Summary and perspectives
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This thesis addresses genetic screening for familial hypercholesterolemia (FH) and is divided into four parts.

Part 1 focuses on the genetic diagnosis of FH and the required steps to confirm that the specific genetic sequence variant is indeed pathogenic. In Chapter 2 we investigate a large pediatric population with an unequivocal autosomal dominant hypercholesterolemia (ADH) phenotype to assess the molecular basis of hereditary hypercholesterolemia and to define the percentage of individuals with unexplained dyslipidemia. Of the 269 children who had a definite FH-diagnosis according to strict clinical criteria, 255 (95%) carried a functional mutation (LDLR 95%; APOB 5%). Thus, in the vast majority of children with an ADH phenotype, a causative mutation can be identified, strongly suggesting that most of the large-effect genes underlying ADH have been identified to date. Chapter 3 further elaborates on the clinical FH patients from Chapter 2 in whom no causal mutation was found. We perform whole exome sequencing in selected individuals within a family with a convincing ADH pattern. Accordingly, we describe the discovery of a pathogenic mutation in a region in the Apolipoprotein B gene that was previously not assumed to cause hypercholesterolemia. Chapter 4 presents a prospective study on the reproducibility of 1003 genetic FH test results, based on comparison of test results from the reference DNA laboratory and that from a counter-expertise laboratory. The overall accuracy of the reference laboratory was 99.8%, with two false positive results identified by the counter-expertise laboratory. The erroneous test results were due to switching of blood or DNA samples. In Chapter 5 we design criteria to detect non-pathogenic mutations, based on lipid levels and cholesterol lowering medication use. Subsequently, the proposed criteria are applied to known pathogenic and non-pathogenic LDLR and APOB sequence variants from the FH screening registry. Last, these criteria are applied to assumed pathogenic prevalent mutations in order to discover non-pathogenicity among those sequence variants. Our criteria discriminate prevalent and established pathogenic mutations from non-pathogenic sequence variants. Of the other prevalent 46 DNA variants that were evaluated, three sequence variants emerged as non-pathogenic, according to the proposed criteria. Segregation analysis supported the conclusion to label these three variants as non-pathogenic. In Chapter 6 we compare cardiovascular risk between individuals with established pathogenic FH mutations and those with sequence variants in LDLR that we consider non-pathogenic, based on the criteria formulated in Chapter 5. Both lipid levels and the risk of CAD in individuals carrying a non-pathogenic LDLR variant are similar to those of their healthy relatives. As such, this provides further evidence that the proposed criteria to determine non-pathogenicity did correctly...
label sequence variants. In contrast, FH patients with a pathogenic mutation had a more than threefold higher hazard ratio for CAD compared to unaffected relatives. **Chapter 7** provides the distribution of the most prevalent pathogenic FH mutations in the Netherlands. We demonstrate that almost all common mutations show a clearly marked region of preference. This can be explained by local founder effects plus limited migration, which is also reflected by the fact that neighbouring countries and countries to which the Dutch used to emigrate, share the same mutations. The high prevalence and typical distribution of Dutch homozygous patients can also be understood by these founder effects.

