eine in der Therapie von Fettstoffwechselstörungen erfahrenen/erfahrene Facharzt/Fachärztin.
Die Behandlung mit Alirocumab kann nur fortgesetzt werden, wenn bei einer Laborkontrolle 2 – 3 Monate nach Behandlungsbeginn das LDL-C gegenüber dem Ausgangswert unter der maximal intensivierten lipidsenkenden Therapie um mindestens 40 % gesunken ist bzw. ein LDL-Wert von kleiner als 70 mg/dl erreicht wurde.
Die Aufnahme in den EKO ist befristet und endet mit 30. Juni 2017.

Ab 01. Juli 2017:

Bei primärer Hypercholesterinämie zur Sekundärprävention nach einem akuten atherosklerotisch bedingten, ischämischen kardiovaskulären Ereignis bei PatientInnen mit diagnostisch gesicherter koronarer Herzkrankheit und/oder peripherer arterieller Verschlusskrankheit und/oder zerebraler arterieller Verschlusskrankheit

- wenn aufgrund des **sehr hohen kardiovaskulären Risikos eine zusätzliche Senkung von LDL-C medizinisch erforderlich** ist,

und

- wenn eine professionelle Ernährungsberatung erfolgt, der arterielle Blutdruck kontrolliert und der Blutzucker auf ein HbA1c kleiner **8 %** eingestellt ist sowie eine Tabakrauchabstinenz angestrebt wird, und

- wenn über mindestens 3 Monate mit der maximal verträglichen Dosierung einer intensivierten LDL-C senkenden Therapie mit Atorvastatin bzw. Rosuvastatin, jeweils in Kombination mit Ezetimib (oder Ezetimib mit oder ohne Colesevelam bei Statinunverträglichkeit) ein LDL-Wert von kleiner als 100 mg/dl nicht erreicht werden kann, oder wenn diese Behandlungen kontraindiziert sind.

Eine Unverträglichkeit gegenüber Statinen gilt jedenfalls als belegt, wenn Therapieversuche mit mehreren Statinen - jedenfalls auch Atorvastatin und Rosuvastatin - zu Myopathien und einem Anstieg der Kreatinin-Kinase auf mindestens das Fünffache des oberen Normwertes führten oder wenn durch ein Statin eine schwere Hepatopathie aufgetreten ist. Diagnose, **Erhebung der Familienanamnese** und Erstverordnung durch ein spezialisiertes Zentrum von einem Facharzt/einer Fachärztin für Innere Medizin mit dem Additivfach Endokrinologie und Stoffwechselerkrankungen.

Die Liste der für die Erstverordnung in Frage kommenden Einrichtungen wird vom Hauptverband erstellt und unter [www.hauptverband.at] publiziert. Regelmäßige Kontrollen durch entsprechende Fachabteilung bzw. Zentrum bzw. durch einen/eine in der Therapie von Fettstoffwechselstörungen erfahrenen/erfahrene Facharzt/Fachärztin. Die Behandlung mit Alirocumab kann nur fortgesetzt werden, wenn bei einer Laborkontrolle 2 – 3 Monate nach Behandlungsbeginn das LDL-C gegenüber dem Ausgangswert unter der maximal intensivierten lipidsenkenden Therapie um mindestens 40 % gesunken ist bzw. ein LDL-Wert von kleiner als 70 mg/dl erreicht wurde.

Die Aufnahme in den EKO ist befristet und endet mit 30.6.2018.

Ab 01. Juli 2018:

Bei primärer Hypercholesterinämie zur Sekundärprävention nach einem akuten atherosklerotisch bedingten, ischämischen kardiovaskulären Ereignis bei PatientInnen mit diagnostisch gesicherter koronarer Herzkrankheit und/oder peripherer arterieller Verschlusskrankheit und/oder zerebraler arterieller Verschlusskrankheit

- wenn aufgrund des sehr hohen kardiovaskulären Risikos eine zusätzliche Senkung von LDL-C medizinisch erforderlich ist,

und

- wenn eine professionelle Ernährungsberatung erfolgt, der arterielle Blutdruck kontrolliert und der Blutzucker auf ein HbA1c kleiner 8 % eingestellt ist sowie eine Tabakrauchabstinenz angestrebt wird,

und

- wenn über mindestens 3 Monate mit der maximal verträglichen Dosierung einer intensivierten LDL-C senkenden Therapie mit Atorvastatin bzw. Rosuvastatin, jeweils in Kombination mit Ezetimib (oder Ezetimib mit oder ohne Colesevelam bei Statinunverträglichkeit) ein LDL-Wert von kleiner als 100 mg/dl nicht erreicht werden kann, oder wenn diese Behandlungen kontraindiziert sind.

