The spectrum of premature atherosclerosis: from single gene to complex genetic disorder
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Summary
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In this thesis, the genetic component of premature atherosclerosis is investigated. The first part focuses on Familial Hypercholesterolemia (FH), a single gene disorder leading to hypercholesterolemia and to premature cardiovascular disease (CVD). In FH patients the influence of accompanying metabolic and genetic factors and the effect of treatment was assessed. The second part presents the investigation of a wide variety of novel metabolic and genetic risk factors for atherosclerosis in a patient cohort with premature CVD. The implications of the findings for our understanding of the pathogenesis of atherosclerosis and the prevention of premature CVD are discussed.

Part I

Chapter 1 is a clinical report of two young patients demonstrating that FH may cause sudden cardiovascular complications and death. Timely diagnosis of FH, assisted by taking a careful history, alertness for anginal complaints, a thorough physical examination and early administration of cholesterol lowering medication may prevent myocardial infarctions and save lives in these patients. Mutations in the genes encoding the LDL receptor or apoprotein B-100 are responsible for FH. Mutation screening in families facilitates the identification of individuals with FH in order to start lipid lowering therapy at an early stage. In Chapter 2 the frequency and the geographical distribution of 13 known mutations in a cohort of 1223 Dutch FH patients is described. Although the genetic basis of FH is definitely monogenic there is a wide variation in the onset and the severity of symptoms of CVD in FH patients, even when they share the same mutation. It was unknown whether this variation was reflected in the intima media thickness (IMT) of the peripheral arterial walls, as a marker of the extent of atherosclerosis. We measured IMT by quantitative B-mode ultrasound in 142 FH patients without and in 106 FH patients with clinical manifestations of CVD. The results of this study are described in Chapter 3. The IMT in all FH patients were severely thickened compared to normals and the mean IMT of, in particular, the common femoral artery is thicker in patients with CVD compared to those without.

It was shown in prospective studies that assessment of IMT could predict coronary artery disease and IMT measurement became a well validated surrogate marker in
clinical intervention trials. Therefore, we designed a statin intervention trial to answer the question whether aggressive cholesterol lowering with statins could alter intima media thickening to a greater extent than conventional therapy in patients with FH. The acronym of the study was ASAP-study (The Atorvastatin versus Simvastatin on Atherosclerosis Progression study). The study results are described in Chapter 4. The ASAP study was a double-blind clinical trial in 325 patients with FH. Patients were given either atorvastatin 80mg (n=160) or simvastatin 40mg (n=165) daily, on an intent-to-treat basis. The primary endpoint was the change of IMT over 2 years of follow-up. The overall baseline IMT, combining the measurements of the common and internal carotid artery and the carotid bulb on both sides, was 0.93 mm (SD 0.22) and 0.92 mm (0.21) in the atorvastatin and simvastatin groups, respectively. After treatment with atorvastatin for 2 years, IMT decreased (-0.031 mm [95% CI -0.007 to -0.055]; p=0.0017), whereas in the simvastatin group it increased (0.036 [0.014-0.058]; p=0.0005). The change in IMT differed significantly between the two groups (p=0.0001). Atorvastatin showed greater reductions in cholesterol concentrations than did simvastatin. Both drug regimens were equally well tolerated. We concluded that aggressive LDL-cholesterol reduction was accompanied by regression of carotid IMT in patients with FH, whereas conventional LDL-cholesterol lowering was not. This implicates that FH patients should be treated with high dose statin therapy to reduce LDL-cholesterol sufficiently to obtain atherosclerosis regression.

In recent years it was shown that inflammatory and thrombotic mechanisms play a pivotal role in atherogenesis and its clinical sequelae and that the beneficial effect of statins on cardiovascular morbidity and mortality may not only result from LDL-cholesterol reduction but also from processes that modify these mechanisms. We were interested if these so called pleiotropic effects of statins would influence the outcome of high dose statin treatment in FH patients. Therefore we evaluated whether high dose simvastatin therapy would modulate markers of inflammation, coagulation and fibrinolysis and whether the alterations in these markers were associated with changes in the IMT of the arterial wall as a marker for atherosclerosis progression in FH patients.