**Part 2** of this thesis focuses on the variable phenotype of patients with confirmed FH mutations. We evaluate several factors which may explain why some mutation carriers do not express a severe FH phenotype. In **Chapter 8** we describe our efforts to highlight why we observe such a large proportion of mutation carriers not expressing an FH phenotype. We focus on the effect of mutation severity in a large cohort of 26,406 individuals tested for assumed pathogenic LDLR or APOB variants. The selection of subjects tested for the severe class 1 mutations in the LDLR did result in a marked separation of LDL-C levels between carriers and non-carriers. Together, our findings indicate that the overlap in terms of LDL-C levels between those with molecularly proven FH and unaffected relatives is to a large extent due to the high prevalence of modestly severe LDL-receptor mutations in the Netherlands. Therefore, cascade screening using LDL-C specific cut-off values would be associated with an unacceptably low sensitivity in our country. In the next chapter, **Chapter 9**, we explore whether such individuals without a penetrant FH mutation are at increased CAD risk or not. We assess the atherosclerotic burden among FH patients without high LDL-C levels, compared to classical FH patients with hypercholesterolemia and controls in a large cross-sectional study, evaluating carotid intima-media thickness by ultrasound. Our findings suggest that individuals who carry pathogenic mutations in LDLR or APOB, but do not exhibit the severely elevated LDL-C phenotype, possess carotid arterial walls of similar thickness as their unaffected relatives. **Chapter 10** investigates the relationship between plasma levels of proprotein convertase subtilisin kexin type 9 (PCSK9) and LDL-C phenotype in FH patients and controls. PCSK9 is a natural inhibitor of the LDL-receptor and we hypothesized that low plasma levels of PCSK9 would associate with a less severe FH phenotype. This hypothesis is tested in the study population from the cross-sectional study described in **Chapter 9**. Our findings demonstrate that PCSK9 levels are significantly lower in normcholesterolemic FH patients than in controls or FH patients with hypercholesterolemia. In conclusion, plasma PCSK9 levels likely contribute to phenotypic variability in FH heterozygotes.
A reasonable assumption would be that low plasma PCSK9 activity might lead to lower LDL-C levels in heterozygous FH. In Chapter 11 we sequence the genes encoding PCSK9, APOB and ANGPTL3 to address whether monogenic dominant loss-of-function mutations in those genes underlie a paradoxical phenotype in carriers of an FH mutation with normal cholesterol levels. Specific mutations in PCSK9, APOB and ANGPTL3 are known to result in a hypobetalipoproteinemia phenotype. Therefore, we hypothesize that carriehship of such loss of function mutations would associate with a less severe FH phenotype in individuals with a concurrent pathogenic FH mutation. Only a small proportion, 75 (1.6%) patients of a total of 4,669 genetic FH patients show the rare paradoxically phenotype, with LDL-C levels below the 50th percentile for age and gender prior to lipid-lowering therapy. APOB mutations, resulting in truncated APOB, and an established loss-of-function PCSK9 mutation were found in 5 (6.7%) probands and 1 (1.3%) proband, respectively. Variants in ANGPTL3 that could completely neutralize the hypercholesterolemic phenotype were not found. In Chapter 12 we describe two individuals with an extreme FH phenotype, i.e. with severe xanthomatosis, and focus on the concurrent mutations in the LDLR and 7-alpha-hydroxylase genes that explain their extraordinary features. In Chapter 13, we determine coagulation factor VIII (FVIII) levels in individuals that underwent cascade screening for genetic FH. FVIII plays a crucial role in the coagulation cascade, but the factors determining its level, are hitherto largely unknown, but LDL-receptor activity is supposed to contribute to its regulation in plasma. We hypothesize that patients with FH would have higher FVIII levels than their unaffected relatives and test this in the study population from the cross-sectional study described in Chapter 9. We demonstrate that patients with heterozygous FH had on average a significant 9% higher FVIII level than unaffected relatives. This finding supports the hypothesis derived from previous findings, suggesting that the LDLR might have a suppressing role on FVIII levels.

In Part 3 of this thesis we focus on access to life insurance after genetic FH diagnosis. Chapter 14 reviews the reported problems with access to insurance after genetic FH. In addition, it describes the advent of the guidelines for insurance companies that were designed to reduce genetic discrimination in our country. These guidelines stipulate that applicants with FH should be accepted at normal rates if the LDL-C level is < 4,0 mmol/l in the absence of additional risk factors. In Chapter 15 we describe our efforts to compare life insurance acceptance rates before and after FH diagnosis and before and after the guidelines, as described in Chapter 14, were issued. We hypothesize that access to life insurance has improved for FH patients after the guidelines were issued. Our findings show that the vast majority (86.3%) of FH
Summary and perspectives

patients are accepted without complications after the advent of guidelines. As such, the unconditional acceptance for life insurance has much improved for patients with FH when compared with a decade ago. Nevertheless, individuals with genetic FH are accepted more often with additional conditions than those without genetic FH. This difference appears to be determined to a greater extent by variations in cholesterol levels than by mutation carriership.

