high risk of CAD development, choice of lipid lowering therapy, and several other cardiovascular diseases (e.g., Alzheimer's disease).

Present analyses of apoE genotypes are based on phenotyping or genotyping. ApoE phenotyping exploits differences in isolectric points. ApoE genotyping requires DNA extraction, followed by PCR and restriction analysis using the known restriction enzymes for apoE. The restriction analysis is performed with a commercially available kit (based on a restriction endonuclease method). Control for the restriction analysis is performed using a control sample provided with the kit. The kit includes the necessary enzymes, buffers, and primers for the PCR amplification of apoE. The amplified apoE fragments are then digested with the restriction enzyme, and the resulting fragments are separated on an agarose gel. The resulting bands are visualized by staining with ethidium bromide and UV transillumination.

Summary
Summary

Chapter 1: General Introduction
Chapter 1 is meant as a general introduction to atherosclerosis and the ensuing coronary artery disease (CAD). It gives special attention to atherogenesis, lipoprotein metabolism and nutritional factors. The chapter opens with the presentation of an integrated model of atherogenesis with a central role for endothelial activation and dysfunction. The latter results from exposure of the endothelium to so-called, CAD risk factors. These are composed of genetic factors, environmental factors and the thrifty phenotype. The risk factors initiate in a concerted action a pathophysiological cascade that leads to atherosclerosis development. The final clinical outcome is CAD, stroke, aneurysm or peripheral vascular disease (notably intermittent claudication). Activation of the endothelium causes enhanced leukocyte adhesion, increased coagulation and impaired fibrinolysis, migration and proliferation of smooth muscle cells, impaired endothelium-dependent vasorelaxation and increased vascular permeability. All of these processes are characterised by mutual enhancement. The ensuing atherosclerosis causes endothelial damage, which in turn initiates endothelial activation, and thereby contributes to atherosclerosis progression.

Circulating lipids, notably cholesterol located in low density lipoproteins (LDL), are important modifiable CAD risk factors. LDL-cholesterol lowering is therefore one of the main therapeutic targets in both primary and secondary CAD prevention. High triglycerides, low concentrations of high density lipoproteins (HDL) and predominance of small dense LDL are other important lipid risk factors, but their concentrations are in fact often related. It stresses the importance of understanding the (patho)physiological basis of lipoprotein interrelationships.

The ‘Seven Countries Study’ indicated that serum cholesterol levels do not fully explain CAD mortality. The discrepancy is likely to indicate the importance of nutrition as one of the major mediators in CAD development. Nutritional factors, in particular the dietary fatty acid composition, influence plasma lipoprotein levels, but dietary fatty acids also mediate some of the key events in endothelial dysfunction, such as coagulation. Micronutrients, especially (pro) vitamins like vitamin E and β-carotene, may protect lipoproteins from oxidation. Others, notably vitamin B₆, vitamin B₁₂ and folic acid, influence circulating levels of homocysteine, which is considered deleterious to the endothelium.

Chapter 2: Apolipoprotein E analysis
Apolipoprotein E (apo-E) physiology and pathophysiology, the importance of apolipoprotein E isotyping in diabetes mellitus and atherosclerosis, and the current apo-E isotyping methods are reviewed in chapter 2.1.

Apo-E plays a central role in the clearance of lipoprotein remnants by serving as a ligand for LDL and apo-E receptors. Three common alleles (apo-e2, apo-e3 and apo-e4) give rise to 6 genotypes. Apo-E3 is the ancestral isofomt, the so-called ‘wild type’. Apo-E polymorphism influences serum triglycerides, and may account for as much as 12 and 4% of the interindividual variance of circulating apo-B and total cholesterol, respectively. Establishment of apo-E/E2 confirms the diagnosis familial dysbetalipoproteinemia (FD). Apo-E isotyping may also be of importance for patients with diabetes mellitus (who are at high risk of atherosclerosis).
ensuing coronary protein metabolism integrated model reaction. The latter factors. These are phenotype. The risk that leads to aneurysm or activation of the endothelium and impaired red endothelium these processes sclerosis causes reduction, and thereby proteins (LDL), is therefore one prevention. High predominance levels do not fully importance of nutrition in particular the dietary fatty rection, such as vitamin B₁₂ and carotenoids.

high risk of CAD development), choice of lipid lowering therapy, and several non-atherosclerotic diseases (e.g. Alzheimer's disease).

