General introduction

Cardiovascular diseases (CVD) are the leading cause of death worldwide. Whereas the precise etiology of atherosclerosis, the underlying pathology of CVD, is not fully understood, the risk factors of developing CVD are well-known. The most important non-modifiable risk factors are age, gender, and genetics. Modifiable risk factors associated with CVD are unfavorable lifestyle parameters (e.g., smoking, lack of physical activity, and low intake of fruits and vegetables), diabetes, abdominal obesity, hypertension and increased cholesterol levels. The INTERHEART study showed that modifiable risk factors account for most of the risk of myocardial infarction and abnormal blood lipids were identified as the most important risk factors. Several lipid abnormalities can be discriminated: increased total cholesterol, low-density lipoprotein cholesterol (LDL-c), triglycerides or decreased high-density lipoprotein cholesterol (HDL-c). In human blood, cholesterol is primarily transported in LDL but also in HDL. Increased plasma LDL-c levels are causally related to the pathogenesis of atherosclerosis. After damage to vascular endothelium – for example because of exposure to classical risk factors – circulating monocytes accumulate at the site of the injury and invade the vascular wall. In the intima, the excessive uptake of LDL in monocyte-derived macrophages leads to the formation of lipid-laden macrophages (foam cells). These foam cells form the culprit of early atherosclerotic lesions known as fatty streaks and ultimately of vulnerable plaques that are prone to rupture. Atherothrombotic events ultimately underlie ischemic events in the heart and brain.

In contrast to LDL-c, HDL-c levels are inversely related to CVD and it is generally assumed that this is related to HDL mediated transport of cholesterol from the lipid-laden vascular wall to the liver for excretion (a process known as reverse cholesterol transport). Unfortunately, pharmaceutical interventions to modulate HDL metabolism to decrease atherosclerosis have failed. This is not described in the context of this thesis but elsewhere (for a recent review, see Balder et al).

This first chapter provides a short summary of LDL biology and an update on pharmaceutical means to modulate cholesterol levels in LDL in humans.

Low-density lipoproteins (LDL)

Metabolism
The liver is the central organ in cholesterol metabolism. Both dietary and de novo synthesized cholesterol and triglycerides are packaged in apolipoprotein (apo) B containing very-low density lipoproteins (VLDL) that are secreted into the circulation. In peripheral tissues such as the heart and skeletal muscle, the triglycerides in VLDL are hydrolyzed by the action of lipoprotein lipase (LPL), where the released fatty acids can serve as energy source whereas in adipose tissue the fatty acids can be stored for later use. The lipolysis of triglycerides in VLDL

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renders smaller particles with higher density such as intermediate-density lipoprotein and ultimately cholesterol rich LDL. The latter are subsequently taken up by the liver through the action of the low-density lipoprotein receptor (LDLR). High plasma levels of LDL-c are associated with the accumulation of LDL in monocyte derived macrophages (resulting in the formation of foam cells) in the vascular wall, the culprit of atherosclerotic lesions.

**Genetics**

Over the years, genetic research has helped resolving why humans can present with either very high LDL-c or very low LDL-c in blood. This research has helped us to understand LDL metabolism but importantly also to provide pharmaceutical tools to modulate cholesterol levels and reduce CVD risk. Besides extremely low and high LDL-c levels, other types of hyperlipidemia have been reported as well, for example familial hyperchylomicronemia (increase in chylomicrons) and dysbetalipoproteinemia (increase in IDL). However, the paragraphs given below will focus on the (genetic) causes of low and high LDL-c levels only.