In Part 4 of this thesis, we focus on treatment of FH patients, in particular the current treatment options, the need for additional treatment modalities and two novel treatment strategies. Chapter 16 provides a review of current and future treatment modalities for FH. Lowering LDL-C is the mainstay of treatment for FH. First choice agents are HMG-CoA-reductase inhibitors (statins). Side effects, less efficacy or contraindications such as pregnancy may preclude some patients from chronic high dose statin treatment. Moreover, treatment of FH often requires therapeutic modalities that lower LDL-C over 50%. Therefore, either combination of statins with other drugs or high dose statin therapy is frequently required. Combination therapy is currently often prescribed to FH patients, with agents targeting cholesterol absorption, such as ezetimibe, or bile acid sequestrants. In addition, several therapies are in an advanced stage of clinical development for FH patients. These therapies include LDL-C lowering with APOB, PCSK9 or MTP inhibition and HDL-raising strategies with CETP inhibition. In Chapter 17, we take a close look at cholesterol lowering medication use two years after genetic FH diagnosis. Our findings suggest that the genetic diagnosis of FH leads to an increased proportion of patients that start or intensify cholesterol-lowering medication, and consequently, to a robust decrease in LDL-C levels. The attained LDL-C levels are lower than those reported in a previous survey which could reflect the effect of more stringent lipid target levels. However, only a minority of the patients was treated with a potent drug regimen to reach set targets. In Chapter 18, we describe our effort to calculate the effectiveness of the genetic screening program in preventing CAD. Based on data from Chapter 17, we could assume that 85% of actively screened patients received statin treatment after genetic FH diagnosis. In addition, we assume that such cholesterol lowering intervention would yield a relative risk reduction of 76%, based on the reported CAD risk reduction through statin treatment from a large Dutch cohort of clinically diagnosed FH patients. Based on these two assumptions, it is calculated that 65% of the untreated FH patients, who are free of CAD at diagnosis, could be protected against a first CAD event as a consequence of genetic testing followed by statin prescription. In fact, the prediction model demonstrates that three untreated individuals free
Summary and perspectives

from CAD would need to be diagnosed with FH in order to protect one individual against a CAD event. Chapter 19 investigates the LDL-C goal target achievement in relation to the prescribed treatment regimen among FH patients referred to five large outpatient lipid clinics. The data of 1249 FH patients were available. Nearly all patients (96%) were on statin treatment. Only a small proportion of FH patients (21%) reached the LDL-C treatment target of <2.5 mmol/l. In Chapter 20 we examine the efficacy and tolerability of one possible treatment regimen that could further decrease LDL-C levels in FH. In a 12-week randomized controlled trial, we evaluate the effect on LDL-C levels of adding the bile acid sequestrant colesevelam to a maximally tolerated regimen of a statin and ezetimibe in 86 FH patients with FH with an LDL-C level above target. Our findings show that colesevelam added to a combination of a statin and ezetimibe is associated with significantly but modestly improved LDL-C concentrations compared with a placebo, with a mean reduction of 12% in the colesevelam treated group compared to the placebo. Colesevelam is generally well-tolerated. As outlined in Chapter 21, we perform a post-hoc analysis of the Treating to New Targets (TNT) study to test the hypothesis whether high PCSK9 activity would be associated with higher CAD incidence in statin treated patients. The TNT-study is a randomized trial that compared the efficacy of high- versus low-dose atorvastatin in preventing CAD. To this end, we measure PCSK9 plasma levels, among a subset of patients from the TNT-study with stable CAD treated with atorvastatin 10 mg daily, who were randomized to either continue with 10 mg or be up-titrated to 80 mg of atorvastatin, and followed during a median period of 5 years for cardiovascular events. Our findings indicate that PCSK9 levels at randomization positively associate with cardiovascular events in patients treated with low-dose atorvastatin. These findings lend support to the development of PCSK9 inhibiting strategies, also for statin treated FH patients.

