method, the statistical calculations and the time that maternal samples are taken, are crucial for establishing such a correlation. These results are of importance for the appropriate management of pregnancies and neonates.

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n-3 fatty acids and cardiovascular disease

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The coronary athero-thrombogenic risk profile and the acute coronary syndrome; the role of n-3 polyunsaturated fatty acids (PUFA)

Cardiovascular risk has been associated with established risk factors such as dyslipidemia, hypertension, smoking, obesity, insulin-resistance / impaired glucose tolerance, physical inactivity, age, gender and genetics.[1] Mild to moderate hyperhomocysteinemia has emerged as an independent cardiovascular risk factor,[4,5,6] probably associated with endothelial dysfunction, and may exert unfavorable effects on the antithrombotic properties of the endothelium.[2] Finally, recent research adds support to the view that atherosclerosis is an inflammatory process.[3]

The acute coronary syndrome usually results from erosion or rupture of a vulnerable atherosclerotic plaque leading to acute coronary occlusion.[3] A thin fibrous cap, surrounding the lipid core with inflammatory cells, is the only structure separating the blood compartment with

its coagulation factors from the prothrombotic material in the lipid core.[3] Erosion or fissure of the fibrous cap allows contact between coagulation factors and tissue factor, the major initiator of the extrinsic coagulation pathway, which contributes strongly to the thrombogenicity of a ruptured plaque.[3] Moreover, the endothelium plays a key role in vascular homeostasis, and endothelial dysfunction is of major importance in the development of a vulnerable plaque.

The complex vascular biology preceding the acute coronary syndrome provides several possible therapeutic targets for n-3 PUFA, demonstrating anti-atherothrombogenic and anti-inflammatory properties, stabilizing the vulnerable atherosclerotic plaque and limiting the consequences of its disruption.

A vast number of publications show an increasing interest in the role of n-3 PUFA in the prevention and management of coronary heart disease. Several issues are, however, under discussion. The optimal intake of omega 3 fatty acids is not firmly established. Recent clinical studies have produced conflicting results, and concerns have been raised about environmental contamination of fish and possible deleterious effects.

This article reviews the current evidence regarding n-3 PUFA and cardiovascular disease, the possible mechanism of action of these fatty acids, results of clinical trials and potential future research strategies.

Essential PUFA

n-3 and n-6 PUFA – chemistry and origin

PUFA are hydrocarbon chains with two or more double bonds in the carbon chain. The omega-3 (n-3) and omega-6 (n-6) PUFA families (Figure 1) are characterized by having their first carbon-carbon double bond in the third and sixth position, respectively, from the methyl (-CH_3) or omega (n-) end of the molecule.[4]

According to accepted terminology, the number of carbon atoms in a PUFA molecule is designated by the first figure, while the number of double bonds is given by the second figure.

n-6 PUFA are mainly derived from plants and vegetable oils, while the n-3 PUFA with 18 carbon atoms are found in vegetable foods. However, very long-chain PUFA (with 20 or more carbon atoms) are made by phytoplankton and via the food chain transported to fatty fish and marine animals. The main members of those long chain n-3 PUFA are eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). The n-3 and n-6 PUFA are essential fatty acids which cannot be synthesized by the human body, but must be ingested.[5] Enzymatic desaturation and elongation of PUFA occur,[5] but only small amounts of EPA and DHA are derived from dietary α-linolenic acid (C18:3n-3).[5] Thus, the body content of EPA and DHA mainly depends on the amounts ingested.