Present analyses of apo-E isoforms are based on phenotyping or genotyping. Apo-E phenotyping exploits differences in isoelectric points. Isoelectric focusing (IEF) uses gels that contain pH 4-7 ampholytes and urea. Serum is directly applied, or prepurified by delipidation, lipoprotein precipitation or dialysation. IEF is followed by immunofixation/protein staining. Another approach is electro or diffusion blotting, followed by protein staining or immunological detection with anti apo-E antibodies and an enzyme-conjugated second antibody. Apo-E genotyping demonstrates the underlying point mutations. Analyses of polymerase chain reaction (PCR) products are done by allele specific oligonucleotide (ASO) probes, restriction fragment length polymorphism (RFLP), single stranded conformational polymorphism (SSCP), the primer-guided nucleotide incorporation assay, or denaturing gradient gel electrophoresis (DGGE). Detection with primers that either or not initiate amplification is performed with the amplification refractory mutation system (ARMS). Detection of rare mutants is important, since most of those that are presently known cause FD. Selection of a routine method, however, is not based on analyses of rare mutants and neither phenotyping nor genotyping guarantees their detection. Combination of techniques may be the first choice for further investigation of patients with FD when, on the basis of a single technique, the condition can not be explained by the apo-E/E₂ isoform. Sequencing provides ultimate proof.

The discussion on discrepancies between the outcomes of phenotyping and genotyping does not seem settled. It appears that the disparity is caused by: IEF methods that do not adequately separate posttranslational apo-E variants, storage artefacts or faint IEF bands. Genotyping is usually less laborious than phenotyping and is obviously unaffected by posttranslational variants. PCR/RFLP is preferred for routine applications, since it does not necessitate multiple PCR reactions (as with primer-guided nucleotide incorporation assays and ARMS), does not require detection by hybridisation (ASO-probes), allows less stringent experimental conditions (SSCP, DGGE, ARMS), and provides an internal control (a 38 basepair fragment) that minimises the risk of misinterpretation caused by faint bands.

In chapter 2.2 we describe the discovery by serendipity of a novel apo-E mutation in a patient with clinical features of FD. The DNA of this patient was used in a comparison study of two different apo-E genotyping methods. We observed a persisting, unexplained discrepancy between the outcomes of both methods: PCR/RFLP resulted in the apo-e₂/e₂ genotype, whereas use of a commercially available kit (based on a minisequencing method) indicated the apo-e₂/e₃ genotype. We hypothesised that the observed discrepancy resulted either from an altered restriction site (other than the common C/T substitutions) or a mutation at the annealing site of one of the primers, as used in the restriction isotyping method. Sequencing of a PCR product that included the possible mutation sites, demonstrated a G-insertion in codons 95 or 96 (⁴⁹AAG→AAG-GGA-G). The ensuing frameshift results in a premature stop codon at codon 146 (AAG→TAA), which is located in the receptor binding domain. The discrepancy between the two apo-E genotyping methods is conceivable, since the G-insertion causes a mismatch at the 3' end of one of the restriction isotyping primers and consequently the inability of this primer to become extended. The G-insertion is likely
to be located on the apo-ε3 allele, since this allele was not amplified with the apo-E restriction isotyping method.

Chapter 3: Public health
In chapter 3.1 we tried to find an explanation for the high CAD risk of Trinidadians of East-Indian descent. Trinidadian Indians have a 2.3-2.4 fold higher relative risk of CAD development, compared with their African counterparts. Although they have higher diabetes mellitus prevalence, higher LDL-cholesterol and triglycerides, and lower HDL-cholesterol, these risk factors do not explain their excessive CAD mortality. Since both apo-E2 and apo-E3 are associated with increased CAD risk, we investigated the apo-E genotype distributions and umbilical plasma lipid indices in a consecutive sample of 300 newborns in Trinidad. The apo-E genotype distributions of Trinidadian Africans and Indians were compared with literature data of populations with similar ethnic backgrounds. We also investigated the association of the apo-E genotypes with plasma lipid indices of Trinidadian newborns. These results were compared with data from a group of 234 consecutive Curacao newborns.