INTERPRETATION AND FUTURE PERSPECTIVES

As outlined in chapter 1, effective screening makes sense for FH patients, because they constitute an important public health problem, the disease can be accurately diagnosed, and effective treatment exists. The demonstration of a causative mutation possibly provides the only unequivocal diagnosis. It is pivotal for effective genetic cascade screening that aims to identify all patients with FH in a region to ascertain a causal mutation.
Summary and perspectives

As outlined in chapter 3, routine genetic FH analysis should be expanded, also including exon 26 of APOB, based on our discovery of the segregation of specific mutations in that region with an FH phenotype. In addition, we believe that with a strict clinical selection for FH a causal mutation can be identified in the vast majority of FH patients. Our findings strongly suggest that most of the large-effect genes underlying FH are known to date, because 95% had a pathogenic LDLR of APOB mutation among our pediatric cohort with clinical FH according to strict criteria. Nevertheless, the use of whole exome sequencing and exclusion linkage analysis may be a successful approach when attempting to identify additional causal gene variants in small-sized FH families in whom LDLR/APOB/PCSK9 mutations were excluded.

A pathogenic mutation can be used to perform genetic cascade screening. The findings from chapter 4 show that blood handling during DNA extraction and preparation of the DNA for mutation-analysis should always be performed with utmost accuracy and guarded by standard operating procedures. Because of the low error rate, we do not recommend routine use of duplicate testing. Furthermore, good clinical judgment and critical evaluation of mutation test results remain essential to conclude on genetic FH status. In cases of discrepancies between clinical probability of FH versus DNA test results, a re-analysis should be considered.

Once a novel sequence variant is identified and assumed to cause FH, it is essential to determine whether that variant is indeed pathogenic. In vitro tests are laborious and hardly ever performed in clinical routine. In silico analysis, i.e. web-based tools predicting the effect on LDLR activity or APOB100 binding properties, are used frequently to determine whether or not a sequence is pathogenic. However, our findings show that in silico analysis does not correspond well with the co-segregation data, with specificity below 50% (chapter 6). We formulate criteria based on lipid levels and medication use to discriminate pathogenic and non-pathogenic sequence variants. Both segregation analysis (chapter 5) and clinical outcomes (chapter 6) substantiate the proposed criteria. Even though the prevalence of these newly discovered non-pathogenic sequence variants was low, the fact that these non-pathogenic variants were identified underline that novel sequence changes in LDLR and APOB should be interpreted with caution before being incorporated into the cascade screening program. We believe that the uniform criteria we formulate to identify nonpathogenic sequence changes could have future wider relevance, especially with regard to recently initiated genetic cascade screening programs in other countries. Thus, re-evaluation of functionality of prevalent sequence variants seems a reasonable approach to select only those FH mutations for genetic cascade screening that are associated with increased LDL-C levels, and consequently increased CAD risk.
Part 2 of this thesis focuses on the variable phenotype of FH patients. Particularly for patients with genetic FH without a severe phenotype, it is crucial to confirm that the sequence variant is pathogenic (chapters 5, 6 and 8). In addition, a lack of FH phenotype is, not surprisingly, most often observed in patients carrying the less severe mutations. Our findings from chapter 8 indicate that the overlap of LDL-C levels between FH causing mutation carriers and non-carriers is to a large extent determined by the severity of the mutation. Furthermore, low PCSK9 activity could contribute to lower LDL-C levels in FH patients (chapters 10 and 11). In contrast, an extreme FH phenotype in heterozygous FH patients should raise the suspicion of additional genetic mutations, such as homozygous FH, compound heterozygosity or hypercholesterolemic mutation in other genes involved in lipid metabolism (chapters 4 and 12). The role of loss-of-function mutations in PCSK9 and ANGPTL3 potentially correcting the FH phenotype in genetically diagnosed patients remains unclear (chapter 11). Since our study cohort from chapter 11 is small, we can only speculate on whether heterozygous loss-of-function mutations in PCSK9 or ANGPTL3 alone are not sufficient enough to induce a major reduction in LDL-C or that the prevalence of PCSK9 or ANGPTL3 mutations is rare in general. Together, the results of our genetic analysis focusing on life long low activity of APOB or PCSK9 are interesting with respect to currently ongoing clinical trials in FH patients with APOB or PCSK9 inhibiting agents (chapter 11).