The functions of n-3 and n-6 PUFA

Dietary n-3 PUFA act via several mechanisms. They are incorporated into the phospholipids of cell membranes, altering the membrane's biochemical and physical properties and influencing its fluidity, permeability, electrophysiological characteristics and membrane-bound receptors and enzymes.[6] Furthermore, PUFA have been shown to interact with cellular nuclear elements and may modulate gene expression.[7]

n-3 PUFA are substrates for the synthesis of eicosanoids,[6] including prostaglandins, thromboxanes, leukotrienes and hydroxy fatty acids, which possess important vasoactive regulatory properties, such as regulation of platelet aggregability, endothelial cell motility, cell growth and chemotaxis.[6] Replacing arachidonic acid (C20:4n-6) by n-3 PUFA with 20 carbon atoms in the synthesis of eicosanoids has the potential to induce changes towards eicosanoid products leading to a more vasodilatory state, reduced inflammatory responses in the injured vessel wall and less potent platelet aggregation.[6]

As a result, mechanisms proposed for the protective role of n-3 PUFA against cardiovascular disease include improved lipid profile, especially reduced serum triglycerides, antithrombotic, anti-inflammatory, antihypertensive, anti-arrhythmic effects, improved vascular endothelial function, increased plaque stability, increased paraoxonase levels and improved insulin sensitivity. Anti-atherothrombogenic and anti-inflammatory effects of n-3 PUFA in relation to coronary occlusive disease are outlined below.[6,7]

n-3 PUFA and atherothrombogenic risk modulation

It was in the 1970s that Bang and Dyerberg *et al.*[8] founded the area of n-3 PUFA research with their reports of beneficial anti-atherothrombogenic effects of high concentrations of n-3 PUFA in Greenland Eskimos. As compared to Danes, Greenland inuits had lower serum concentrations of total- and low density lipoprotein-cholesterol, triglycerides and very low density lipoprotein- cholesterol and higher levels of high density lipoprotein cholesterol. Since then various studies have confirmed several favorable effects of n-3 PUFA supplementation, particularly EPA and DHA, on a variety of factors of the athero-thrombogenic risk profile,

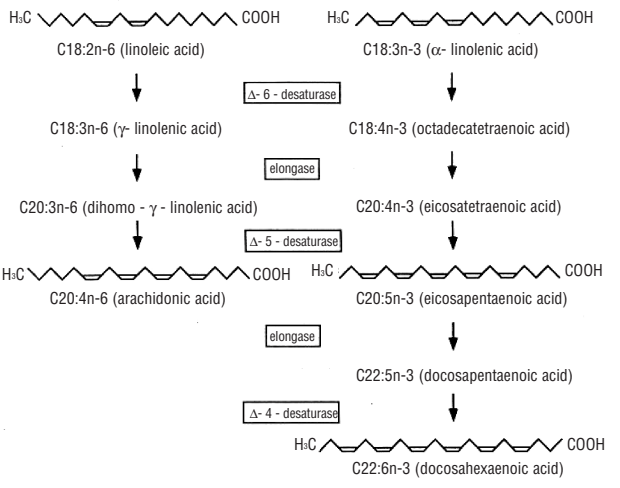

Figure 1. n-6 and n-3 PUFA series.

including dyslipoproteinemia, hypertension and impaired glucose tolerance / hyperinsulinemia.[6,7] These changes might be mediated through a beneficial shift in insulin resistance. The effect of n-3 PUFA on insulin as a mediator of the metabolic syndrome needs to be clarified, but there is some support for a relationship between the content of n-3 PUFA in membrane lipids and the action of insulin.[6]

The anti-platelet properties of high doses of n-3 PUFA, and the longer bleeding time, as initially described by Bang and Dyerberg, have been confirmed by numerous studies.[6,7] Reports on the influence of n-3 PUFA on other procoagulant and fibrinolytic factors are, however, divergent.

n-3 PUFA, inflammation and endothelial dysfunction in coronary artery disease

Inflammation and endothelial dysfunction are closely linked to processes related to the formation and disrupture of the vulnerable atherosclerotic plaque and thrombotic artery occlusion.[3] The anti-inflammatory properties of n-3 PUFA are of major importance.[6,7] A change in leukotriene formation towards less potent inflammatory (more inert) mediators is induced by partial replacement of arachidonic acid by n-3 PUFA in inflammatory cell membranes.[6] Moreover, n-3 PUFA supplementation has resulted in reduced production of the pro-inflammatory cytokines interleukin-1 and tumor necrosis factor from mononuclear cells.[6] Cellular adhesion molecules released from the endothelium, such as intercellular adhesion molecule-1, vascular cellular adhesion molecule-1 and E-selectin, are responsible for the attachment of leukocytes to the endothelium prior to their penetration into the subendothelial space. These markers reflect the functional state of the endothelium. Their expression may be down-regulated by n-3 PUFA, as demonstrated mostly in studies *ex vivo*.[7] Moreover, beneficial effects on the vasoregulatory secretagogues of the endothelium, such as nitric oxide and prostacyclin, have been obtained by n-3 PUFA.[6]