**Monogenic hypercholesterolemia**

Hypercholesterolemia can be caused by mutations in single genes. Mutations in LDLR can be categorized into four functional classes: 1) defective synthesis; 2) transport defective; 3) binding defective; and 4) internalization defective. More than 1,200 mutations in LDLR have been documented, of which almost 80% were expected to be functional. Another gene associated with hypercholesterolemia is APOB. Mutations in the LDLR-binding region can reduce LDL clearance from the circulation. Before the next-generation sequencing era, studying APOB was restricted to only the exons that were known to encode for the domain of APOB known to interact with the LDLR. The most frequently reported mutation is Arg3500Gln. However, several functional mutations outside the LDLR-binding region have also been reported to cause hypercholesterolemia. The third gene associated with hypercholesterolemia is proprotein convertase subtilisin/kexin type 9 (PCSK9). When PCSK9 is bound to LDLR, the receptor is degraded – instead of recycled to the cell membrane – upon endocytosis of LDL. As a consequence, the number of LDLRs present on the cell surface is reduced leading to a reduction of the cellular uptake of LDL. So indirectly, gain-of-function mutations in PCSK9 increase plasma cholesterol levels. Together LDLR, APOB and PCSK9 are candidate genes for autosomal dominant familial hypercholesterolemia (FH). Most individuals with heterozygous FH carry mutations in LDLR (~90%), while in approximately 5% functional variants are detected in APOB. Functional mutations in PCSK9 are only present in 1% of the target populations. These prevalence rates vary geographically.

FH is the most common monogenic disease in men. The frequency of FH in the general population is expected to be approximately 1 in 200 to 1 in 250. FH is characterized by increased LDL-c levels, familial predisposition for CVD, premature atherosclerosis, and ultimately cholesterol rich LDL. The latter are subsequently taken up by the liver through the action of the low-density lipoprotein receptor (LDLR). High plasma levels of LDL-c are associated with the accumulation of LDL in monocyte derived macrophages (resulting in the formation of foam cells) in the vascular wall, the culprit of atherosclerotic lesions.

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cutaneous deposition of yellowish cholesterol-rich material (tendon xanthomas and xanthelasma). There is also one known cause of autosomal recessive hypercholesterolemia which is the result of mutations in low-density lipoprotein receptor adaptor protein 1 (LDLRAP1). This mutation is extremely rare. In 10 – 40% of individuals with a clinical diagnosis of FH, no causative mutation can be identified. This leaves room for other studies to explore novel genes to be involved in FH.

Polygenic hypercholesterolemia
Increased LDL-c levels can also have a polygenic origin. In such cases the increased LDL-c levels are not caused by high impact rare mutations, but because of a combination of several small impact genetic variants each contributing to elevated LDL-c levels. It has been reported that approximately 13% of individuals with severe hypercholesterolemia may have a polygenic origin.

Hypocholesterolemia
Several diseases are characterized by hypocholesterolemia. Familial hypobetalipoproteinemia is a monogenic disorder characterized by reduced plasma LDL-c levels and therefore longevity syndrome. Loss-of-function mutations in APOB or PCSK9 are known to cause familial hypobetalipoproteinemia. Complete deficiency of APOB, in patients with abetalipoproteinemia, leads to the inability to synthesize chylomicrons and VLDL. Besides very low LDL-c levels, patients with this disease suffer from fat-soluble vitamins deficiency, developmental delay and gastrointestinal symptoms. Besides mutations in APOB, abetalipoproteinemia can also be the consequence of deleterious mutations in the gene encoding for microsomal triglyceride transfer protein (MTTP). MTTP is important for the assembly of apoB-containing particles.

Another disease characterized by low LDL-c levels is combined hypolipidemia. Besides low LDL-c levels, HDL-c and triglyceride levels are decreased as well. This disease is caused by mutations in angiopoietin-like 3 (ANGPTL3). Two family members with combined hypolipidemia were found to carry two distinct mutations in ANGPTL3. ANGPTL3 inhibits lipoprotein and endothelial lipases and thereby increases triglycerides and HDL-c.

Secondary causes of increased LDL-c
Many patients at increased risk for CVD present with increased LDL-c levels in combination with increased triglycerides and reduced HDL-c for which the etiology is not clear but related to combination of genetic as well as environmental factors. Several known secondary causes of elevated LDL-c include type 2 diabetes mellitus, hypothyroidism, chronic kidney disease, and cholestatic liver disease.
Pharmaceutical interventions to reduce LDL-c

HMG-CoA reductase inhibitors

HMG-CoA reductase inhibitors or statins are widely used drugs to reduce LDL-c in primary and secondary prevention. Statins increase hepatic LDLR expression which results in LDL-c lowering in plasma. Statins can (depending on the type and dosage) reduce LDL-c with 10 – 60%. Meta-analyses have shown that statins reduce all-cause mortality, and combined fatal and non-fatal CVD endpoints. These trials have shown that (1) low-cost statin treatment reduces cholesterol by more than 2.0 mmol/l (if LDL-c ≥ 4.0 mmol/l), (2) each 1.0 mmol/l reduction in LDL-c is associated with a proportional reduction of about 25% in the rate of cardiovascular events, irrespective of the LDL-c at presentation; and (3) lowering LDL-c by 2.0 mmol/l for 5 years in 1000 patients will prevent 100 (10%) cardiovascular events in patients at high risk of CVD, and 50 (5%) cardiovascular events in patients at lower risk.