An important dilemma in our country is the medical management of individuals with molecular FH but without severe hypercholesterolemia. Our findings from chapter 9 suggest that the risk of cardiovascular disease in patients with FH is to a large extent related to LDL-C levels and not to the presence of a mutation per se. Consequently, we cautiously suggest that individuals with an FH genotype without expression of hypercholesterolemia may not require a pharmaceutical intervention that is as aggressive as the standard for subjects with FH.

Our findings from chapter 13 suggest that LDLR might have a suppressing role on FVIII levels. If FH patients indeed have higher FVIII levels, then the increased risk of CAD would not only be determined by an increased rate of atherosclerotic plaque formation but also by an enhanced athero-thrombotic tendency in case of, for instance, plaque rupture. Additionally, if LDLR activity is indeed a determinant of FVIII levels, then statin treatment would induce an anti-thrombotic effect, potentially because upregulating LDLR expression would result in a lower FVIII. Future studies that prospectively assess whether statin treatment can prevent the occurrence of venous thrombo-embolic events are eagerly awaited.
As outlined in **part 3**, access to life insurance depends more on the actual phenotype of FH than on the genotype. More importantly, the unconditional acceptance for life insurance has much improved for patients with FH when compared with a decade ago. Our findings may reduce the perception of discrimination based on genetic FH. It may enhance the participation rate in the screening program for FH, because it may encourage relatives to be tested, rather than being deterred by concerns about life insurance.

As outlined in **Part 4** of this thesis, lowering LDL-C is the mainstay of treatment for FH. First choice agents are statins. Initiation of statin treatment after genetic FH diagnosis is predicted to contribute to the prevention of the majority of CAD events in these patients (**chapters 17 and 18**). Although statin therapy is rather efficacious in lowering LDL-C and CAD prevention, combination therapy is often required to achieve LDL-C target levels. Our results from **chapter 19** emphasize the need for better monitoring, better utilization of available medication and for new treatment options in FH to further decrease LDL-C levels. Currently, often used additional treatment options to lower LDL-C further include ezetimibe in particular and -the less often prescribed- bile acid sequestrants. Our findings from **chapter 20** show that the addition of the bile acid sequestrant colesevelam yields a modest improvement of LDL-C levels in FH patients with LDL-C levels above target levels, despite statin and ezetimibe treatment. As such, we believe that only a minority of FH patients can be motivated by their physicians to use 6 tablets of colesevelam each day in addition to the treatment regimen with a statin and ezetimibe, in current medical practice. Therefore, several other novel therapies in an advanced stage of clinical development raise higher expectations for treating FH patients not reaching acceptable LDL-C levels. Our findings from **chapter 21** lend support to the development of PCSK9 inhibiting strategies for statin treated FH patients, because on-statin PCSK9 plasma levels were shown to be positively associated with recurrent CAD events in patients with stable CAD disease.

In conclusion, effective cascade screening is very worthwhile for FH, because it is an important public health problem, it can be accurately diagnosed, and effective treatment exists. Ascertaining a causal mutation is pivotal for effective genetic cascade screening aimed at identifying all patients with FH in a particular region. Genetic FH diagnosis and subsequent initiation or intensification of treatment contributes to the prevention of the majority of CAD events in these patients. In fact, the prediction model from **Chapter 18** demonstrates that three untreated individuals free from CAD
would need to be diagnosed with FH in order to protect one individual against a CAD event. Accordingly, genetic cascade testing for FH, as is currently being performed in the Netherlands, should also be considered in other countries.