Eine Unverträglichkeit gegenüber Statinen gilt jedenfalls als belegt, wenn Therapieversuche mit mehreren Statinen - jedenfalls auch Atorvastatin und Rosuvastatin - zu Myopathien und einem Anstieg der Kreatinin-Kinase auf mindestens das Fünffache des oberen Normwertes führten oder wenn durch ein Statin eine schwere Hepatopathie aufgetreten ist.

Diagnose, Erhebung der Familienanamnese und Erstverordnung durch ein spezialisiertes Zentrum von einem Facharzt/einer Fachärztin für Innere Medizin mit dem Additivfach Endokrinologie und Stoffwechselerkrankungen. Die Liste der für die Erstverordnung in Frage kommenden Einrichtungen wird vom Hauptverband erstellt und unter **www.hauptverband.at/erstattungskodex_stoffwechsel** publiziert. Regelmäßige Kontrollen durch entsprechende Fachabteilung bzw. Zentrum bzw. durch einen/eine in der Therapie von Fettstoffwechselstörungen erfahrenen/erfahrene Facharzt/Fachärztin.

Die Behandlung mit Alirocumab kann nur fortgesetzt werden, wenn bei einer Laborkontrolle 2 – 3 Monate nach Behandlungsbeginn das LDL-C gegenüber dem Ausgangswert unter der maximal intensivierten lipidsenkenden Therapie um mindestens 40 % gesunken ist bzw. ein LDL-Wert von kleiner als 70 mg/dl erreicht wurde.

Die Aufnahme in den EKO ist befristet und endet mit 30.6.2019.

Kommentar: Die Erstattung von Praluent® (Alirocumab) war in den Monaten Juli und August 2019 nicht im Rahmen des Gelben Bereiches / RE1 möglich.

Ab 01. September 2019:

Bei primärer Hypercholesterinämie zur Sekundärprävention nach einem akuten atherosklerotisch bedingten, ischämischen kardiovaskulären Ereignis bei PatientInnen mit diagnostisch gesicherter koronarer Herzkrankheit und/oder peripherer arterieller Verschlusskrankheit und/oder zerebraler arterieller Verschlusskrankheit

- wenn aufgrund des sehr hohen kardiovaskulären Risikos eine zusätzliche Senkung von LDL-C medizinisch erforderlich ist, und

- wenn eine professionelle Ernährungsberatung erfolgt, der arterielle Blutdruck kontrolliert und der Blutzucker auf ein HbA1c kleiner 8 % eingestellt ist sowie eine Tabakrauchabstinenz angestrebt wird, und

- wenn über mindestens 3 Monate mit der maximal verträglichen Dosierung einer intensivierten LDL-C senkenden Therapie mit Atorvastatin bzw. Rosuvastatin, jeweils in Kombination mit Ezetimib (oder Ezetimib mit oder ohne Colesevelam bei Statinunverträglichkeit) ein LDL-Wert von kleiner als 100 mg/dl nicht erreicht werden kann, oder wenn diese Behandlungen kontraindiziert sind.

Eine Unverträglichkeit gegenüber Statinen gilt jedenfalls als belegt, wenn Therapieversuche mit mehreren Statinen - jedenfalls auch Atorvastatin und Rosuvastatin - zu Myopathien und einem Anstieg der Kreatinin-Kinase auf mindestens das Fünffache des oberen Normwertes führten oder wenn durch ein Statin eine schwere Hepatopathie aufgetreten ist.

Diagnose, Erhebung der Familienanamnese und Erstverordnung durch ein spezialisiertes Zentrum von einem Facharzt/einer Fachärztin für Innere Medizin mit dem Additivfach Endokrinologie und Stoffwechselerkrankungen. Die Liste der für die Erstverordnung in Frage kommenden Einrichtungen wird vom Hauptverband erstellt und unter www.hauptverband.at/erstattungskodex_stoffwechsel publiziert.