Overall, statins are safe, but some side effects may occur, including myopathy (common), hepatic dysfunction (rare), and new-onset diabetes mellitus (rare). Approximately 10 – 20 individuals treated per 1000 patients for five years reported to suffer from side effects. Of note, the harmful effects of statin therapy can usually be reversed by discontinuation of statin use, whereas the consequences of cardiovascular events are devastating and irreversible.

Ezetimibe

Ezetimibe decreases the absorption of dietary and biliary cholesterol in the intestine. Its mechanism involves inhibition of Niemann-Pick C1 like 1 protein. Ezetimibe is mostly used when cholesterol targets are not met with statins but the drug can also be used in patients who do not tolerate statins. Daily ezetimibe at a dose of 10 mg lowers LDL-c by approximately 17%, and on top of statin treatment ezetimibe can lower LDL-c by 14%. The IMPROVE-IT trial showed that ezetimibe combined with statin treatment, is more effective in reducing cardiovascular risk, compared to statin treatment alone in individuals with acute coronary syndrome. In this trial, and others as well, ezetimibe was well-tolerated.

Proprotein convertase subtilisin/kexin type 9 inhibitors

Since the discovery that gain-of-function mutations in PCSK9 cause FH, and that individuals with loss-of-function mutations in PCSK9 are protected from CVD due to lifelong low LDL-c levels, PCSK9 inhibitors have been rising stars. PCSK9 is produced in the liver and is involved in the recycling of LDLR. In the presence of PCSK9, LDLR is broken down. If PCSK9 is inhibited through e.g. monoclonal antibodies, this results in the presence of more LDLR at the cell surface of hepatocytes which results in lowering plasma LDL-c levels. PCSK9-inhibitors can lower LDL-c by 60% if used on top of maximum statin therapy. A meta-analysis of 24 trials showed that PCSK9-inhibitors reduce all-cause mortality and cardiovascular mortality.
A downside of PCSK9-inhibitors to date are the high costs.37 As the monoclonal antibodies are subcutaneously injected this can lead to unwanted injection site reactions, neurocognitive events, and ophthalmologic events.36 Since 2015, two PCSK9-inhibitors (alirocumab and evolocumab) were approved by the U.S. Food and Drug Administration. PCSK9 inhibitors can be prescribed to patients at high risk of CVD who do not reach therapeutic targets on maximally tolerated statin therapy and ezetimibe, or if in case of statin intolerance.39, 40 Although these data are very promising, long-term follow up data on efficacy and safety are still required.

Instead of using monoclonal antibodies against PCSK9, PCSK9 levels can also be reduced by means of small interfering (si)RNA molecules. siRNAs facilitate the PCSK9 mRNA degradation, which ultimately results in lower levels of PCSK9 protein. A single injection of inclisiran significantly reduced PCSK9 and LDL-c levels, after 84 days.41 Inclisiran was furthermore tested in a phase II randomized controlled trial (ORION-1 study; n = 501). After 180 days inclisiran was found to lower plasma LDL-c levels and PCSK9 levels.42 Further long-term results are awaited (ORION-3 study; ClinicalTrials.gov Identifier: NCT03060577).

Mipomersen
Mipomersen is an antisense oligonucleotide complementary to coding regions of APOB and results in reduced VLDL production by the liver. Because LDLRs are not involved in the working mechanism, this antisense treatment is especially of interest for patients suffering from for example LDLR null mutations meaning that they are not able to produce LDLRs (and thus not responsive to statin therapy). Analyzing data from randomized controlled trials showed that LDL-c levels were reduced by 25 – 39% on maximally tolerated statin treatment. The most common side effects like injection site reactions, flu-like symptoms and increased liver enzyme levels are common, leading to a high discontinuation rate.43, 44 Concomitant with blocking of VLDL production, an increased risk of liver steatosis is unwanted side-effect of this drug.45