Although homocysteine may exert unfavorable effects on the antithrombotic properties of the endothelium, the influence of n-3 PUFA on homocysteine has not been well investigated. In a randomized trial we observed a reduction of homocysteine after treatment with a high-dose concentrate of n-3 PUFA as compared to corn oil for 1 year following a myocardial infarction.[9]

However, relatively small sample sizes, different doses and compositions of n-3 PUFA, different intervention periods and study designs can make the results of intervention studies with n-3 PUFA difficult to interpret. Thus, the impact of n-3 PUFA supplementation on the complex processes involved in vascular injury and repair still remains an unsettled issue.

n-3 PUFA, lipid peroxidation and pollutants

Concern has been raised regarding the potential deleterious effects of n-3 PUFA interventions, especially as n-3 PUFA may have a potential to increase oxidative stress, resulting in the formation of lipid peroxides. Different experimental settings in studies evaluating the impact of n-3 PUFA on oxidative stress of the vascular system may explain the contradictory results of these studies. We found a modest increase in lipid peroxidation, measured as thiobarbituric acid-malondialdehyde complex by high performance liquid chromatography, in subjects on a high-dose concentrate of n-3 PUFA for 1 year following a myocardial infarction.[10]

Environmental contamination of fish by dioxins, polychlorinated biphenyls and mercury is another increasing worry related to long-term n-3 PUFA supplementation. A recent study[11] showed that mercury in fish may counteract the beneficial cardioprotective effects of n-3 PUFA. This issue might be related to an optimum dose of n-3 PUFA intake, as high doses of n-3 PUFA might exceed an optimal threshold level,[12] leading to a lack of clinical benefit that might be due to lipid peroxidation or accumulation of other toxic substances.

n-3 PUFA and cardiovascular disease

Primary prevention studies

The pioneer studies of Greenland Eskimos who consumed a diet rich in n-3 PUFA, reported a lower coronary mortality in this population[8] than in Danish control subjects. Greenland Eskimos also had a more favorable lipid profile.[8] Additionally, longer bleeding times and reduced platelet aggregability were described in Greenland inuits. These findings were related to the shift in eicosanoid synthesis towards metabolites with less vasoconstrictive and pro-thrombotic properties.

During the following years several prospective epidemiological studies noted an association between fish intake and a lower risk of mortality from coronary artery disease.[13-14] However, recently, the Japan EPA Lipid Intervention Study (JELIS),[15] mainly a primary prevention trial comparing a daily dose of EPA 1800 mg in combination with statins versus a statin alone, including 18 645 patients with a total cholesterol ≥ 6.5 mmol/L (20% with coronary artery disease), reported a 19% reduction in the primary outcome in the n-3 PUFA group (p=0.011) after a mean follow-up of 4.6 years. The primary outcome included sudden cardiac death, fatal and non-fatal myocardial infarction, and other non-fatal events such as unstable angina pectoris, angioplasty, stenting or coronary artery by-pass grafting, but the benefit was mainly a statistically significant reduction of unstable angina pectoris (relative risk reduction 28%; 95% confidence interval [CI] 0.55-0.95, p=0.019), while no favorable effect was observed on myocardial infarction, total death or sudden death.

Secondary prevention trials

Several trials have assessed the effects of marine-derived omega-3 fatty acids on death from coronary artery disease during secondary prevention following a myocardial infarction. Subjects advised to consume fatty fish after recovery from a recent infarct had a 29% reduction in overall mortality (p< 0.05) during a 2- year follow-up as compared to those not so advised in the Diet and Reinfarction Trial (DART),[16] mainly due to a

reduction in deaths from coronary artery disease.