In the ASAP study population we determined highly sensitive C-reactive protein (hs-CRP), the best and most extensively studied marker of inflammation, at baseline and after 1 and 2 years of treatment. The results are described in Chapter 5. We could show that atorvastatin 80mg reduces hs-CRP levels to a greater extent than simvastatin 40mg and that the extent of hs-CRP reduction is associated with the regression rate of the atherosclerotic process as measured by IMT. The results of our
study might implicate that the benefits of high dose statin therapy are a consequence of the reduction of both LDL-cholesterol and hs-CRP levels. We also studied the long-term effects of atorvastatin and simvastatin on fibrinogen levels in relation to the progression of IMT in the ASAP study population and describe the results in Chapter 6. Fibrinogen, an independent risk factor for CVD is modulated by statin therapy, which might have clinical consequences. We showed, indeed, that fibrinogen level was increased significantly both by treatment with 80mg atorvastatin (0.09 g/l (3.6%, p = 0.0104)) and with 40mg simvastatin (0.11 g/l (3.8%, p = 0.0077)). These small changes, however, were not related to changes in the IMT that occurred over a 2 year period. The fact that the increase of plasma fibrinogen was not related to progression of atherosclerosis is important in cardiovascular risk management and implies that the choice for a lipid lowering agent has to be made on the capacity to improve the lipoprotein profile and not on the modulation of fibrinogen levels.

We also investigated whether high dose simvastatin therapy would modulate markers of coagulation and fibrinolysis and whether alterations of these markers are associated with changes in IMT. In Chapter 7 the results of the study are described. We treated 106 FH patients with 80mg simvastatin for two years. At baseline and after 6 months of treatment parameters of coagulation and fibrinolysis were evaluated and IMT changes were calculated after 2 years of therapy. Simvastatin therapy produced small, but significant changes in a number of hemostatic parameters. An increase was observed for fibrinogen, coagulation factor VIII, von Willebrand Factor, D-dimer and plasminogen activator inhibitor type 1, whereas prothrombin fragment 1+2 and prothrombin were decreased. Nevertheless, all these alterations in coagulation and fibrinolysis parameters were not correlated with IMT changes over a 2 years treatment period. So we concluded that both the coagulation and the fibrinolysis system in patients with FH were significantly affected by 80mg simvastatin, but that these changes were not associated with either pro- or regression of IMT.

We already mentioned that the FH phenotype is influenced by additional genetic and metabolic factors. One of these genetic factors is a common D9N substitution in the gene encoding lipoprotein lipase. This mutation has been associated with reduced levels of HDL-cholesterol and elevated triglycerides. We screened a cohort of 2091 FH for the D9N mutation and found 94 (4.5%) carriers. The FH patients who carried the D9N mutation had significant higher triglycerides and lower HDL-cholesterol levels and, moreover, they were at higher risk for CVD (odds ratio=2.8; 95% CI, 1.43 to 5.32; p=0.002). We described these study results in Chapter 8 and
concluded that the common D9N lipoprotein lipase mutation, present in 4.5% of Dutch FH heterozygotes, leads to increased triglycerides and decreased HDL-cholesterol levels and to increased risk for CVD. This again supports the hypothesis that the observed large variation in clinical manifestations in FH is explained by additional genetic risk factors acting in conjunction with the LDL receptor defect.

Part II

Premature atherosclerosis has a strong familial component. However, single gene defects, such as FH, account only for a small fraction of less than 5% of premature CVD, indicating that the genetic basis of CVD is complex; not only inherited but also acquired or environmental factors play a significant role in its pathogenesis. It is becoming increasingly clear that the analysis of genetic and biological risk factors, together with lifestyle and environmental factors may contribute significantly to our understanding of the predisposition to CVD. We performed several studies to unravel some of the components that may predispose to premature CVD.