We found that Trinidadian Africans have higher apo-ε2 and apo-ε3, and lower apo-ε4 allele frequencies as compared with their Indian counterparts. The apo-E genotype distribution of Trinidadian Africans resembles to a certain extent that of counterparts in Curacao and Sudan, but not that of counterparts in Nigeria and the USA. Trinidadian and Singapore Indians had comparable apo-E genotype distributions. Trinidadian term appropriate for gestational age newborns of African and Indian descent had comparable umbilical plasma lipid indices, except for lower lipoprotein (a) [Lp(a)] and ‘adapted lipid tetrad index’ in newborns of Indian descent. Correlation analysis between apo-E genotypes and umbilical lipid indices revealed an apo-B increasing effect of apo-ε3 and an apo-B decreasing effect of apo-ε2 in both Trinidadian and Curacao Africans.

Our data showed that apo-E polymorphism in Trinidadian and Curacao newborns of African descent exerts similar effects on plasma lipoprotein metabolism. The apo-B increasing effect of apo-ε4, and the combination of apo-ε3 homozgyosity with FD precipitating factors may therefore contribute similarly to their CAD development. APO-E polymorphism related CAD risk may especially apply to Trinidadians of African descent, because of their high apo-ε2 and apo-ε3 allele frequencies, but not to Trinidadian Indians, who have low apo-ε2 and apo-ε3 allele frequencies. Trinidadian Indians also had lower Lp(a) and adapted lipid tetrad index. We conclude that the apo-E polymorphism and umbilical plasma lipid indices do not explain the higher CAD incidence of Trinidadian Indians, compared with their African counterparts.

The study does not provide us with an explanation for the high CAD risk of Trinidadian Indians. Various studies suggested that the increased risk of Asian Indians results from genetic factors (e.g. predisposition of insulin resistance), or altered environmental factors (e.g. emigration and urbanisation), or their combination. As yet poorly understood cause may be found in the relation between low birth weight and the risk of insulin resistance and CAD at later life. Ethnicity of Trinidadian newborns is indeed a significant factor in birth weight, with children from Indian mothers having lower birth weights, compared with Africans and Mixed. According to the ‘thrifty phenotype hypothesis’ developed by Hales and Barker, it seems possible that we are
In chapter 3.2, we investigated classical lipid risk factors, apo-E genotypes and other CAD risk factors of CAD patients in Curacao in a case-control study design. Apo-E genotype distributions of CAD patients were compared with those of age, race and sex matched controls and those of newborns. In addition, we studied the association between apo-E genotypes with lipid indices.

Compared with controls, male CAD patients had higher cholesterol, triglycerides, LDL-cholesterol, apo-B and decreased HDL-cholesterol and HDL-cholesterol/cholesterol. Other CAD risk factors were increased fasting glucose and HbA1c, decreased creatinine clearance, and increased prevalences of Lp(a) >500 mg/l, renal disease, hyperhomocysteinaemia, diabetes mellitus type II (DM-II), positive CAD family history and cigarette smoking. Male CAD patients had higher plasma cr-tocopherol. Compared with controls, female CAD patients had higher fasting plasma glucose, HbA1c and DM-II prevalence. Predicting factors for CAD development in the whole CAD group were DM-II, cigarette smoking, and apo-ε4/ε4 and apo-ε4/ε4 genotypes. The apo-ε4 allele was associated with lower HDL- and higher LDL-cholesterol.

This study shows that commonly known lipid risk factors (high total cholesterol, LDL-cholesterol and triglycerides, and low HDL-cholesterol and HDL-cholesterol/total cholesterol) and other risk factors (positive family history, cigarette smoking) are involved in CAD development in Curacao. Most patients were males, confirming male gender as a CAD risk factor. We conclude that classical atherogenic lipid risk factors and cigarette smoking are associated with CAD risk in Curacao. Diabetes mellitus type II may contribute considerably to CAD development, notably in women. The apo-ε4 allele, possibly by induction of a more atherogenic lipid profile, is likely to be a risk factor. There is a need for local studies on the improvement of diabetic control, reference values of Lp(a) and homocysteine, apo(a) phenotypes, causes of hyperhomocysteinaemia, and dietary influences on CAD development in subjects with the apo-ε4 allele.