In the most important trial of the effects of n-3 PUFA on cardiovascular disease outcomes, the open label GISSI-Prevenzione trial,[17] 11 324 patients surviving a myocardial infarct within the preceding 3 months were randomized to receive a daily dose of one gelatin capsule of n-3 fatty acids (≈ 850 mg/day as EPA plus DHA ethyl esters in an EPA to DHA ratio of 1.2:1), 300 mg vitamin E, the combination, or neither for 3.5 years. The primary outcome was a composite of death, non-fatal myocardial infarction and stroke. The trial showed a modest, but statistically significant 15% reduction in the risk of the primary outcome among patients receiving n-3 PUFA (p=0.023). Subgroup analyses showed a reduction of the relative risk of cardiovascular death by 30% (p=0.024) and of sudden cardiac death by 45% (p=0.01) in patients receiving n-3 PUFA. However, it has been argued that the GISSI-Prevenzione trial was not designed to evaluate sudden cardiac death, and that its statistical power was insufficient. The results of these subgroup analyses should, therefore, be interpreted with caution.

Meta-analyses

Based upon a high quality meta-analysis of 41 cohort studies (including more than 563 218 participants) and 48 randomized intervention trials, in which 36 913 participants with and without cardiovascular disease received n-3 PUFA for at least 6 months, Hooper *et al*.[18] concluded that n-3 PUFA do not have a clear effect on total mortality or combined cardiovascular events.

The results of that review differ from those of another systematic review by Bucher *et al.*,[19] who found significant protective effects on fatal myocardial infarction (RR 0.7; 95% CI 0.6-0.8), sudden death (RR 0.7; 95% CI 0.6-0.9) and total mortality (RR 0.8; 95% CI 0.7-0.9) in subjects with coronary artery disease receiving dietary / non-dietary n-3 PUFA, with at least 6 months follow-up. However, the incidence of non-fatal myocardial infarction was not influenced by n-3 PUFA. Among the 15 806 patients included in this meta-analysis, 72% belonged to the GISSI-Prevenzione study.

Studies on anti-arrhythmic effects

Experimental work in animal models have shown that n-3 PUFA alter the electrophysiological properties of the heart,[20] and it has been suggested that stabilization of cell membranes might explain the anti-arrhythmic and anti-fibrillatory effects of n-3 PUFA.[21] The early effect of n-3 PUFA on total mortality and sudden death, as demonstrated by the time-course analysis of the results of the GISSI-Prevenzione study, supported a hypothesis of an anti-arrhythmic potential of n-3 PUFA.[22]

Recently, three randomized clinical trials[20,23,24] have been performed in patients with an implantable cardioverter defibrillator (ICD), to evaluate the anti-arrhythmic effects of n-3 PUFA. In the study by Raitt *et al.*[23] no significant decrease in total mortality was observed among 200 patients with an ICD and a recent episode of sustained ventricular tachycardia or ventricular fibrillation receiving EPA/DHA 1.3g/day as compared to olive oil for 2 years. Surprisingly, in the study by Raitt *et al.*[23] recurrent episodes of ventricular tachycardia or fibrillation occurred more frequently in patients on n-3 PUFA, and the time to event was reduced. In contrast to these findings, Leaf *et al.*[20] observed a trend towards an increase in time to recurrent ventricular tachycardia or fibrillation or death in the n-3 PUFA group in the Fatty Acid Arrhythmia Trial (FAAT) in which 402 patients with an ICD were randomized to either 2.6 g/day n-3 PUFA or olive oil for 1 year. Moreover, Brouwer and co-workers[24] found no strong evidence of a protective effect of omega-3 PUFA given at a daily dose of 0.9 g for 1 year as compared to that of sunflower oil, against ventricular arrhythmias in 546 patients with ICD, although a trend towards decreased ventricular arrhythmias or death was noted in the subgroup of patients with a prior myocardial infarction. The disparate results of these studies may be related to different doses of n-3 PUFA used and background dietary fish intake, as well as heterogeneous groups of patients. Based on data collected at 1 year in these trials, a meta-analysis by Jenkins *et al.*[25] showed no overall effect of n-3 PUFA on the relative risk of ICD discharge or mortality. Obviously, these ICD populations differed from the GISSI-Prevenzione population. Consequently, the mechanisms of arrhythmia may also have been different. Post-infarction patients are more prone to ischemia-related arrhythmias, while patients with an ICD are more likely to suffer scar-related malignant arrhythmias.