Chapter 1 gives the results of a study we started in 1983. At that time aspirin treatment after myocardial infarction was not yet common, so we still had the opportunity to perform a study on platelet aggregation with a follow-up period of five years in 94 myocardial infarction survivors not using aspirin. We showed that spontaneous platelet aggregation in vitro predicted coronary events and mortality in patients who survived myocardial infarction. Since then, large scale clinical intervention studies have demonstrated the beneficial affect of aspirin treatment on cardiac morbidity and mortality in patients with CVD and nowadays anti-platelet drugs are routinely recommended and prescribed. Platelets play a pivotal role in atherosclerosis and its complications, with the platelet glycoprotein IIb/IIIa surface receptor as central point in the process of platelet aggregation. The PI41/A2 polymorphism of the platelet glycoprotein IIIa is associated with platelet dysfunction, but the clinical relevance for CVD is disputed. We therefore studied this polymorphism in relation to CVD, angiographic progression of CVD and the response to aspirin in 753 CVD patients, participants of the Regression Growth Evaluation Statin Study, a placebo-controlled lipid lowering regression trial with a follow up of 2 years and in 222 controls. The study results are described in Chapter 2. The PI41 allele frequencies in patients and controls were 0.17 and 0.14 respectively, in accordance with Hardy-Weinberg equilibrium. Angiographic progression measured by quantitative coronary
angiography after 2 years was unrelated to the platelet glycoprotein IIIa P1A genotype. Placebo treated patients, however, receiving aspirin therapy who carried the P1A2 allele showed a significantly greater loss of mean segment diameter than patients not on aspirin therapy (0.17 versus 0.07, p=0.03). This difference was not observed in the statin treated patients. We concluded that the glycoprotein IIIa P1A polymorphism is not associated with CAD nor with CAD progression. However, patients carrying the P1A2 allele may not benefit from aspirin therapy alone and should possibly be prescribed different antiplatelet medication. Before our findings are translated to therapeutic decisions, they should be confirmed in larger, prospective studies.

Not only genetic variation in platelets but also in other components of the haemostatic system were claimed as risk factors for CVD. Characterising the genetic markers for thrombosis would enable us to establish individual profiles of thrombotic risk, and might eventually result in new and individualised prognostic and therapeutic measures. In Chapter 3 we review the published data that relate gene abnormalities of the haemostatic system with arterial thrombosis. The overall picture was rather disappointing since no convincing evidence exists in favor of the notion that haemostatic gene variations play a significant role in arterial thrombosis. We performed a study on a sequence variation in the 3'-untranslated region of the prothrombin gene (20210 G→A), that was recently claimed to be associated with elevated plasma prothrombin levels and an increased risk for venous and arterial thrombosis. We examined the prevalence of the 20210 A allele in the prothrombin gene in 400 healthy controls and in 263 patients with proven premature CVD. The 20210 A allele was found in 1% of controls and in 2.7% of patients in the patient group, (RR for the 20210 A allele of 2.7 (95% CI 0.8-9.4)). All heterozygotes in the patient group were found to have had a myocardial infarction (RR for MI of 4.2 (95% CI 1.2-14.6)). Plasma prothrombin levels, the level of TAT complexes as well as of prothrombin fragment 1+2 were higher in carriers than in non-carriers. Our findings suggest that the 20210 G→A mutation in the prothrombin gene is a genetic risk factor for myocardial infarction, associated with excessive thrombin generation, which may contribute to the understanding of its role in and arterial disease as we described in Chapter 4.