Regelmäßige Kontrollen durch entsprechende Fachabteilung bzw. Zentrum bzw. durch einen/eine in der Therapie von Fettstoffwechselstörungen erfahrenen/erfahrene Facharzt/Fachärztin. Die Behandlung mit Alirocumab kann nur fortgesetzt werden, wenn bei einer Laborkontrolle 2 - 3 Monate nach Behandlungsbeginn das LDL-C gegenüber dem Ausgangswert unter der maximal intensivierten lipidsenkenden Therapie um mindestens 40 % gesunken ist bzw. ein LDL-Wert von kleiner als 70 mg/dl erreicht wurde.

Kommentar: Die zeitliche Befristung des Regeltextes wurde entfernt.

6.4 Case Report Form (Example)

EudraCT Study Code: 2018-000981-12	Patient Number:		Subject initials: (First-/Last-Name)	

CASE REPORT FORM

STUDY NAME: ALIROCKS
EudraCT number: 2018-000981-12

CLINICAL TRIAL SITE/UNIT:	Clinical Department of Angiology
PRINCIPAL INVESTIGATOR:	Günther Silbernagel

Subject Initials (First- /Last Name):	
Patient Number:	

I am confident that the information supplied in this case record form is complete and accurate data. I confirm that the study was conducted in accordance with the protocol and any protocol amendments and that written informed consent was obtained prior to the study.

Investigator's Signature: .....

Date of signature: | | | | | | | | | |
D d m m m y y y y

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CRF Version 1, 18. JUNE 2018, ALIROCKS Study

EudraCT Study Code: 2018-000981-12	Patient Number:		Subject initials: (First-/Last-Name)	

Screening

INFORMED CONSENT

Please note: written informed consent must be given before any study specific procedures take place or any current therapy is discontinued for the purposes of participation in this study.

Has the subject freely given written informed consent? Yes ☐ No ☐

Date (signed): _____ (DD MMM YYYY)

DEMOGRAPHIC DATA

Age (yrs): ☐☐ Sex: Female ☐ Male ☐

Inclusion Criteria	**Yes**	**No***
1 Patient scheduled for treatment with Alirocumab (Praluent®) in clinical routine (After approval of cost coverage by insurance company)?	☐	☐
2 Has the subject willingly given written informed consent?	☐	☐
3 No previous treatment with PCSK9 antibodies?	☐	☐

*If any inclusion criteria are ticked no then the patient is not eligible for the study.

Exclusion Criteria	**Yes***	**No**
1 Patient's age is below 18 years?	☐	☐
2 Pregnancy (Pregnancy test at screening visit negative)?	☐	☐
3 Breast-feeding?	☐	☐
4 Impossibility to perform magnetic resonance imaging of the carotid artery? (e.g. Claustrophobia, carotid stent)	☐	☐

* If any exclusion criteria are ticked yes then the patient is not eligible for the study.

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EudraCT Study Code: 2018-000981-12	Patient Number:		Subject initials: (First-/Last-Name)	

VISIT 1 (Baseline):

Date: _____
DD MMM YYYY

A. Concomitant Diseases (Baseline):

SMOKING HABITS

Current or former smoker? *Yes ☐ No ☐

* How many cigarettes per day and years?

Per day: ____ For how many years (average): ____

Specify, duration and substance (approx.): ----------

A cigarillo is 2 cigarettes and a cigar is 4 cigarettes. Loose tobacco estimates that 25 grams (1 oz) has been approximated to be the equivalent of 50 cigarettes. One pipe is equivalent to 2½ cigarettes. Smoking pack years: One pack year is equivalent to 20 cigarettes smoked daily for one year.

ALCOHOL CONSUMPTION

Does the subject consume alcohol? *Yes ☐ No ☐

*If yes, how many units per week and years?

Per week: ____ For how many years (average): ____

Is there any relevant concomitant disease in the following systems?

Code	System	*Yes	No		Code	System	*Yes	No
1	Dyslipidemia				9	Neoplasia		
2	Cardiovascular				10	Neurological		
3	Endocrine				11	Psychological		
4	Gastro-intestinal				12	Immunological		
5	Genito-urinary				13	Dermatological		
6	Respiratory				14	Allergies		
7	Haematological				15	Eyes, ear, nose, throat		
8	Musculo-skeletal				00	Other		

If *YES for any of the above, enter the code for each condition in the boxes below and give further details (including dates).