Studies on relationships between intake of n-3 PUFA and less life-threatening forms of arrhythmia, such as atrial fibrillation and premature ventricular complexes, have also given equivocal results. Thus, after 35 years of research the question of whether n-3 PUFA prevent heart disease remains unanswered, and an anti-arrhythmic effect of n-3 PUFA remains unproven although the idea is still viable and is being actively investigated in further trials.

Risks and benefits from n-3 PUFA – an optimum dose threshold effect

Previous prospective and randomized studies suggesting lower risks of coronary heart disease death and sudden death as a result of n-3 PUFA ingestion have been reviewed by Mozaffarian and Rimm.[26] Across different studies, a modest consumption (≈ 250-500 mg/day) of EPA and DHA, as compared to little or no intake, was shown to lower the relative risk by 25% or more. Intakes above this did not substantially affect coronary heart disease mortality, suggesting a threshold of effect.

At intakes of up to 250 mg/day, the relative risk of coronary heart disease mortality was 14.6% lower (95% CI, 8% to 21%) for each 100 mg/day of EPA/DHA. At higher intakes, little additional risk reduction was found (0% change for each 100 mg/day; 95% CI, –0.9% to +0.8%). This threshold-related effect may explain the minor benefits in the secondary prevention group in JELIS.[15] The background fish intake of a median of 900 mg/day of EPA and DHA in that study was associated with very low coronary heart disease death rates (87% lower than in comparable Western populations), and additional n-3 PUFA intake yielded little further reduction in the death rate. It has, therefore, been concluded that most of the population is already above the thresh-

old for maximum mortality benefits. Furthermore, Mozaffarian and Rimm[26] argue that the heterogeneity of the effects of n-3 PUFA intake on cardiovascular disease outcomes may be related to the impact of varying doses over time on specific risk factors, such as triglycerides. With typical dietary intakes, anti-arrhythmic effects predominate, reducing the risk of cardiac death within weeks.

The omega-3 index as a new risk factor

Hypothesizing that increased electrical stabilization of myocardial cells is related to incorporation of n-3 PUFA into the cell membranes of myocytes, the levels of EPA and DHA in the cell membrane of myocytes could provide information on the risk of sudden cardiac death. Recently, Harris[27] has presented evidence that EPA and DHA in red blood cells, expressed as weight percent of total fatty acids, the omega-3 index, might be regarded as a reflector of the omega-3 fatty acid content of myocardial cells. Furthermore, Shacky and Harris[27] have claimed that the omega-3 index may be considered a risk factor for sudden cardiac death, as an omega-3 index > 8% is associated with a 90% lower risk of sudden cardiac death, as compared to that in subjects with an omega-3 index < 4%.

Future and ongoing randomized trials with cardiovascular end-points

Among several ongoing or planned large-scale randomized studies in Europe on the effect of n-3 PUFA on different cardiovascular end-points,[28] only the Omega trial has been designed to examine the effect of 1 g/day n-3 PUFA versus placebo on sudden cardiac death as the primary end-point in 3800 patients with a recent myocardial infarction. In the GISSI-Heart Failure, randomizing almost 7000 patients with class II-IV heart failure to n-3 PUFA, rosuvastatin, both active drugs or placebo (double-blind 2x2 factorial design), the primary end-point is all-cause mortality or hospital admissions for cardiovascular reasons. Moreover, two large-scale randomized trials are planned in diabetic patients to investigate the effects of n-3 PUFA on serious cardiovascular events: the ASCEND trial (A Study of Cardiovascular Events in Diabetes) intends to randomize 10 000 diabetic patients to low-dose aspirin versus PUFA 1 g/day, both or placebo (2x2 factorial design), while the ORIGIN (the Outcome Reduction with Initial Glargine Intervention) trial aims to randomize 10 000 diabetic patients with cardiovascular disease to insulin glargine versus n-3 PUFA 1 g/day, both or placebo, in an open-label 2x2 factorial design.