Microsomal triglyceride transfer protein inhibitors
Microsomal triglyceride transfer protein (MTTP) is an important protein for the synthesis of apoB-containing lipoproteins in the liver and small intestine. Lomitapide inhibits MTTP leading to a reduction of lipoproteins in blood. In 29 patients with homozygous FH, LDL-c was reduced by 50% and triglycerides were reduced by 65%. The most common side effect was severe diarrhea, and the use of a specific low lipid diet during treatment is mandatory. Four patients had increased liver enzymes of more than five times upper limit of normal.46 For patients with extremely high LDL-c levels, who are statin intolerant, lomitapide could be a useful addition to lipid-lowering therapy. However, these patients should be closely monitored because of possible liver steatosis.47 To overcome the hepatotoxicity, two novel PCSK9 inhibitors can be prescribed to patients at high risk of CVD who do not reach therapeutic targets on maximally tolerated statin therapy and ezetimibe, or if in case of statin intolerance.39, 40 Although these data are very promising, long-term follow up data on efficacy and safety are still required.

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agents, JTT-130 and SLx-4090 – which only inhibit lipoprotein synthesis in the intestine – were developed. The results look promising and phase II clinical trials are currently running (ClinicalTrials.gov Identifier: NCT01675154).

Angiopoietin-like 3 (ANGPTL3)
Individuals with heterozygous ANGPTL3 loss-of-function mutations present with lower triglycerides and LDL-c levels and 39% lower odds of CVD in comparison to controls.\textsuperscript{22} Inhibition of ANGPTL3 using a monoclonal antibody, evinacumab, successfully reduced triglycerides, total cholesterol and atherosclerotic lesion size in mice. In phase I trial, evinacumab has been shown to reduce fasting triglyceride levels (up to 76%) and LDL-c levels (up to 23%) in a dose dependent manner.\textsuperscript{48} In another phase I trial, antisense oligonucleotides targeting ANGPTL3 mRNA in the liver reduced atherogenic lipoproteins during six weeks of treatment.\textsuperscript{46} Targeting ANGPTL3 is interesting because of reduction in atherogenic remnant lipoproteins which are generally not reduced with any of the above described drugs.

Aims and scope of this thesis
This short introduction sets the stage for the current thesis. The chapters presented in this thesis describe novel data under three main topics.

The first part, which includes chapters 2 and 3, focuses on the discrepancy between clinical practice and guideline recommendations regarding statin treatment. There seems to be a huge undertreatment (no treatment despite guideline recommendations) of statins in both primary and secondary prevention. Our studies show that over the past decade no progress in the identification and treatment with statins of individuals with increased cardiovascular risk have been made in the Netherlands. On the other side, overtreatment (statin treatment despite no guideline recommendation) is a common phenomenon as well. Most of these individuals have a low short-term estimated CVD risk while current guidelines do not advocate statin treatment in such individuals.

The second part of this thesis describes lipid reference values for adults (Chapter 4) and children (Chapter 5). In adults, blood lipids appear to be highly age- and gender-specific. LDL-c levels rise drastically with ageing. Current guidelines, however, do not take age and gender into account in the identification of severe hypercholesterolemia leaving the young underexposed. The data presented in these studies are of utmost importance to CVD treating clinicians.

The third and last part of this thesis describes genetic and lifestyle parameters associated with dyslipidemia, especially non-LDL dyslipidemia, hypo- and hypercholesterolemia. Chapter 6 shows the surprising high prevalence of non-LDL dyslipidemia in the Lifelines

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population, also in the young. Furthermore, we observe that non-LDL dyslipidemia is strongly associated with a poor lifestyle. In Chapter 7 we show a high prevalence of monogenic origins of extreme LDL-c phenotypes in apparently healthy women (selected from Lifelines) which is likely related to the use of age- and gender specific lipid values. Interestingly, we also identified a strong polygenic component for hypocholesterolemia while there was no association with lifestyle parameters. In contrast, hypercholesterolemia in women was associated with poor lifestyle scores. Chapter 8 describes the generation of a murine Stap1 knockout model that was generated to investigate this novel candidate gene in LDL metabolism. The last chapter (Chapter 9) critically discusses the means to improve cardiovascular risk treatment with a special focus on the young.