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Concomitant Diseases – Details:	
Code	Details (including dates)

B. Concomitant Medication (Baseline):

Dosage and Units needed only when ***Disease Code 1*** (Dyslipidemia)

Medication	Total Daily Dose	Units	Disease Code	Start Date *(MM/DD/YYYY)*	Stop Date *(MM/DD/YYYY)*	Continuing
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐

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C. Medical History of Cardio-Vascular Events / Interventions (Baseline):

Date (MM/DD/YYYY)	Details

D. Laboratory Values (Baseline):

☐ Yes, the laboratory value reports are filed in the medical history folder (source data) AND in the patients study folder.

E. Brachial Artery Flow-Mediated-Dilatation (FMD) and Carotid Intima-Media-Thickness (IMT) at Baseline:

Date: _____
DD MMM YYYY

	Value	Unit
Mean Brachial Artery FMD:		
Mean Carotid IMT:		

F. Cardiovascular Magnetic Resonance (CMR) in Carotid Arteries at Baseline:

Date: _____
DD MMM YYYY

	Value	Unit
Mean Carotid Vessel Wall Fractional Anisotropy:		

EudraCT Study Code: 2018-000981-12	Patient Number:		Subject initials: (First-/Last-Name)	

G. Determination of Post-Prandial Lipemia at Baseline (Optional)*: Only if the patient is willing to participate and informed consent form is signed accordingly.

☐ Yes, the laboratory value reports per time points are filed in the medical history folder (source data) AND in the patients study folder.

☐ Not applicable. The patient does not participate in the fat-tolerance test.

H. First Praluent® (Alirocumab) Injection and Dosage:

Date: _____ (DD MMM YYYY) **Dosage:** _____ **mg**

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VISIT 2 (Week 10):

Date: _____ (DD MMM YYYY)

A. Changes in Concomitant Diseases (Week 10):

Concomitant Diseases Details	
Code	Details (including dates)

EudraCT Study Code: 2018-000981-12	Patient Number:		Subject initials: (First-/Last-Name)	

B. Changes in Concomitant Medication (Week 10):

Dosage and Units needed only when ***Disease Code 1*** (Dyslipidemia)

Medication	Total Daily Dose	Units	Disease Code	Start Date *(MM/DD/YYYY)*	Stop Date *(MM/DD/YYYY)*	Continuing
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐
				__/__/____	__/__/____	☐

C. Changes in Medical History of Cardio-Vascular Events / Interventions (Week 10):

Date *(MM/DD/YYYY)*	Details

D. Laboratory Values (Week 10):

☐ Yes, the laboratory value reports are filed in the medical history folder (source data) AND in the patients study folder.

EudraCT Study Code: 2018-000981-12	Patient Number:		Subject initials: (First-/Last-Name)	

E. Brachial Artery Flow-Mediated-Dilatation (FMD) and Carotid Intima-Media-Thickness (IMT) at Week 10:

Date: _____
DD MMM YYYY

	Value	Unit
Mean Brachial Artery FMD:		
Mean Carotid IMT:		

F. Cardiovascular Magnetic Resonance (CMR) in Carotid Arteries at Week 10:

Date: _____
DD MMM YYYY

	Value	Unit
Mean Carotid Vessel Wall Fractional Anisotropy		

G. Determination of Post-Prandial Lipemia at Week 10 (Optional)*: Only if the patient is willing to participate and informed consent form is signed accordingly.

☐ Yes, the laboratory value reports per time points are filed in the medical history folder (source data) AND in the patients study folder.

☐ Not applicable. The patient does not participate in the fat-tolerance test

H. Last Praluent® (Alirocumab) Injection and Dosage before Visit 2 (Week 10):

Date: _____ **Dosage:** _____ **mg**
DD MMM YYYY

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EudraCT Study Code: 2018-000981-12	Patient Number:				Subject initials: (First-/Last-Name)		

Monitoring:

Source Date Verification (SDV) has been completed for:

Tick box below:

☐ Informed Consent Forms

☐ Study Inclusion and Exclusion Criteria

☐ Mean carotid vessel wall fractional anisotropy. (Primary Endpoint)

☐ Brachial Artery Flow-Mediated-Dilatation (FMD)

Monitor's Signature: ----------

Date of signature:

D	d	m	m	m	y	y	y	y