Patients with CAD are advised to augment their dietary polyunsaturated fatty acid (PUFA) intakes at the expense of saturated fatty acids (SAFA). Linoleic acid (LA) is the quantitatively most important dietary PUFA in Western countries. In chapter 3.3 we investigated whether the dietary LA intake of the Curacao CAD patients (see chapter 3.2) was higher as compared to their controls. For this we measured the plasma cholesterol ester (CE) fatty acid composition, which reflects the dietary fatty acid composition of the preceding weeks. By comparing the CE fatty acid compositions of the controls with those reported in the literature, we tried to identify CAD risk factors that are related to the Curacao dietary fatty acid composition.

Patients with CAD and controls had minor differences in CE fatty acids. The groups notably did not differ in CE LA, which suggests that both have the same dietary PUFA/SAFA ratio. Their CE LA content suggests that the dietary PUFA/SAFA ratio is far below the recommended value of 1, as issued by the American Heart Association. Comparison with data reported for The Netherlands, Greenland and Crete showed that the dietary fatty acid composition in Curacao is typically Western with a high intake of SAFA,
Summary

a low intake of monounsaturated fatty acids and the consumption of LA as the predominant PUFA. The small difference between CE eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contents of Curacao and The Netherlands suggests that intake of EPA and DHA from fish in Curacao is not likely to be very much higher than the low intake in The Netherlands. Cretans have lower CAD incidence than may be expected from their major risk factors. It is explained by their higher dietary intake of oleic acid (from olive oil) at the expense of LA which leads to a higher dietary α-linolenic/linoleic acid (ALA/LA) ratio. The low CE ALA/LA ratio in Curacao, as compared with Crete, points at a low ALA/LA ratio in the diet, which is consistent with the local use of predominantly corn- and sunflower oils.

We conclude that reduction of dietary SAFA, augmentation of fish consumption, and an increase of the ALA/LA ratio are likely to be of benefit to both primary and secondary CAD prevention in Curacao.

Chapter 4: Nutrition

Hyperhomocysteinemia is an independent risk factor for premature atherosclerosis. Plasma homocysteine levels are dependent on genetic (mutations in enzymes involved in homocysteine metabolism) and nutritional (folic acid, vitamin B₉, and vitamin B₁₂) factors. In chapter 4.1 we investigated the effect of a short term supplementation of vitamin B₁₂ and subsequently folic acid on the fasting plasma homocysteine concentrations of 103 apparently healthy Dutch volunteers, aged 20-75 years. Plasma folic acid, vitamin B₁₂ and homocysteine and whole blood vitamin B₉ were measured at baseline, and after supplementation with vitamin B₁₂ and folic acid.

Baseline folic acid concentrations of the participants were above the lower limit of the reference range. Plasma homocysteine was inversely related to plasma folic acid and vitamin B₁₂ at that moment. Plasma homocysteine did not change after vitamin B₁₂ supplementation and only one participant exhibited a significant plasma homocysteine decrease in that period. Plasma homocysteine dropped significantly following folic acid supplementation. Those who exhibited the biggest absolute plasma homocysteine decreases had the lowest initial folic acid concentrations. Forty participants (40%) exhibited significant plasma homocysteine decreases in that period. Plasma homocysteine was still related to plasma vitamin B₁₂ at the study end, which may be caused by 19 subjects with vitamin B₁₂ concentrations in the low-normal or below-normal range. We conclude that notably the folic acid status was the cause of not exhibiting the lowest possible homocysteine concentrations in the present study population. This may also be true for the vitamin B₁₂ status, though to a lesser extent, but not for the vitamin B₉ status.