Despite advances in the understanding of the cardioprotective effects of n-3 PUFA since the pioneering work of Bang and Dyerberg, several areas of uncertainty still remain. To understand the effects of n-3 PUFA on cardiovascular disease, we definitely need more high quality, large-scale, randomized, controlled clinical trials of long duration, also reporting possible harmful effects. Finally, the above mentioned studies could contribute to define the appropriate role of n-3 PUFA in the prevention of cardiovascular disease outcomes. Further research is also justifiable in order to define an optimum dose of n-3 PUFA and to fully understand the mechanisms of action of these fatty acids.

In this issue of Haematologica, Serena Del Turco[29] and co-authors present an original paper reporting the results of a randomized, double blind study comparing the effect of a high dose of concentrated n-3 PUFA or olive oil, administered for 12 weeks in patients with a previous myocardial infarction, on circulating levels of microparticles. Several interesting findings of the study include a reduction of the generation and pro-coagulant activity of platelet-related microparticles by n-3 PUFA. The authors observed a longer clotting time after n-3 PUFA treatment, especially when pre-incubating microparticles with factor XII- deficient plasma, indicating a major contribution by the intrinsic system. The reduced shedding of microparticles may explain the beneficial effects of n-3 PUFA on the thrombogenic potential which is bound to be increased in a post-infarction population with reduced ejection fraction and atherosclerosis. These findings are of great interest and may inspire further research within this challenging scientific field.

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Endothelial cell protein C receptor and the risk of venous thrombosis

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The natural anticoagulant protein C (PC) pathway plays a major role in regulating coagulation and inflammation.[1-3] PC is activated at the endothelial surface when thrombin binds to thrombomodulin (TM), a transmembrane glycoprotein, that transforms thrombin into a potent activator of PC. In the presence of its cofactor protein S, activated protein C (APC) inactivates factors Va and VIIIa, thereby down regulating the thrombin feedback loop. Thrombomodulin is a vascular endothelial cell receptor present in many cells and tissues. Its density is higher in small vessels and capillaries, however, and this is why PC activation was believed to occur mainly in these vessels. However, thrombosis in subjects with hereditary PC or PS deficiency is not restricted to the microcirculation but can also affect larger vessels, suggesting that the PC pathway is also active in zones of lower TM density. This apparent paradox was resolved by the discovery of an endothelial cell receptor specific for PC – the endothelial cell protein C receptor (EPCR) – which increases the PC activation rate by thrombin-TM complexes[4] and is most abundant in large vessels.[5] Functional studies performed *in vitro* showed a ~20-fold increase in the PC activation rate by membrane thrombin-thrombomodulin complexes when PC was bound to its receptor.[6] This increase results from a significant effect of EPCR on the Michaelis-Menten constant (Km) for PC activation by the thrombin-thrombomodulin complex. Indeed, without EPCR intervention, this Km is significantly higher (1 μM) than the circulating concentration of PC (60-70 nM). By presenting PC to the thrombin-TM complex (Figure 1), EPCR reduces the Km and allows the interaction to occur.

Compared to the effect of thrombin binding to TM, which results in a >1,000-fold increase in the PC activation rate,[7] the 20-fold increase in the PC activation rate after PC binding to EPCR might seem rather inconsequential. Yet, EPCR is even more important for PC activation than suggested by *in vitro* studies. Indeed, in a baboon model, APC generation induced by thrombin infusion fell by 88% when the animals were pre-treated with anti-EPCR antibodies that blocked the PC/EPCR interaction.[8] The crucial role of EPCR was further confirmed by studies of *PROCR* knock-out mice:[9] complete invalidation of the gene led to intrauterine death by fibrin deposition in embryonic trophoblast giant cells, leading to thrombosis at the maternal-embryonic interface.

EPCR is a 46-Kda type 1 transmembrane glycoprotein