Very recently we discovered a mutation in the ABCC6 gene (R1141X), a cause of Pseudoxanthoma Elasticum (PXE), to be associated with a strong increase in the prevalence of CAD. This finding is described in Chapter 5. PXE is an inborn disorder, the hallmark of which is dystrophic mineralization of elastic tissue of the skin, retina and arterial walls. Cardiovascular manifestations of PXE include
accelerated atherosclerosis, which results in myocardial infarction at a young age. Recently, we and others have identified mutations in the gene coding for the ABCC6 transporter in PXE patients with ocular and skin involvement. In The Netherlands, as in the rest of Europe, a particular premature truncation variant ABCC6 (R1141X) was found in a large cohort of PXE patients. Given the association between CVD and PXE we hypothesized that heterozygosity for this ABCC6 mutation could also confer an increased risk for CVD. Therefore, we conducted a case-control study of 441 patients under the age of 50 years with definite CAD and 1057 age- and sex-matched population-based controls who were free of CAD. Strikingly, the prevalence of the R1141X mutation was 4.2 times higher among patients than among controls (3.2% vs. 0.8%; p < 0.001). Consequently, among subjects with the R1141X mutation, the odds ratio for a coronary event was 4.23 (95% CI: 1.76 - 10.20, p=0.001). Thus, the presence of the R1141X mutation in the ABCC6 gene is associated with an increased risk of premature CAD. The exact biological function of ABCC6, however, is presently still unknown, as is the functional relationship of this transmembrane transporter to the pathogenesis of the PXE phenotype. In addition, we could not find a relation between this mutation and other major CAD risk factors, suggesting that this mutation in the ABCC6 transporter is operating through a novel pathway in atherogenesis. This intriguing finding will be an important issue in future research.

Another study on the genetic basis of CVD focused on thrombospondin and is described in Chapter 6. Thrombospondins form a family of multidomain extracellular matrix proteins with related sequences but diverse tissue distribution and a wide variety of functions. The fact that thrombospondins are involved in the modulation of a range of processes in the vessel wall makes them plausible risk factors for premature CAD. Variation in the genes encoding for this protein family could therefore modulate this CAD risk. Recently, three polymorphisms in thrombospondin-1, -2, and -4 (TSP-1 N700S, TSP-2 T→G substitution in the 3′-untranslated region, TSP-4 A387P) were proposed to modulate the risk of premature CAD. It was our objective to verify this hypothesis in an independent cohort. In a case-control study among 503 patients < 50 years with symptomatic CAD and 1071 age- and sex-matched population-based controls free of CAD we demonstrated that a relationship between the TSP-1 N700S polymorphism and premature CAD was highly unlikely, that the TSP-2 polymorphism was associated with reduced risk of premature myocardial infarction and that for the TSP-4 A387P polymorphism further studies are required to elucidate its role in premature CAD. It has also been proposed that iron accumulation may contribute to atherogenesis
by increasing free radical formation and oxidative stress. Epidemiological studies in which the association of iron status with atherosclerosis was assessed raised conflicting results. To test whether genetic haemochromatosis is associated with increased atherosclerosis, we determined the prevalence of two mutations in the HFE gene related to haemochromatosis (G845A and C187T) in 265 consecutive patients with premature CVD and in 272 healthy controls. The G845A mutant allele had a frequency of 0.07 among controls and 0.04 among patients. The frequency of the C187T mutant allele was 0.11 in patients and 0.14 in controls. The compound heterozygous state for these mutations is associated with iron overload and genetic haemochromatosis. In our study 5/265 patients (1.1%) and 9/272 controls (3.3%) were compound heterozygotes. These findings, described in Chapter 7, do not support an association between haemochromatosis and atherogenesis.

In recent years, mild hyperhomocysteinemia is increasingly considered a novel risk factor for CVD, as was demonstrated by numerous epidemiological studies, but whether the relationship between hyperhomocysteinemia and CVD is causal is still subject of debate. The plasma level of homocysteine is influenced by both environmental and genetic factors. The C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene, which renders the enzyme thermolabile and less active, is a genetic cause of hyperhomocysteinemia. Moreover, the TT-genotype has been claimed to be a strong genetic risk factor for atherosclerosis. We performed a case-control study in 257 patients with premature CVD and in 272 healthy controls, we found a strong correlation between MTHFR genotype and plasma homocysteine levels, but the MTHFR genotype distribution was not different between patient and control groups. Therefore we concluded in Chapter 8 that the MTHFR genotype does not influence the risk for CVD.