Since atherosclerosis risk may increase continuously with increasing plasma homocysteine, it seems advisable to keep plasma homocysteine levels as low as possible by augmentation of the folic acid intake. A beneficial effect of lowering plasma homocysteine on atherosclerosis risk is, however, as yet unproven and should come from randomised prospective intervention studies with healthy subjects. It will on the other hand take much time before their results will be available when such studies aim at hard clinical endpoints. Fortification of flour and cereals with 140 or 350 μg/100 g has been estimated to increase folic acid intakes by 160-170 or 280-350 μg/day, which is likely to cause a 4.8-5.4 % reduction in mortality from coronary artery disease in women and a 6.6-8.8 % reduction in men, respectively. It may therefore be advisable
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not to wait for the outcome of randomised intervention studies, but meanwhile aim at a folic acid intake that causes no further plasma homocysteine decrease. This intake has been estimated at 350-400 µg/day. Since at present only 6% of the Dutch population has daily folic acid intakes ≥400 µg, the above advice translates practically into an augmented consumption of folic acid rich or folic acid fortified foodstuffs, or the intake of folic acid supplements.

In chapter 4.2 we used the data of the study in chapter 4.1 to establish a new cut-off value for plasma folic acid. In addition to fasting plasma homocysteine concentrations, we present data on plasma homocysteine concentrations 6 h after oral administration of methionine (oral methionine tolerance test; OMTT).

The diagnosis of subclinical folic acid deficiency is important, since it is increasingly becoming clear that this condition may contribute to CAD development in the long run. Clinical chemical reference values are usually determined by calculation of the 95% confidence interval of data from apparently healthy subjects. Individuals with subclinical vitamin deficiencies cannot be excluded from the reference population on clinical grounds, because they do not exhibit symptoms that can be related directly to their suboptimal vitamin status. A solution to the above vicious circle is to define suboptimal vitamin status on biochemical grounds, i.e. by the measurement of a vitamin-dependent function (i.e. a functional marker).

In our study, the plasma homocysteine concentration served as a functional marker to establish the cut-off value of the plasma folic acid concentration. For this, we investigated at baseline its relation with fasting plasma homocysteine and plasma homocysteine at 6 h during OMTT, and also its relation with the decline of fasting homocysteine following 7 days of supplementation with a pharmacological dose of folic acid. The finally selected plasma folic acid cut-off value was defined as the concentration at, or below, which an individual has a significantly higher chance to lower its plasma homocysteine following folic acid supplementation, compared with the chance to exhibit such a decrease when the plasma folic acid concentration exceeds this value. The results of the three approaches suggested a cut-off value of 10 nmol/l.

The presently suggested plasma folic acid cut-off value contrasts clearly with our locally used reference range of 4-30 nmol/l. This reference range reflects the 95% confidence interval of apparently healthy subjects, and it was consequently not surprising that the newly selected population had values above the lower limit of this range. We now find that 32 subjects (31%) have values below the new cut-off value of 10 nmol/l. From those who had values below 10 nmol/l, 66% significantly decreased their fasting plasma homocysteine upon folic acid supplementation, whereas this was the case for 28% with values above 10 nmol/l. Values above 10 nmol/l, therefore, do not necessarily imply that the lowest homocysteine levels are always reached.

Since there is compelling, though circumstantial, evidence that low folic acid and high homocysteine are associated with atherosclerosis risk, we advise to adopt plasma folic acid cut-off values that are based on functional grounds, and not on the 95% confidence interval of the folic acid concentration of an apparently healthy population.

Oxidation of LDL is considered to play an important role in the pathogenesis of CAD. The anti-oxidants vitamin E (α-tocopherol) and β-carotene may protect LDL from oxidation, although beneficial effect of intervention with these (pro)vitamins in primary
Summary

... and secondary CAD prevention is not consistently found. Adipose tissue serves as storage site for α-tocopherol and β-carotene. Little is known on the rapidity and extent by which human adipose tissue accumulates α-tocopherol and β-carotene during supplementation and to what extent adipose tissue contributes to circulating levels during fat mobilisation, as occurs during fasting. In chapter 4.3 we investigated the influence of fasting on the levels of α-tocopherol in plasma, erythrocytes and platelets, and on plasma β-carotene before, during and after oral α-tocopherol and β-carotene supplementation. For this, six apparently healthy adults were subjected to 17 h feed-fasting experiments at various days before, during and after supplementation with α-tocopherol and β-carotene. We also monitored the kinetics of α-tocopherol and β-carotene accumulation in and disappearance from adipose tissue.

Supplementation increased α-tocopherol and β-carotene in all compartments, except for β-carotene in adipose tissue. Discontinuation caused a rapid return to baseline, except for adipose tissue α-tocopherol and plasma β-carotene. Fasting caused linear increases of free fatty acids (reflecting adipose tissue mobilisation), consistent (but small) increases of plasma α-tocopherol and inconsistent increases of plasma β-carotene. The different behaviours of α-tocopherol and β-carotene are most probably due to their different chemical properties, causing different distribution among lipoproteins and consequently different organ distributions and organ accumulation rates. After its entry in the circulation, the amphiphatic α-tocopherol, located in the surface of chylomicrons, is likely to become rapidly distributed among a large body surface area, composed of the surfaces of all plasma lipoproteins, cellular membranes and finally the surfaces of adipose tissue triglyceride droplets. The highly apolar β-carotene will also enter the circulation in chylomicrons, but it will be located in the core. Consequently β-carotene, like retinol esters, will predominantly be targeted to the liver via the uptake of chylomicron remnants. Subsequent secretion of β-carotene via VLDL explains its predominant plasma localisation in LDL and its prolonged residence in plasma due to its (slow) LDL-receptor mediated uptake in organs.

We conclude that adipose tissue α-tocopherol and β-carotene contents are likely to reflect their long term intakes, but the storage pools do not seem able to maintain high α-tocopherol and β-carotene contents in circulating lipoproteins and cells following a short-term supplementation. Maintenance of high contents may necessitate regular high oral intakes.

Epidemiological studies show an inverse relation between the intake of long-chain-polyunsaturated fatty acids of the ω3 series (LCPUFAω3; abundant in fish oil) and CAD risk. In addition, many fish oil supplementation trials suggest beneficial effects on important mediators in CAD development. These effects are largely ascribed to EPA (20:5ω3), which competes with arachidonic acid (AA; 20:4ω6) for incorporation into cellular phospholipids, and thereby alters the eicosanoid balance to a state of less platelet aggregation and less vasoconstriction.

LCPUFA of the ω3 and ω6 series derive either from the diet or synthesis from the parent essential fatty acids ALA (18:3ω3) and LA (18:2ω6). Synthesis occurs by alternating desaturation and chain elongation reactions, in which the first step, Δ6-desaturation, is rate limiting. Some studies with both humans and rats indicated that administration of γ-linolenic acid (GLA; 18:3ω6) augments LCPUFAω3 status,
Summary

probably by its ability to stimulate the conversion of ALA to LCPUFAω3 through augmented Δ6-desaturation activity. The ‘Seven Countries Study’ showed that the Cretan diet is associated with lower CAD mortality rate, despite comparable serum cholesterol levels. The Cretan diet is characterised by a high consumption of oleic acid from olive oil, at the expense of LA from sunflower oil, resulting in a high ALA/LA ratio. Beneficial effects of an increased ALA/LA ratio were demonstrated in the ‘Lyon Diet Heart Study’. The intervention comprised adaptation to the Cretan diet, and caused a 70% reduction of all cardiac events in patients who recovered from myocardial infarction. The beneficial effect may derive from many factors, including augmented LCPUFAω3 status.

In chapter 4.4 we investigated whether a short term supplementation with low dose GLA augments the LCPUFAω3 status of 15 apparently healthy adults. Eight of them took GLA daily during 4 weeks and the remaining 7 took ALA daily to investigate the influence of each of these fatty acids separately. In the consecutive 4 weeks all 15 took ALA + GLA. The ALA dose was chosen to reach the same dietary ALA/LA ratio as used in the Lyon Diet Heart Study. Our results indicate that ALA and GLA supplementation caused statistically significant increases of ALA and GLA in nearly all studied compartments, that GLA supplementation caused an increase of dihomo-γ-linolenic acid in all studied compartments, but that the supplements had negligible effects on the levels of AA, EPA and DHA.

We conclude that LCPUFAω3 status can not be improved by supplementation of low dose GLA, ALA or GLA + ALA. From literature data, it can be concluded that augmentation of the LCPUFAω3 status by ALA supplementation is poor compared with fish oil. The discrepancy may be explained by preferential oxidation of ALA, an inhibitory effect of dietary AA from the omnivorous diet (since AA exerts negative feed-back inhibition on the desaturating enzymes), or by the low dietary ALA/LA ratio.